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The actin cytoskeleton as a therapeutic target for the prevention of relapse to methamphetamine use

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Abstract

A high rate of relapse is a defining characteristic of substance use disorder for which few treatments are available. Exposure to environmental cues associated with previous drug use can elicit relapse by causing the involuntary retrieval of deeply engrained associative memories that trigger a strong motivation to seek out drugs. Our lab is focused on identifying and disrupting mechanisms that support these powerful consolidated memories, with the goal of developing therapeutics. A particularly promising mechanism is regulation of synaptic dynamics by actin polymerization within dendritic spines. Emerging evidence indicates that memory is supported by structural and functional plasticity dendritic spines, for which actin polymerization is critical, and that prior drug use increases both spine and actin dynamics. Indeed we have found that inhibiting amygdala (AMY) actin polymerization immediately or twenty-four hours prior to testing disrupted methamphetamine (METH)-associated memories, but not food reward or fear memories. Furthermore, METH training increased AMY spine density which was reversed by actin depolymerization treatment. Actin dynamics were also shifted to a more dynamic state by METH training. While promising, actin polymerization inhibitors are not a viable therapeutic, as a multitude of peripheral process (e.g. cardiac function) rely on dynamic actin. For this reason, we have shifted our focus upstream of actin polymerization to nonmuscle myosin II. We and others have demonstrated that myosin IIb imparts a mechanical force that triggers spine actin polymerization in response to synaptic stimulation. Similar to an actin depolymerizing compound, pre-test inhibition of myosin II ATPase activity in the AMY produced a rapid and lasting disruption of drug-seeking behavior. While many questions remain, these findings indicate that myosin II represents a potential therapeutic avenue to target the actin cytoskeleton and disrupt the powerful, extinction-resistant memories capable of triggering relapse.

Keywords

addiction; substance use disorder; memory; treatment

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Memory in SUD Relapse

Substance use disorder (SUD) is a chronic, relapsing disorder with limited treatment options. Although somewhat effective replacement therapies exist for opiate and nicotine dependence, there are no FDA-approved pharmacotherapies for stimulant abuse or relapse. Many of the challenges facing the development of effective SUD pharmacotherapies stem from the complexity of the disorder, which is characterized by diverse, interrelated emotional and physical aspects. One particularly recalcitrant issue associated with SUD is the deeply engrained and highly motivating associations for drug-associated stimuli that develop during drug use. Reminders of these associations can trigger immediate and overwhelming motivation, both conscious and unconscious, to seek the drug. Perhaps most troubling, former substance users are susceptible to these relapse triggers even after seemingly successful rehabilitation and prolonged drug-free periods. Triggers of these memories are numerous and often unconscious or abstract (e.g. craving for cocaine at the sight of a snow-capped mountain). This makes them difficult to predict and treat with current behavioral modification strategies, such as exposure therapy, that rely on active retrieval of each trigger, followed by manipulation of the memory. Retrieval-dependent treatment strategies include attempts to target extinction of the drug-associated memory (i.e. exposure therapy), by accelerating learning of a new, non-reward contingency for the associated stimuli, or reconsolidation, by preventing the return of the associative memory to storage after retrieval [1–5].

Taken together, this suggests that it might be more efficacious to devise a retrieval-independent approach. However, this is not without its own challenges. Retrieval-dependent strategies exist because the process of active recall allows for the selective targeting of the memory of interest, rather than a broad induction of amnesia. In order to overcome this barrier, a mechanism unique to the storage of a drug-associated memory would need to be identified. By turning to the physical site of memory storage in the brain, dendritic spines, we recently stumbled upon such a mechanism [6].

Why Actin: Memory

Dendritic spines are small, but highly dynamic post-synaptic structures on dendrites that receive the majority of the brain's excitatory input (Fig. 1). There is a growing consensus that dendritic spines serve as a structural storage site for memories. They enable input-specific biochemical and electrical isolation of synapses, which facilitates signal integration and information storage at the time of memory formation. Furthermore, the successful formation of a long-term memory is dependent on the ability of spines to undergo volumetric and functional changes [7, 8]. Calcium influx into a spine, following either long-term potentiation (LTP) stimulation or glutamate receptor activation, leads to spine enlargement and synapse stabilization (Fig. 2). This structural and functional plasticity is regulated by polymerization of actin, a cytoskeletal protein found in abundance within spines [9] [10–17]. Actin polymerization is the highly dynamic process by which actin monomers (G-actin) become linked into complex, branched filaments (F-actin) of the cytoskeleton, allowing dendritic spines to change size and shape on the order of seconds following stimulation [18]. Importantly, disruption of F-actin dynamics at the time of

learning prevents the formation of long-term memory. Current studies report that these learning-associated F-actin dynamics are under tight temporal control, such that actin stabilizes just as rapidly as the polymerization was triggered [16, 19–22]. As a result, inhibiting actin polymerization just minutes *after* learning no longer interferes with long-term memory [19].

Why Actin: Drugs of Abuse

SUD is considered by many to be a memory disorder [23, 24]. As discussed above, drug users form long-lasting and highly motivating associative memories for environmental stimuli present at the time of drug use. In humans, non-human primates and rodents, encountering drug-associated stimuli following withdrawal can elicit feelings of intense craving and/or drug seeking, with concomitant activation of a number of brain areas, including the prefrontal cortex (PFC), nucleus accumbens (NAc) and amygdala (AMY) [25–32]. Drug users frequently report that these feelings of craving or motivation for the drug intensify as abstinence periods progress, usually peaking after a month of abstinence [33]. Interestingly, the first 30 days of withdrawal in animal models is marked by dynamic changes in NAc L-DOPA (L-3,4-dihydroxyphenylalanine) and dopamine levels, resulting in a more sensitized state following a month of withdrawal, as compared to one week. Such temporal dopamine effects may support enhanced craving and/or drug seeking with time [34–36]. Furthermore, structural plasticity accompanies long withdrawal periods from cocaine or amphetamine, as evidenced by spine density increases in the PFC and NAc [37]. Morphine exposure, on the other hand, decreases NAc and cortical spine density [38]. Recent evidence indicates that these drug-associated changes in spine density correspond to associative drug conditioning rather than non-associative drug sensitization [39]. Indeed, prior cocaine self-administration training increased spine density and F-actin dynamics in the NAc and spine density in the neocortex [40–44]. While these studies investigated drug-associated synaptic and actin dynamics following a long period of withdrawal, there is evidence that some of these changes also take place after shorter withdrawal periods [6, 45]. Indeed, withdrawal is slowly emerging as a highly dynamic period that promotes drug seeking by intensifying drug-environment associative memories, in part, through actin-mediated structural plasticity.

Why Actin: Bridging the Gap between Memory and SUD

Given the critical role of the NAc and ventral tegmental area (VTA) in reward seeking and dopamine release, it is, perhaps, not surprising that stimulants trigger synaptic structural plasticity in these regions. However, the amygdala (AMY), a structure often associated with anxiety and both positive and negative emotionally arousing memories, also undergoes stimulant-induced structural plasticity [6, 46–49]. Interestingly, these AMY changes appear to rely upon the stimulant being delivered in a novel environment, such that an association forms. AMY-dependent associative memories are major motivators of drug seeking, driving both reward seeking and avoidance of withdrawal symptoms [50–53]. Moreover, expression of aversive withdrawal memories is supported by AMY actin polymerization [54]. Taken together, these findings suggest that drugs of abuse are capable of altering F-actin dynamics throughout the brain.

We recently reported a surprising role for F-actin dynamics in a subregion of the AMY, the basolateral amygdala complex (BLC), in supporting memories associated with the highly addictive stimulant, methamphetamine (METH) [6]. We found that directly depolymerizing BLC actin with a naturally occurring toxin, Latrunculin A (LatA), prior to retrieval produced a long-lasting disruption of METH-associated conditioned place preference (CPP) memories, but not memories for food reward. Unexpectedly, this manipulation was equally effective whether administered minutes before testing, or in the home cage an entire day before testing. This indicates that LatA's effect on the drug-associated memory does not require a retrieval session. Rather, the actin depolymerizer targets the memory in storage. Furthermore, depolymerized actin also blocked reinstatement of context-induced METH self-administration, an animal model of drug seeking with strong face validity. Analysis of spine density revealed an increase in BLC spine density with METH training that was reversed by LatA. LatA's mechanism of action is to sequester actin monomers, preventing their incorporation into F-actin filaments. Thus, the drug is ineffective when actin is stable, as occurs shortly after a typical learning event (e.g. fear and food reward memories). Given the unique ability of LatA to disrupt drug-associated memories *days* after learning in the absence of retrieval, we hypothesized that the actin cytoskeleton fails to stabilize, remaining in an active, cycling state that is susceptible to depolymerization.

To begin to address this, we recently analyzed the ratio of actin monomers (G-actin) to filaments (F-actin) two days after METH CPP training. In other cell types, higher rates of actin cycling are associated with the presence of more actin monomers (44). Consistent with this, we observed a shift towards monomeric (G-) actin in the BLC of METH, but not saline, CPP-trained animals (Fig. 3; METH: $F_{(1,7)} = 11.29$; $P < 0.05$; Saline: $F_{(1,7)} = 1.19$; $P > 0.05$), with no difference in total actin (Saline: 31879.8 ± 2772.3 , METH: 31409.8 ± 2403.8 ; $F_{(1,7)} = 0.02$; $P > 0.05$). This evidence, in conjunction with the ability of actin depolymerization to disrupt drug seeking further suggests that the actin cytoskeleton supporting a METH-associated memory may be in a constitutively active state of cycling, rather than being triggered by the act of memory retrieval.

Is Actin Druggable?

The ability of a single actin depolymerization treatment to produce a long-lasting disruption of METH-associated memories independent of retrieval initially seems like a promising therapeutic target for the selective disruption of relapse-inducing triggers. Indeed, actin is also a seemingly promising target for other indications, such as cancer. However, the actin isoform implicated in adult neuronal structural plasticity, β -actin, is ubiquitously expressed throughout the body and critical to a multitude of processes in the periphery (e.g. cell division, cardiac function) [13, 55–58]. Indeed, the body's fundamental reliance on actin polymerization is why pharmaceutical companies have limited their pursuit of actin-targeting drugs to indications calling for topical treatment, such as glaucoma. Because an actin depolymerizing agent is not a viable therapeutic option, we are actively investigating upstream regulators of the synaptic actin cytoskeleton. Nonmuscle myosin II (NMII), which is highly expressed in neuronal tissue, is one such potential target.

Why Myosin

The 143 member myosin family is made up of seventeen different classes of molecular motors with tissue-specific properties. NMII is the most common class of myosin found in neuronal tissue, with three distinct isoforms of the heavy chain, which bears the actin-binding site and ATPase activity (NMIIA (*Myh9*), NMIIB (*Myh10*) and NMIIC (*Myh14*)) [59, 60]. It is well established that NMII regulates F-actin dynamics in growth cones of developing neurons by driving retrograde actin flow [16, 61]. To do this, NMII's ATPase motor protein binds to actin filaments, causing them to contract and sever. Subsequently, this NMII-driven action replenishes the pool of G-actin monomers, thereby facilitating actin polymerization in growth cones [61–63]. Recently, Rex et al. (2010) reported that the NMIIB ATPase motor is also a critical regulator of structural plasticity following LTP induction and NMDA receptor stimulation, through direct action on dendritic spine actin [16]. Similar to the results of these NMIIB genetic manipulations, pharmacologic inhibition via Blebbistatin (Blebb), disrupts LTP stability [16, 64]. Rex et al. (2010) went on to show that myosin II activity and NMIIB, in particular, is required for hippocampus-dependent memory formation [16]. In a follow up study, Gavin et al. (2011) established that NMIIB is also required for amygdala-dependent memory formation [20].

Taken together, these studies establish that myosin II activity reorganizes the actin cytoskeleton to support the stable expression of LTP, as well as the formation of memories reliant upon the hippocampus and amygdala. In light of this, we have begun to test the hypothesis that NMIIB is uniquely required for the stabilization of drug-associated memories, such that it can be targeted in lieu of direct depolymerization of actin. Indeed, we recently reported that administration of Blebb directly into the BLC prior to testing disrupted METH CPP [6].

Unfortunately, the only available NMII inhibitor is Blebb and it has a number of properties in need of improvement [65]. For instance, not only does Blebb lack NMII isoform selectivity, but it also hits skeletal, smooth and, perhaps, cardiac muscle myosin IIs. However, *in silico* assessments indicate that Blebb should have high brain penetrance and it has been shown to be tolerated by rodents when delivered intravenously [66]. Given that Blebb was found through a relatively small-scale screen, the potential for improving Blebb through derivitization and/or identifying novel NMII inhibitors remains high [65].

Future Questions

While NMII represents a potential target for safely targeting central nervous system (CNS) actin polymerization, a multitude of questions remain. At the forefront are questions related to what regulatory mechanisms could be responsible for the constitutively cycling actin that is unique to the storage of METH-associated memories. While memories of fear, food reward and METH are all known to be long-lasting, evidence suggests that they are supported by different states of F-actin, cycling versus stable. We and others have previously demonstrated that fear learning triggers a brief window of F-actin dynamics [16, 20]. This limits the efficacy of actin depolymerizing agents to a brief window surrounding learning. One possible contributor to this unique feature of cycling F-actin associated with

METH memories is withdrawal-induced release of neuromodulators, such as dopamine or brain-derived neurotrophic factor (BDNF), into the AMY. Both are capable of regulating functional and structural plasticity [67–69] and importantly, they are both released into the BLC during periods of cocaine withdrawal [70, 71]. Therefore, it is possible that the actin cytoskeleton supporting METH-associated memory initially stabilizes, but is later reactivated upon forced abstinence from METH at the completion of training.

As stated previously, drugs of abuse activate brain regions involved in memory processes, including the AMY, hippocampus, NAc, PFC and the dorsal striatum. This naturally leads to the question of actin dynamics in other regions of the brain. Is actively cycling actin unique to the AMY, or is it occurring in other memory centers of the brain? Further, are memories associated with other drug classes similarly susceptible to actin depolymerization? It is known that withdrawal from cocaine and development of aversive associations with morphine withdrawal both involve actin polymerization [44, 54, 72]. However, very little is known about F-actin dynamics associated with drug-related memories in these regions. There is evidence, as detailed above, that many drugs of abuse are capable of altering spine density. For instance, studies performed by Robinson and Kolb show increases in spine density in the NAc and PFC following cocaine and amphetamine injections, while spine density decreases after morphine treatment [37, 38]. Indeed, the persistent, neurobehavioral effects of repeated exposure to these drugs may stem from their ability to reorganize the actin cytoskeleton of dendritic spines. Although it is not yet known if actin has a prolonged period of cycling in these regions after exposure to drugs of abuse, as in the AMY, one can speculate that memories associated with any drugs capable of altering actin dynamics may be susceptible to selective, retrieval-independent disruption.

In seeking a potential SUD therapeutic, one must consider that users seeking treatment will have different drug consumption histories. This raises the important question of how drug history, such as amount of drug use, period of use and polydrug use, might affect the efficacy of targeting the actin cytoskeleton. One way to address this is through what is known as extended access self-administration, in which animals are given six or more hours of daily access to self-administer the drug of choice, rather than the standard two hours [73, 74]. Ferrario et al (2005) demonstrated that following both limited and extended access to cocaine, there was an increase in dendritic spine density in NAc and the medial PFC, with a further enhancement in spine density in the NAc with extended access [75]. This data suggests that an increase in drug intake, as seen with the extended access model, may have a similar, but perhaps even more substantial, impact on synaptic reorganization as the limited access protocol. We have previously shown a correlation between spine density and the strength of a METH-associated memory, but that memory strength does not affect the efficiency with which actin depolymerization disrupts METH-associated memories [6]. Thus, we have reason to believe that memories formed through extended access to METH will be similarly susceptible to actin depolymerization. Clearly, more research in this area is still needed.

In designing any therapeutic, safety is a primary concern. In the case of targeting drug-associated memories to mitigate the risk of SUD relapse, we have directed our search for a pharmacotherapy upstream of actin. Our initial results indicate that NMII inhibition in the

BLC produces similar results to that of direct actin depolymerization with LatA. Indeed, myosin II represents an attractive therapeutic target because its distribution is more tissue-specific, with the NMII isoforms being abundantly expressed within the CNS, including the AMY. Further, systemic inhibition with Blebb is tolerated by rodents [66]. An additional benefit of our discovery, from a safety perspective, is that it appears a single manipulation of the actin cytoskeleton is efficacious. This precludes the need for chronic treatment, mitigating the associated safety and off-target concerns.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague-Dawley rats (300–350g) were obtained from Charles River Laboratories and adult male C57BL/6. All animals were housed under a 12:12 light/dark cycle, with food and water ad libitum. 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 for 3–5 days prior to the start of behavioral conditioning.

Behavioral Procedures

Conditioned Place Preference—Animals were trained using an unbiased conditioned place preference (CPP) procedure. The box consisted of three chambers (Med Associates) – two of equal size and one smaller, center chamber. The chambers differed in terms of their visual, tactile and olfactory cues.

The CPP protocol consisted of three phases: Pretesting, training and testing. During the pretest, rats were given free access to the apparatus for 15 min and the time spent in each chamber was recorded. During training, Methamphetamine hydrochloride (Sigma-Aldrich) was delivered IP (1mg/kg at 1ml/kg) to rats prior to placement in the CS+ chamber for 30 min (counterbalanced between chambers within groups). On alternating sessions, rats were injected with an equivalent volume of 0.9% saline and confined to the opposite chamber for 30 min. Animals received five pairings with METH and saline during twice daily training sessions (am and pm), such that rats received METH (1mg/kg) and saline each day. Time of day for METH treatment was counterbalanced within groups. Tissue was collected 48 hrs after the last day of training.

G/F Actin Assay

G- and F-actin were measured using an *in vivo* assay kit for actin polymerization (Cytoskeleton). Briefly, fresh BLC tissue punches were rapidly obtained and immediately placed in warmed (37°C) LAS2 buffer for homogenization. The homogenized tissue lysate was kept at 37°C during the entire fractionation process to preserve the state of F-actin. Following a 10 min incubation for cell lysis, the lysates were centrifuged at 2,000 rpm for 5 min. The supernatant was then transferred into centrifuge tubes and spun in a Beckman Coulter Ultracentrifuge with a SW-55 rotor at 55,000 rpm for 1hr. Immediately following fractionation, the tubes were placed on ice. The pellets (F-actin containing fraction) were resuspended to the same volume as the supernatant (G-actin containing fraction) in ice-cold

Milli-Q water containing 10 μ M Cytochalasin D and left on ice for 1hr to dissociate the actin pellet. The fractions were stored at –20 for Western blot analysis.

Equal volumes of each fraction, as well as total actin, were mixed with SDS-PAGE buffer, heated for 5 mins at 95°C and then loaded onto a 12% SDS-PAGE gel and transferred to a PVDF membrane. The blots were blocked for 1hr at room temperature in 5% non-fat dry milk-TBST (Tris-buffered saline with 0.1% Tween20) and exposed to anti-actin primary antibody (Cytoskeleton, 1:1000) overnight at 4°C. After washes, blots were incubated in secondary antibody (Promega, anti-rabbit IgG HRP-conjugated, 1:5000) for 1hr at room temperature. Protein expression was assessed by chemiluminescence and exposure to Biomax Light film (Eastman Kodak Co). Image J software (NIH) was used to quantify band intensities. Relative protein expression was calculated by normalizing the integrated band density values for G- and F-actin fractions to the values determined for total actin.

Statistical Analysis

One-way analysis of variance and repeated measures ANOVAs were used to analyze all behavioral data, with post hoc tests performed when appropriate.

ABBREVIATIONS

AMY	Amygdala
BDNF	Brain-derived neurotrophic factor
BLC	Basolateral amygdala complex
Blebb	Blebbistatin
CPP	Conditioned place preference
CNS	Central nervous system
L-DOPA	L-3,4-dihydroxyphenylalanine
LatA	Latrunculin A
LTP	Long-term potentiation
METH	Methamphetamine
NAc	Nucleus accumbens
PFC	Prefrontal cortex
SUD	Substance use disorder
VTA	Ventral tegmental area

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Figure 1.
Thy1-GFP(m) expression in dendrites and spine processes of the basolateral amygdala complex imaged at 4× and 40×.

Figure 2.

Memory formation is dependent on structural and functional synaptic plasticity. Following learning or LTP induction, NMDA receptor activation initiates actin polymerization, or elongation, which drives dendritic spine enlargement and stabilization.

Figure 3.

METH-associated memories are associated with a dynamic actin cytoskeleton during the maintenance phase. Animals underwent Saline or METH CPP training then the ratio of monomeric (G-) and filamentous (F-) actin in the BLC was assessed. METH CPP potentiated BLC G-actin compared to F-actin.