Methamphetamine Learning Induces Persistent and Selective Nonmuscle Myosin II-Dependent Spine Motility in the Basolateral Amygdala

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Nonmuscle myosin II inhibition (NMII) in the basolateral amygdala (BLA), but not dorsal hippocampus (CA1), selectively disrupts memories associated with methamphetamine (METH) days after learning, without retrieval. However, the molecular mechanisms underlying this selective vulnerability remain poorly understood. A known function of NMII is to transiently activate synaptic actin dynamics with learning. Therefore, we hypothesized that METH-associated learning perpetuates NMII-driven actin dynamics in synapses, leading to an extended window of vulnerability for memory disruption. We used time-lapse two-photon imaging of dendritic spine motility in acutely prepared brain slices from female and male mice following METH-associated learning as a readout of actin–myosin dynamics. Spine motility was persistently increased in the BLA, but not in CA1. Consistent with the memory disrupting effect of intra-BLA NMII inhibition, METH-induced changes to BLA spine dynamics were reversed by a single systemic injection of an NMII inhibitor. Intra-CA1 NMII inhibition, on the other hand, did not disrupt METH-associated memory. Thus, we report identification of a previously unknown ability for spine actin dynamics to persist days after stimulation and that this is under the control of NMII. Further, these perpetual NMII-driven spine actin dynamics in BLA neurons may contribute to the unique susceptibility of METH-associated memories.

Key words: actin; addiction; cytoskeleton; dendritic spine; drug seeking; hippocampus

Significance Statement

There are no Food and Drug Administration-approved pharmacotherapies to prevent relapse to the use of stimulants, such as methamphetamine (METH). Environmental cues become associated with drug use, such that the memories can elicit strong motivation to seek the drug during abstinence. We previously reported that the storage of METH-associated memories is uniquely vulnerable to immediate, retrieval-independent, and lasting disruption by direct actin depolymerization or by inhibiting the actin driver nonmuscle myosin II (NMII) in the BLA or systemically. Here we report a potential structural mechanism responsible for the unique vulnerability of METH-associated memories and METH-seeking behavior to NMII inhibition within the BLA.

Introduction

Dendritic spines, actin-rich postsynaptic compartments, are dynamic and enable input-specific biochemical and electrical isolation of synapses to facilitate information storage (Kasai et al., 2003; Araya et al., 2006a; Dumitriu et al., 2007). During learning, spines undergo structural and functional changes to stabilize synapses and, ultimately, memory (Lai et al., 2012; Hayashi-Takagi

et al., 2015). Consistent with this, there is a tight connection between the physical geometry of spines and the ability to transform experiences into long-term memory (Xu et al., 2009; Yang et al., 2009; Makino and Malinow, 2011). Actin polymerization, the elongation and complex branching of filamentous actin, drives the spine structural plasticity that is required for functional plasticity and learning (Kim and Lisman, 1999; Smart and Halpain, 2000; Matsuzaki et al., 2001; Star et al., 2002; Noguchi et al., 2005, 2011).

Long-term potentiation (LTP) and newly formed memories become impervious to actin depolymerization shortly after the underlying synaptic plasticity occurs (Mantzur et al., 2009; Rehberg et al., 2010; Rex et al., 2010). This is attributed to rapid stabilization of the actin cytoskeleton through the cessation of polymerization and recruitment of actin capping and stabilizing proteins (Lamprecht, 2014). However, we recently discov-

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ered that memories associated with the commonly abused stimulant, methamphetamine (METH), remain uniquely susceptible to depolymerization days after learning (Young et al., 2014). Indeed, a single infusion of the actin depolymerizer Latrunculin A (LatA) into the basolateral amygdala (BLA) results in an immediate, long-lasting, and retrieval-independent loss of METH-associated memory and associated drug-seeking behavior. This memory loss is accompanied by a return of BLA spine density to pre-METH conditioning levels. LatA influences populations of dynamic, but not stable, actin by sequestering monomers (Morton et al., 2000; Yarmola et al., 2000). Thus, the susceptibility of a METH-associated memory days after learning suggests that METH may interfere with actin stabilization in BLA spines.

The motivation of drug-associated memories is triggered, in large part, by activation of the amygdala (Childress et al., 1999; Miller and Marshall, 2005; Otis et al., 2015), making the ability to rapidly and selectively disrupt amygdala-dependent METH-associated memories a potential therapeutic avenue (Young et al., 2015). Because the critical role of actin in numerous cellular processes limits its therapeutic potential, we shifted focus to the molecular motor ATPase, nonmuscle myosin II (NMII), a driver of actin polymerization (Ryu et al., 2006; Koskinen et al., 2014; Omotade et al., 2017). NMII is a hexameric protein made of two heavy chains (MHC), two regulatory light chains (RLCs), and two essential light chains. The MHCs are the workhorse, bearing both the ATPase and actin-binding sites, and motor heads that bind actin and move it through physical force. We have demonstrated that NMII drives synaptic actin polymerization to promote and stabilize LTP and long-term memory formation in CA1 and the BLA (Rex et al., 2010; Gavin et al., 2012). In addition, through genetic and pharmacologic manipulations, we have established that targeting NMII in the BLA or systemically is well tolerated and recapitulates the METH-associated memory, drug-seeking, and spine density effects of direct actin depolymerization (Young et al., 2016, 2017).

The rapid and persistent impact of NMII inhibition on METH-associated memory is surprisingly specific, having no such effect on memories associated with footshock, food reward, or other drugs of abuse (nicotine, morphine, cocaine, or mephedrone; Briggs et al., 2017). The underlying mechanism responsible for this selectivity is largely

Figure 1. Motility of CA1 and BLA spines decreases with age. **A**, CA1 dendritic spine motility from naive young animals (P16–P21) and early adolescent animals (P28–P35). Small lighter colored circles represent the movement of each individual spine over 1 h, while the large darker circles represent the average of 30 spines from each slice (P28–P35: $n = 210$ spines, 7 slices, 5 animals; P16–P21: $n = 210$ spines, 7 slices, 4 animals). **B**, CA1 spine motility organized by animal and slice. Slices from female animals are in light green and gray, males are depicted by dark green and black. **C**, Cumulative distributions of CA1 spine movements from P16–P21 and P28–P35. **D–F**, Spine movements measured in BLA slices from P16–P21 and P28–P35 (**D**, **E**), and corresponding cumulative distributions (**F**; P28–P35: $n = 260$ spines, 8 slices, 5 animals; P16–P21: $n = 240$ spines, 8 slices, 5 animals). **G**, **H**, Comparison of BLA and CA1 spine movements at P16–P21 and P28–P35. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

unknown to date. The impact of actin depolymerization on BLA spine density via LatA or NMII inhibition with blebbistatin (Blebb) indicates a connection to spine actin. Therefore, we hypothesized that the selective susceptibility of METH-associated

Figure 2. Cluster analysis of CA1 and BLA age-related spine movements. **A**, All CA1 and BLA spine movements underwent cluster analysis. **B**, **C**, Distribution of spine movements within each group across three clusters. Error bars represent SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

memory is due to NMII-dependent sustained spine actin dynamics. As a first step toward addressing this, we used time-lapse two-photon imaging of actin-dependent BLA spine motility, as measured by the dynamic extension and retraction of the spine head from the dendrite. CA1 spine motility was measured for comparison. Like the BLA, CA1 is a critical component of the neural circuit supporting drug-associated memories, and we have reported spine density increases in CA1 with METH learning, as in the BLA (Kutlu and Gould, 2016; Briggs et al., 2017). However, METH memories do not have the same vulnerability to NMII disruption in CA1. And, unlike BLA spines, METH-induced CA1 spine density increases remain intact after systemic Blebb treatment (Briggs et al., 2017). Therefore, we anticipated that METH conditioning would result in a NMII-dependent increase in spine motility in the BLA, but not in CA1.

Materials and Methods

All animal procedures were conducted in accordance with the Scripps Research Animal Care and Use Committee and national regulations and policies.

Animals

Male and female heterozygous Thy-GFPm mice (The Jackson Laboratory) were bred onsite, and male and female C57BL/6 mice were birthed onsite from pregnant females obtained from The Jackson Laboratory. All animals were weaned at postnatal day 22 (P22) to P23 and housed in groups of four, separated by sex. All animals were handled 3 d prior to the start of training on P28. To minimize stress, handling consisted of scooping all animals in a single cage into the experimenter's hands at once for 1 min, followed by each mouse being scooped and handled individually for 1 min. Additionally, each animal was scruffed during the second handling session to habituate them to the handling associated with injections.

Drug

For actin-stabilizing imaging experiments, 200 nM jaspakolinide (Jasp; Tocris Bioscience) in 0.02% DMSO was bath applied to slices. During

training, mice received 1 mg/kg doses of methamphetamine hydrochloride (Sigma-Aldrich). For systemic Blebb infusions mice received a 10 mg/kg, i.p., dose of racemic blebbistatin (TSRI) diluted to 1 mg/kg in a vehicle (Veh) of 0.9% DMSO/25% hydropropyl β -cyclodextrin. Vehicle animals received the vehicle without Blebb. For intrahippocampal CA1 infusions, the active (–) or inactive (+) Blebb enantiomer (R&D Systems) was infused at a concentration of 90 ng/ μ l in 10% DMSO and 0.9% saline. The delivery rate was 0.1 μ l/min over 2 min. Injectors were left in place for 1 min after completion of the infusion to allow for sufficient diffusion of drug away from the needle tips.

Surgery

Mice were anesthetized using isoflurane gas and implanted bilaterally with 22 guide cannulae (Plastics One) 1 mm above CA1 (anteroposterior, –2 mm relative to bregma; ML, $\pm$ 1.5 mm relative to bregma; DV, –0.9 mm from the skull). Metacam (1.5 mg/ml, 1–2 drops orally) was administered immediately after surgery.

Behavior

Adolescent mice were trained as previously described (Young et al., 2017). Conditioned place preference (CPP) consisted of two phases, pretesting and training, followed by imaging 1–3 d later. Pretesting was conducted over 2 consecutive days. Animals received an intraperitoneal injection of saline before freely exploring all three CPP chambers for 30 min. Either the white or black chamber was assigned as the METH-paired chamber [conditioned stimulus (CS+)] for each animal based on their least preferred chamber during the final 15 min of the second pretest session. There were no initial differences between groups for the amount of time spent in either the white or black chamber. Over the next 4 consecutive days, animals were trained twice daily in 30 min training sessions. Animals either received saline in the CS chamber in the mornings and METH in the CS+ in the afternoons or were assigned the opposite training schedule. One to three days following the final day of training, animals were killed for acute tissue collection. After testing, the brains were postfixed overnight using 4% paraformaldehyde so that cannula tract location could be verified. Correct injector placement in the CA1 of the hippocampus was confirmed for all animals in the study.

Spine motility

Acute slice preparation. Acute coronal brain slices (350 μ m thick) were extracted and sliced in cold cutting solution composed of the following (mM): 119 choline Cl, 22 D-glucose, 4.3 MgSO₄, 2.5 KCl, 1 NaH₂PO₄, 1 CaCl₂, and 26.2 NaHCO₃. Adolescent animals (P28–P36) were gravity perfused with cold cutting solution for 40 s before brain extraction and slicing. Following slicing, brain slices were continuously bubbled with 95% O₂/5% CO₂ and incubated at 34°C for 30 min then room temperature for 30 min in artificial CSF (aCSF) composed of the following (mM): 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 2.5 CaCl₂, 1 NaH₂PO₄, 0.2 Trolox, 11 glucose, and 26.2 NaHCO₃. For imaging, slices were continuously perfused with oxygenated aCSF at 1.5–2 ml/min.

Two-photon imaging. Two-photon imaging of the basolateral amygdala complex (BLC) and CA1 was conducted with a multiphoton laser-scanning microscopy (FV1000MPE-TWIN, Olympus), equipped with a water-immersion objective lens (ULTRA 25 $\times$, numerical aperture 1.05, Olympus) and Fluoview software (Olympus). To excite eGFP, we used a Ti:sapphire laser and collected emitted photons that passed through a 500–550 nm bandpass filter with an external nondescanned detector. To measure spine dynamics, we imaged slices in XYZT dimension (45–60 μ m z-stack; 60 min time series; 5 min between stacks; Clement et al., 2012). The imaging parameters used were as follows: excitation = 930 nm; power at sample = $\sim$ 15 mW; pixel dwell time = 2 μ s; x–y scaling = 0.201 μ m/pixel; and z-scale = 1 μ m; no averaging. For the effects of actin stabilization, vehicle (aCSF with 0.02% DMSO) or aCSF + Jasp was bath applied to slices. Slices were allowed to stabilize in the imaging chamber and incubate for 30 min before imaging. Slices were then imaged in the XYZT dimension over 1 h with 5 min in between each 45–60 μ m z-stack. **Spine movement analysis.** Spine movements were assessed in both the CA1 and BLC following standard procedure (Majewska and Sur, 2003; Clement et al., 2012). ImageJ software (NIH) was used to process and align images. After z-stack compression of the 12 images (5 min between

Table 1. Comparison of initial dendritic spine length by age and region

	Initial spine length			
	BLA	Statistics	CA1	Statistics
Age-related comparisons (within regions)				
P16–P21	1.52 ± 0.03	Mann–Whitney U test = 29522	1.30 ± 0.02	Mann–Whitney U test = 21603
P28–P35	1.44 ± 0.03	p = 0.299	1.30 ± 0.02	p = 0.719
	Region	Mean	Statistics	
Region-related comparisons (between regions)				
Young (P16–P21)	BLA	1.52 ± 0.03	Mann–Whitney U test = 19580	
	CA1	1.30 ± 0.02	p < 0.0001	
Adolescent (P28–P35)	BLA	1.44 ± 0.03	Mann–Whitney U test = 22676	
	CA1	1.30 ± 0.02	p = 0.0016	

frames), the resulting two-dimensional projections were aligned to compensate for inherit *x–y* drifting during time series imaging using the ImageJ plugin StackReg. Spines used for analysis were selected in an unbiased way with the researcher blinded to the group. Tertiary dendritic segments that were relatively clear of random axonic and dendritic processes were selected for analysis. Along these segments, the movement of the first 10 unobstructed dendritic spines was analyzed for a total of 30 spines per slice. The spine length was measured from the base to the tip of the protrusion in each of the 12 images. Spine movement was expressed as the average change in spine length per minute ($\mu\text{m}/\text{min}$; Lendvai et al., 2000; Majewska and Sur, 2003; Clement et al., 2012).

Statistical analysis

For all studies, the experimenters were blind to the treatment group. The averages of spine movement per slice were found to be normally distributed but all spines per group was not. Therefore, comparisons of groups using slice averages was conducted using *t* tests or one-way ANOVAs, while population comparisons were performed with Kolmogorov–Smirnov (KS) tests. For *post hoc* analysis of ANOVAs, Bonferroni’s multiple-comparison test was used. To determine the presence of subpopulations within the data, for each study BLC and CA1 spine movements across all groups were combined and examined using cluster analysis. It was found that the data clustered unbiasedly into either two or three clusters. Dictating two or three clusters for the cluster analysis of each study had equal levels of good cluster quality. Visual examination of the data when grouped according to either the two or three cluster outputs revealed that three clusters modeled the data better than two clusters. Therefore, all data were analyzed using cluster analysis for three clusters. Cluster comparison within brain region was performed using χ^2 analysis. A Mann–Whitney *U* test or Kruskal–Wallis test was used to examine initial spine lengths. To examine the influence of highly motile spines on group differences, the receiver operating characteristic (ROC) was used to determine a cutoff of highly motile METH spines, which were then removed. Pretest training data were analyzed using Wilcoxon signed-rank tests.

Figure 3. Age-related CA1 and BLA spine movements. **A**, **B**, Representative movements of individual CA1 (**A**) and BLA (**B**) spines with different levels of motility over 1 h. **C**, Examples of spines emerging and disappearing during the imaging period.

Results

BLA and CA1 spine motility is actin dependent and decreases with development

Spine motility has not yet been assessed in the BLA. Therefore, we first examined age-dependent spine motility, as it has been reported to decrease with development elsewhere in the brain and likely acts as an important component of use-dependent synaptic wiring during development (Bonhoeffer and Yuste, 2002). The exact age at which spine motility decreases varies by brain region and preparation (Dunaevsky et al., 1999; Lendvai et al., 2000; Majewska and Sur, 2003; Clement et al., 2012). Therefore, we examined spine movements across two postnatal day ranges, P16–P21 and P28–P35. Acute slices from naive Thy1-GFP(m) mice, containing both CA1 and BLA, were imaged every 5 min for 1 h. The motility of 30 spines per slice was then quantified. Consistent with prior reports on spine motility in the hippocampus (Dunaevsky et al., 1999; Lordkipanidze et al., 2013), CA1 spines displayed more motility at P16–P21, compared with P28–P35 (Fig. 1A; $t_{(12)} = 3.77$, $p < 0.01$; power = 0.93). Before performing any additional analyses, we confirmed that the range of spine motilities observed as a group was represented across spines from all slices analyzed and was, therefore, not biased by a slice with particularly high or low overall motility (Fig. 1B). This was performed for all experiments in the study. To determine whether the shift in spine movements was driven by decreased motility across the whole population of spines or by loss of a subgroup of spines only present in P16–P21 CA1, we examined the cumulative distribution of spine movement. This revealed a shift in the whole population of spines toward decreased movement at P28–P35, as well as the loss of highly motile spines (movement, $\geq 0.030 \mu\text{m}/\text{min}$; Fig. 1C; KS test, $p < 0.0001$). BLA spine movements followed a similar developmental pattern (Fig. 1D; $t_{(14)} = 5.01$, $p < 0.001$, power = 0.99), with an even greater shift in the population of spines toward decreased motility at P28–P35 (Fig. 1F; KS test, $p < 0.0001$).

We next performed unbiased cluster analysis, identifying three clusters. This enabled comparison of the relative representation of spines in low-, mid-, and high-range motility within CA1 and BLA in early life (P16–P21) versus early adolescence (P28–P35; combined overall: $\chi^2_{(6)} = 95.93$, $p < 0.0001$; CA1 overall: $\chi^2_{(2)} = 20.30$, $p < 0.0001$; BLA overall: $\chi^2_{(2)} = 65.82$, $p < 0.0001$; Fig. 2A). χ^2 analysis established age-dependent differences in CA1 for low (cluster 1) and mid-range (cluster 2) motility (cluster 1: $\chi^2_{(1)} = 20.15$, $p < 0.0001$; cluster 2: $\chi^2_{(1)} = 19.14$, $p < 0.0001$; cluster 3: $\chi^2_{(1)} = 1.00$, $p > 0.05$; Fig. 2B), while motility decreased in all three clusters (including high motility) with age for BLA spines (cluster 1: $\chi^2_{(1)} = 65.64$, $p < 0.0001$; cluster 2: $\chi^2_{(1)} = 57.43$, $p < 0.0001$; cluster 3: $\chi^2_{(1)} = 6.58$, $p < 0.01$; Fig. 2C). When comparing regions, BLA spines were more motile than CA1 spines during early life (P16–P21; KS test, $p < 0.05$), while the opposite was true in early adolescence (P28–P35; KS test, $p < 0.0001$; Fig. 1G,H). Additional similarities and differences were observed between CA1 and BLA. The most pronounced difference was in initial spine lengths. BLA spines were longer than those in CA1, and this was not age dependent (Table 1). This is consistent with a prior report using Golgi staining, though a direct comparison was not made in that study (Luczynski et al., 2016). Examining individual spines in early development (P16–P21), a time point when motility was sufficiently elevated to reveal variability in movements between spines over the course of an hour, we found CA1 and BLA spines with low to medium overall motility displayed a remarkably similar range of move-

Figure 4. Actin stabilization of young BLA spines. **A**, Young (P16–P21) BLA slices were incubated in bath-applied Veh or Jasp for 30 min before imaging. **B**, Spine movements and cumulative distributions were measured in the presence of Jasp or Veh (Veh: $n = 210$ spines, 7 slices, 5 animals; Jasp: $n = 210$ spines, 7 slices, 6 animals). Error bars represent SEM. **** $p < 0.0001$.

ment events (Fig. 3A,B). These movements were relatively infrequent and represented small to medium changes in length, rather than, for example, only one to two large movements that averaged to moderate motility over the course of an hour. Differences emerged in spines with overall high motility rates; BLA spines made larger, more frequent movements when compared with CA1 spines (Fig. 3A,B, top). In addition, the appearance and disappearance of spines over the course of imaging, while rare, was observed in both regions (Fig. 3C). It was more prominent in young BLA slices and CA1 slices from both age ranges, occurring in 3% of spines.

Spine motility is actin dependent in the hippocampus and cortex (Fischer et al., 1998, 2000). To confirm that our measurements were a readout of actin-dependent processes within dendritic spines, we measured the effect of the actin stabilizer Jasp on BLA spine dynamics in slices from P16–P21 animals. Indeed, Jasp decreased BLA spine movement (Fig. 4A,B; $t_{(12)} = 5.48$, $p = 0.0001$; Power = 0.99; KS test, $p < 0.0001$). This included an absence of highly motile spines, making it more akin to P28–P35 spine motility distribution (Fig. 1F). We also confirmed there were no group differences in initial spine length for this and all subsequent experiments (Tables 1, 2). Together, these data establish that BLA spine motility is actin dependent and decreases markedly by P28–P35, with a significant loss of highly motile spines ($\geq 0.03 \mu\text{m}/\text{min}$).

METH conditioning increases basal spine motility in BLA, but not CA1

We next assessed BLA spine motility following METH-associated learning as a measure of spine actin dynamics in male and female Thy1-GFP(m) mice. The number of neurons expressing fluorescence increases dramatically with development and, accordingly, we found that P28–P35 is the oldest age at which it is possible to confidently resolve individual dendritic segments and associated

Table 2. Comparison of initial spine lengths by experiment

		Initial spine length			
		BLA	Statistics (Mann–Whitney or Kruskal–Wallis test)	CA1	Statistics (Mann–Whitney or Kruskal–Wallis test)
Jasp slice treatment	Veh	1.56 ± 0.03	$U = 20724$		
	Jasp	1.50 ± 0.03	$p = 0.286$		
METH CPP	Saline	1.48 ± 0.03	$U = 28698$	1.21 ± 0.02	$U = 29214$
	METH	1.48 ± 0.03	$p = 0.947$	1.26 ± 0.02	$p = 0.055$
METH CPP + Blebb treatment	Saline/Veh	1.66 ± 0.03	$H(3) = 7.38$	1.22 ± 0.02	$H(3) = 5.89$
	Saline/Blebb	1.56 ± 0.03		1.24 ± 0.02	
	METH/Veh	1.64 ± 0.03	$p = 0.061$	1.23 ± 0.02	$p = 0.117$
	METH/Blebb	1.59 ± 0.03		1.30 ± 0.02	

spines in the BLA. Importantly, we have previously demonstrated that METH-associated memory is vulnerable to disruption by Blebb days after training in both adult and adolescent (P28–P35) male and female mice (Young et al., 2017).

Mice underwent CPP training with either saline or METH, followed by imaging 1–3 d later (Fig. 5A). Behavioral testing was not performed before imaging to avoid retrieval and/or extinction-induced effects on spine motility. However, our CPP training protocol consistently produces a conditioned place preference for METH in adolescents (Young et al., 2017; see Fig. 12). Similar to P16–P21 motility in naive slices (Fig. 3), differences in individual spine movements were the most pronounced as motility increased (Fig. 6A,B). Additionally, as in naive slices, the appearance and disappearance of spines over the course of imaging was rare following CPP training (1.7–2.9%) and was not influenced by METH exposure.

Spine motility was elevated in the BLA, but not in CA1, 1–3 d after METH training relative to saline controls (Fig. 5B,C; BLA: $t_{(15)} = 2.38$, $p < 0.05$, power = 0.60; CA1: $t_{(16)} = 1.27$, $p > 0.05$, power = 0.22), and motility was equally represented across slices analyzed (Fig. 6C,D). Moreover, every slice imaged from METH-trained animals had spines in the BLA that reached the “high-motility” threshold of $\geq 0.03 \mu\text{m}/\text{min}$ (Fig. 6C), identified in the age-dependent motility experiment in Figure 1. As a reminder, the BLA and CA1 of young (P16–P21), but not adolescent (P28–P35) animals displayed spine motility rates $> 0.03 \mu\text{m}/\text{min}$ (Fig. 1D–F). High-motility spines were not observed in the BLA from the saline condition (Fig. 6C) or in CA1 following saline or METH training (Fig. 6D). Further analysis of spine motility with a KS test of the cumulative distribution of spines revealed that METH training shifted all spine movements in the BLA ($p < 0.0001$), but not in CA1 ($p = 0.06$), toward higher motility and precipitated the appearance of highly motile spines (Fig. 5D,E). Even with highly motile spines identified and removed using ROC to establish a cutoff criteria, METH training still significantly increased BLA spine movements (BLA: $p < 0.0001$, power = 1.0; CA1: $p > 0.05$, power = 0.82; Fig. 5F,G). Thus, the effect of METH on BLA spine dynamics cannot be attributed solely to a subset of highly dynamic spines. However, given the sparse distribution of memories, it is plausible that METH-associated memory is preferentially targeted to highly motile spines.

To examine motility in greater depth, cluster analyses were performed (combined overall: $\chi^2_{(6)} = 33.29$, $p < 0.0001$; BLA overall: $\chi^2_{(2)} = 15.07$, $p < 0.001$; CA1 overall: $\chi^2_{(2)} = 6.58$, $p < 0.05$; Fig. 7A), as in Figure 2. χ^2 analysis showed that METH decreased the number of spines in the low-motility group (cluster 1: $\chi^2_{(1)} = 9.25$, $p < 0.01$) and increased the number of spines in the high-motility group (cluster 3: $\chi^2_{(1)} = 10.59$, $p < 0.001$), relative to spines from saline-treated mice (cluster 2: $\chi^2_{(1)} = 3.33$,

$p > 0.05$; Fig. 7B). In contrast, the high-motility cluster 3 I CA1 was unchanged by METH training (cluster 1: $\chi^2_{(1)} = 5.59$, $p < 0.05$; cluster 2: $\chi^2_{(1)} = 4.11$, $p < 0.05$; cluster 3: $\chi^2_{(1)} = 2.20$, $p > 0.05$; Fig. 7C). Interestingly, BLA spine movement appeared to be restricted to the spine head, as very little neck motility was observed in spines, regardless of treatment during training, and it did not correlate with the overall motility rate of the spine ($F_{(3,116)} = 20.61$, $p < 0.0001$; neck saline vs METH, $p > 0.05$; overall length saline vs METH, $p < 0.01$; Fig. 8). Collectively, these data indicate that METH-associated learning produces a selective and lasting increase in basal spine actin dynamics (i.e., no acute stimulation at the time of imaging) of P28–P35 BLA spine heads that are normally quite stable.

Persistent METH-induced BLA spine motility is NMII dependent

To directly test the hypothesis that METH-induced BLA spine dynamics are sustained by NMII activity, we next measured the effect of Blebb on BLA and CA1 spine motility. Animals underwent saline or METH-associated CPP training, as in Figure 4. One to three days later, and 24 h before imaging, mice were treated with either Blebb or vehicle intraperitoneally, resulting in four groups (Saline/Veh, Saline/Blebb, METH/Veh, METH/Blebb; Fig. 9A). Comparing the spine motility of saline and METH-trained animals given vehicle 1–3 d post-training replicated the results reported in Figure 4. Specifically, METH conditioning increased the overall motility of spines in the BLA, but not in CA1 (BLA: $F_{(3,25)} = 6.69$, $p < 0.01$; METH/Veh vs Saline/Veh, Saline/Blebb, METH/Blebb: $p < 0.001$, power = 0.95; CA1: $F_{(3,24)} = 0.95$, $p > 0.05$, power = 0.23; Fig. 9B,C). The cumulative distribution of spines followed a similar pattern, with a large shift in BLA motility and increased representation of highly motile spines (Fig. 9D, top right; Saline/Veh vs METH/Veh, $p < 0.0001$). CA1 spines showed a small, but significant, shift in the distribution of motility, but no appearance of highly motile spines (Fig. 9E, top right; Saline/Veh vs METH/Veh, $p < 0.05$). Again, all effects were broadly consistent across slices (Fig. 10). Using a protocol that disrupts METH-associated memory (intraperitoneal Blebb 1–3 d post-training; Young et al., 2017), we observed a reversal of METH-associated increases in BLA spine movements (Figs. 9B,D, bottom left, 10; METH/Veh vs METH/Blebb, $p < 0.0001$). As a result, the distribution of spine motility for the METH/Blebb group was identical to BLA spines from the Saline/Blebb group (Fig. 9D, bottom right; Saline/Blebb vs METH/Blebb, $p > 0.05$). Blebb had no effect on the motility of BLA or CA1 spines from saline-trained animals (Fig. 9B,C, and D,E, top left; BLA: Saline/Veh vs Saline/Blebb, $p > 0.05$; CA1: Saline/Veh vs Saline/Blebb, $p > 0.05$). While Blebb had no effect on overall spine motility in CA1 (Fig. 9C), it did reverse the small rightward shift present in METH-trained, vehicle-treated mice (Fig. 9E,

Figure 5. METH conditioning produces a persistent increase in spine motility in BLA, but not in CA1. **A**, Schematic of the experimental design. **B**, **C**, Spine movements in the BLA (**B**) and CA1 (**C**) were measured over 1 h, 1–3 d after saline or METH training. Small circles represent the movement of each spine, while the larger circles with heavier borders are the average of 30 spines per slice (BLA: saline, $n = 240$ spines, 8 slices, 8 animals; METH, $n = 240$ spines, 8 slices, 7 animals; CA1: saline, $n = 270$ spines, 9 slices, 7 animals; METH, $n = 240$ spines, 8 slices, 6 animals). **D**, **E**, Cumulative distributions of spine movements in the BLA (**D**) and CA1 (**E**) following training. **F**, ROC curves were used to determine cutoff for highly motile spines (those in the gray boxes). The cutoff was set as the maximum motility displayed by any spine from the saline-treated condition ($0.030 \mu\text{m}/\text{min}$). **G**, Cumulative distributions of spine movements with highly motile spines removed. Error bars represent SEM. * $p < 0.05$, **** $p < 0.0001$.

Figure 6. BLA and CA1 spine movements following CPP training. *A, B*, Representative movements of individual (*A*) BLA and (*B*) CA1 spines with different levels of motility over 1 h. *C, D*, BLA (*C*) and CA1 (*D*) spine movements by slice and animal. Lighter colored circles denote females, and darker colored circles denote males.

Figure 7. Cluster analysis of spine motility following CPP training. **A**, All BLA and CA1 spines underwent cluster analysis. **B**, **C**, Number of spines in each cluster for BLA (**B**) and CA1 (**C**) spines. Error bars represent SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, χ^2 analyses.

bottom left; METH/Veh vs METH/Blebb, $p > 0.05$). This is further supported by cluster analysis (Fig. 11), which revealed that METH training influenced all three clusters in the BLA, including the development of highly motile cluster 3 and that this population of spine motility was almost entirely lost with Blebb (Figs. 9F, 11; BLA overall: $\chi^2_{(6)} = 63.68$, $p < 0.0001$; Saline/Veh vs Saline/Blebb overall: $\chi^2_{(2)} = 0.01$, $p > 0.05$; Saline/Veh vs METH/Veh overall: $\chi^2_{(2)} = 34.63$, $p < 0.0001$; cluster 1: $\chi^2_{(1)} = 26.60$, $p < 0.0001$; cluster 2: $\chi^2_{(1)} = 13.17$, $p < 0.001$; cluster 3: $\chi^2_{(1)} = 16.63$, $p < 0.0001$; METH/Veh vs METH/Blebb overall: $\chi^2_{(2)} = 23.25$, $p < 0.0001$; cluster 1: $\chi^2_{(1)} = 17.89$, $p < 0.0001$; cluster 2: $\chi^2_{(1)} = 8.51$, $p < 0.01$; cluster 3: $\chi^2_{(1)} = 11.23$, $p < 0.001$; Saline/Blebb vs METH/Blebb: $\chi^2_{(2)} = 3.32$, $p > 0.05$). The most notable difference between the BLA and CA1 following METH training was the complete absence of highly motile spines in CA1 (Figs. 7B,C, 9F,G; CA1 overall: $\chi^2_{(6)} = 7.24$, $p = 0.06$; Saline/Veh vs Saline/Blebb: $\chi^2_{(2)} = 0.05$, $p > 0.05$; Saline/Veh vs METH/Veh: $\chi^2_{(2)} = 5.34$, $p < 0.05$; METH/Veh vs METH/Blebb: $\chi^2_{(2)} = 3.86$, $p < 0.05$; Saline/Blebb vs METH/Blebb: $\chi^2_{(2)} = 0.01$, $p > 0.05$).

We previously shown that systemic intraperitoneal NMII inhibition disrupts METH-associated memory in adult animals (Young et al., 2014, 2016). This disruption is mediated through a selective effect within the BLA, as direct infusions into the BLA, but not CA1, the nucleus accumbens, or the orbital frontal cortex, recapitulate the ability of systemically administered Blebb to disrupt METH-associated memory in adult animals (Young et al., 2014, 2016; Briggs et al., 2017). Similar to adults, we have found that METH-associated memory in adolescent animals, which this study used for technical reasons, is similarly disrupted by systemic NMII inhibition (Young et al., 2017). The spine-imaging data presented support the prediction that intra-CA1 Blebb infusions in adolescents should have no effect on the memory, similar to adults. Indeed, Blebb infused into CA1 of adolescent mice 30 min before testing (Fig. 12A,B, Test 1) had no effect

Figure 8. Comparison of neck motility following training. Neck motility was measured in a subset of high- and medium-motility spines from saline- and METH-treated animals. Error bars represent SEM. ** $p < 0.01$.

on the METH-associated memory then or 24 h later (Fig. 12, Test 2; Veh overall: $F_{(2,27)} = 17.18$, $p < 0.0001$; PreTest vs test 1, $p < 0.001$; PreTest vs test 2, $p < 0.0001$; Blebb overall: $F_{(2,24)} = 4.86$, $p < 0.05$; PreTest vs test 1, $p < 0.05$; PreTest vs test 2, $p < 0.05$). This provides indirect support for the particular importance of the highly motile spines, which resemble early postnatal spine movements and are present in the BLA but not CA1 after METH training, to the storage of METH-associated memory and its unique vulnerability to NMII inhibition.

Discussion

We have previously reported that direct actin depolymerization or NMII inhibition within the BLA results in an immediate, long-lasting and retrieval-independent disruption of METH-associated memory and reversal of METH-induced spine density increases to basal levels (Young et al., 2014, 2016). Interestingly, NMII inhibition within CA1 does not produce the same retrieval-independent disruption of METH-associated memory, nor does it alter METH-induced increases in spine density. Here, we used time-lapse two-photon imaging of spine motility to begin to address the hypothesis that the selective susceptibility of METH-associated memory and spines to NMII inhibition is due to NMII-dependent actin dynamics in BLA spines that uniquely persist well past the acute phase of METH-associated learning.

Because spine motility had not been assessed in the BLA before, we first confirmed that motility rates decrease with age, as has been reported in cultured hippocampal cells and slices, acute dentate gyrus and CA1 slices, and primary visual cortex *in vivo* (Fischer et al., 1998; Dunaevsky et al., 1999; Lendvai et al., 2000; Parnass et al., 2000; Portera-Cailliau et al., 2003; Clement et al., 2012; Lordkipanidze et al., 2013). This also allowed us to compare and deeply characterize spine motility in the BLA and CA1 in early postnatal life and later, in adolescence. BLA spines, which are longer than CA1 spines, were more motile than CA1 at P16–P21. This is not overly surprising, as the amygdala undergoes intense innervation between P18 and P20 (Bouwmeester et al., 2002; Brummelte and Teuchert-Noodt, 2006), a process that occurs earlier in the hippocampus (P14–P16; Morris, 1985). Therefore, it is possible that more similar spine movements would be observed if P16–P21 BLA spines were compared with P14–P16 CA1 spines. At P28–P35, as developmental synaptic refinement is ending, spine motility dramatically decreased in both BLA and CA1. Interestingly, when directly comparing motility rates between the BLA and CA1 at P28–P35, BLA spines were more stable, making the subsequent impact of METH training on BLA motility even more striking. We also confirmed that BLA spine motility is actin dependent, as has been shown in cultured hippocampal cells and slices (Fischer et al., 1998; Dunaevsky et al., 1999; Parnass et al., 2000; Lordkipanidze et al., 2013). This established that spine motility is an appropriate measure to determine

Figure 9. Persistent METH-induced BLA spine motility is NMII dependent. **A**, Schematic of the experimental design. **B**, Spine movements and representative images of P28–P35 BLA slices 24 h after treatment (saline/Veh: $n = 210$ spines, 7 slices, 6 animals; saline/Blebb: $n = 210$ spines, 7 slices, 7 animals; METH/Veh: $n = 210$ spines, 7 slices, 7 animals; METH/Blebb: $n = 240$ spines, 8 slices, 7 animals). **C**, Representative images and CA1 spine movements (saline/Veh: $n = 210$ spine, 7 slices, 6 animals; saline/Blebb: $n = 210$ spines, 7 slices, 5 animals; METH/Veh: $n = 210$ spines, 7 slices, 7 animals; METH/Blebb: $n = 210$ spines, 7 slices, 6 animals). **D**, **E**, Cumulative distribution plots comparing spine motility in the (**D**) BLA and (**E**) CA1 of different treatment groups. **F**, **G**, Summary of cluster analysis of spine movements in the (**F**) BLA and (**G**) CA1. Error bars represent SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

the impact of METH and NMII on spine actin dynamics and that doing so in P28–P35 slices would assess changes to spine motility after the age-dependent decrease in natural motility has occurred. This allowed us to separate METH effects on spine motility from developmental motility thought to be critical to the establishment and refinement of brain circuits.

Spines from the BLA and CA1 of saline-treated animals and CA1 of METH-treated animals were stable, displaying very low levels of motility. However, METH-associated learning produced a dramatic increase in BLA spine dynamics that was present when measured 3 d after training. While consistent with our hypothesis, the result was still surprising, as there is nothing in the literature to suggest that a stimulus-induced increase in spine density, like that produced by METH training, would be accompanied by increased spine motility that would be sustained for days after the stimulus. Changes in spine motility reflect an altered dynamic state that is performed by signaling through the actin cytoskele-

ton. It remains unknown whether changes in spine motility observed after METH exposure are mechanistically linked to the spines that are newly formed (Young et al., 2014, 2016).

In addition to a broad increase in BLA spine motility following METH training, the appearance of a subset of highly motile spines was striking (Figs. 5, 9) in its similarity to what is seen during the postnatal developmental period (Fig. 1). This may have implications for the stability of future memories allocated to these motile spines, outside the window of acute drug exposure. In support of this, we have found that memories associated with morphine or nicotine, which are not susceptible to NMII inhibition on their own, become vulnerable when the same animal undergoes METH training, even if the drugs are administered on different days (Briggs et al., 2018). Given the use of amphetamine-based medications for the treatment of attention deficit hyperactivity disorder, this has public health relevance, and we plan to follow up on the impact of such treatment on general

Figure 10. CA1 and BLA spine motility across different slices and animals. One to two slices were used per animal, and the movement of 30 spines per slice was analyzed.

memory stability. In addition, while it is not yet technically possible to do so, we are very interested in selectively ablating these highly motile spines to determine whether this is sufficient to disrupt METH-associated memory, which would be consistent with the sparsely distributed nature of memory.

Deeper analysis revealed that the spine movements were restricted to the head, as there was no change in spine neck motility. Spine neck motility has been connected to functional changes in spines through the regulation of calcium entry and exit (Araya et al., 2006b), suggesting that such dynamic changes are not occurring within BLA spines altered by METH training. This is consistent with the notion that the persistent motility in response to METH training does not reflect a process actively involved in METH-associated memory maintenance. Rather, we favor the interpretation that METH-associated memory persists despite this lack of normal actin–myosin stabilization and that this aberrant plasticity imparts a unique vulnerability of METH-associated memory to disruption long after learning. This is the foundation of our emerging therapeutic strategy to reduce relapse to METH seeking (Young et al., 2015, 2016).

NMII inhibition disrupts METH-associated memory and reverses BLA spine density with equal efficacy to direct actin depolymerization (Young et al., 2014, 2016, 2017; Briggs et al., 2017). If the persistent BLA spine motility changes observed here are connected to the memory disruption, we would predict the motility to be NMII dependent. And indeed, NMII inhibition reverted BLA spine motility to control levels, without influencing CA1 motility. In addition, we replicated the effects of METH training on BLA motility reported in Figure 4. It is important to note that Blebb had no effect on BLA spine motility under control (saline-treated) conditions. It is possible that this reflects a dose-dependent effect; that is, a higher concentration of Blebb would disrupt motility. Higher doses of Blebb are not tolerated by animals, preventing us from directly testing this possibility. However, we are currently developing Blebb analogs with improved safety profiles that can be used to address this in the future. An alternative explanation to the possibility of a dose-dependent

Figure 11. Cluster analysis of CA1 and BLA spine movements following NMII inhibition. **A**, All CA1 and BLA spine movements were organized into three clusters based on cluster analysis. **B**, Histograms plotting the number of spines in each cluster when the groups were graphed individually.

Figure 12. Intra-CA1 NMII inhibition has no immediate effect on an established METH-associated memory in adolescents. **A**, Schematic of experimental design. **B**, CPP data comparing time spent in the METH-paired CS+ compartment before training (PreTest), 2 d after training, and 30 min after Blebb infusion (Test 1) and 3 d after training (no Blebb; Test 2; Veh, $n = 10$; Blebb, $n = 9$). Error bars represent SEM. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

effect is that NMII is specifically involved in dynamic changes to the spine actin cytoskeleton and the remaining dynamics are driven by actin treadmilling that does not require NMII or by microtubule polymerization (Hu et al., 2008; Gu and Zheng, 2009; Jaworski et al., 2009). This would argue for the particular importance of more motile spines. Indeed, ~75% of all BLA spines display low motility at P28–P35 ($<0.015 \mu\text{m}/\text{min}$) following saline training, with or without Blebb, and the remaining 25% display moderate motility ($0.015\text{--}0.03 \mu\text{m}/\text{min}$; Fig. 9F). However, METH training shifts the distribution of BLA spines such that moderately and highly motile spines (clusters 2 and 3; $0.015\text{--}0.05 \mu\text{m}/\text{min}$) account for 50% of the total spines assessed. Remarkably, administering Blebb after METH training, but at least 24 h before slicing and imaging, results in a very precise return of spine motility distribution to control levels, not below (Fig. 9D, bottom right, cumulative distribution plot). The notion that NMII may not contribute to the low-level motility seen under control conditions is consistent with prior evidence from our group that NMII is activated by synaptic stimulation, rather than serving a housekeeping function (Rex et al., 2010; Ozkan et al., 2015). This is precisely what makes the sustained susceptibility of METH spines to Blebb of interest, as it suggests that NMII remains constitutively active days after METH training. The ability of NMII to drive actin polymerization is dependent upon two critical steps. First, the RLC of NMII must be phosphorylated to drive a conformational change that exposes the head of the heavy chain (MHC) of NMII, which contains the ATP binding site (Wendt et al., 2001). ATP provides the energy for the motor head of the MHC to physically slide actin filaments, driving cytoskeletal rearrangement (Vicente-Manzanares et al., 2009). Blebb works by interfering with the ATP binding site (Kovács et al., 2004). However, Blebb can influence NMII function only if the protein is activated via RLC phosphorylation. Thus, the ability of Blebb to disrupt actin-dependent spine motility days after the stimulation events of METH-associated learning suggests the RLC of NMII may need to remain phosphorylated. This could represent a point of divergence between METH-associated memory representation in the BLA versus CA1, as well as METH-associated memory versus memories for other conditioned stimuli, including footshock, food reward, and other drugs of abuse (Young et al., 2014, 2016; Briggs et al., 2017). Future studies will be directed at identifying the upstream signaling cascade regulating METH-induced NMII RLC phosphorylation, focusing on the possibility that METH recruits a unique set of players in the BLA that interferes with the normal postlearning mechanisms responsible for actin–myosin stabilization.

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