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Targeting the cytoskeleton as a therapeutic approach to substance use disorders

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Abstract

Substance use disorders (SUD) are chronic relapsing disorders governed by continually shifting cycles of positive drug reward experiences and drug withdrawal-induced negative experiences. A large body of research points to plasticity within systems regulating emotional, motivational, and cognitive processes as drivers of continued compulsive pursuit and consumption of substances despite negative consequences. This plasticity is observed at all levels of analysis from molecules to networks, providing multiple avenues for intervention in SUD. The cytoskeleton and its regulatory proteins within neurons and glia are fundamental to the structural and functional integrity of brain processes and are potentially the major drivers of the morphological and behavioral plasticity associated with substance use. In this review, we discuss preclinical studies that provide support for targeting the brain cytoskeleton as a therapeutic approach to SUD. We focus on the interplay between actin cytoskeleton dynamics and exposure to cocaine, methamphetamine, alcohol, opioids, and nicotine and highlight preclinical studies pointing to a wide range of potential therapeutic targets, such as nonmuscle myosin II, Rac1, cofilin, prosapip 1, and drebrin. These studies broaden our understanding of substance-induced plasticity driving behaviors associated with SUD and provide new research directions for the development of SUD therapeutics.

Keywords

Actin; Cytoskeleton; Addiction; Structural plasticity; Medication; Myosin

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1. Introduction

Substance use disorder (SUD) is characterized by an inability to control use of substances like legal or illegal drugs, alcohol, or medications, subserved by genetic, developmental, and social vulnerabilities. In the severe form of the disorder, individuals compulsively seek and use these substances despite negative consequences. The persistence of drug seeking and taking in the face of harm is due, in part, to substance-induced changes to brain systems regulating cognition, motivation, and emotion [1–4]. Thus, many therapeutic developments for this disorder are focused on the goal of restoring these processes by targeting structural and functional plasticity mechanisms at the level of cellular morphology, electrophysiology, neurotransmission and neuromodulation, and circuit dynamics [5]. These efforts have primarily focused on neurotransmitters and neuropeptides, ion channels, receptors, and transporters as means to modulate neural communication. Recently, there has been a growing interest in correcting drug-induced brain plasticity changes by targeting dynamics of the cytoskeleton, the complex of proteins that provide physical structure to cellular compartments such as the post-synapse. This is an intuitively logical approach because cytoskeletal dynamics are central to the structural integrity of a cell, as well as to the mechanical function of critical intra- and intercellular processes, such as cargo transport and neurotransmitter release. Furthermore, cytoskeletal dynamics in dendritic spines and axons as well as the glia are proposed to be critical to information processing, storage, and relay across brain regions [6–8]. Thus, the cytoskeleton presents a viable target to restore maladaptive brain functions in SUD.

As in other eukaryotic cells, the cytoskeleton of neurons and glia is comprised of microtubules, intermediate filaments, and microfilaments. Microtubules are heterodimers of highly conserved α and β tubulins aligned to form protofilaments that form a tubular structure. The dimers function as GTPases to assemble and disassemble the microtubules, and the microtubule associated proteins (MAPs) such as tau, kinesin and dynein regulate the structural and functional properties. Neuronal and glial microtubules primarily support intracellular transport of cargo, such as organelles and vesicles, and provide scaffolding for extension and maintenance of neurite and glial processes. However, evidence also points to their role in neuronal excitability, synaptic plasticity, and cognitive processes (reviewed in [9] and [10]). Neurofilaments are widely spread bundles of ropelike intermediate filaments in neurons made primarily of the three subunit proteins known as NF triplet. They provide mechanical strength and a stable structural framework to neurons. They also regulate vesicle transport, somatic and axonal volume for proper signal conduction, and synaptic plasticity (reviewed in [11]). The analogous structure in glia is made up of the glial fibrillary acidic protein (Gfap) and supports similar functions in the astrocytes. The actin cytoskeleton is comprised of actin monomers arranged as two intertwined strings. Actin interacts with a vast array of proteins, referred to as actin binding proteins (ABPs). These include myosin motors like nonmuscle myosin II (NmII), cross-linkers like fimbrin and fascin, bundling proteins like villin, anchoring proteins like twinfilin1, sequestration proteins like profilin, and small GTPase regulatory proteins like Rho family GTPases. Actin is the primary component of the membrane cytoskeleton and is thus involved in maintaining the distribution of plasma membrane proteins, establishing cell morphology, and interacting with extracellular

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matrix components and neighboring cells. Finally, actin is a critical component of structural plasticity at the synapse supporting cognitive processes (reviewed in [12,13], and [14]).

In this review, we discuss preclinical studies supporting the cytoskeleton in neurons and glia as a general target for the treatment of SUD. We highlight studies that show direct impacts of drugs of abuse on cytoskeletal dynamics and structural plasticity. This is followed by studies targeting the cytoskeleton and its regulators with small molecules and genetic manipulations to curb or reverse drug-seeking and related behaviors. This includes medications development work from our own group aimed at developing a first-in-class small molecule inhibitor of NMII, an upstream regulator of actin, to produce a long-lasting disruption of the motivation for drugs of abuse. Because most studies focus on the actin cytoskeleton and associated proteins in neurons, we dedicate a significant portion of this review to these studies (Section 2). Studies on microtubules and neurofilaments and those on glia are separately discussed in Section 3. A few studies that highlight cytoskeleton effects in polysubstance use context are discussed in Box 1. Finally, a limited number of studies investigating the cytoskeleton in the brain vasculature are discussed in Box 2.

2. A two-way interaction between substances of abuse and neuronal actin dynamics

A large body of evidence suggests that drugs of abuse influence neuronal function and behavior by altering synaptic architecture and neuronal communication through cytoskeleton driven changes in dendritic spine density and morphology (reviewed in [15] and [16]). A great deal of parallel work has focused on the interplay between drugs of abuse and gene expression changes, including the role of transcriptional regulators and epigenetic modifications [17–19]. However, a limited number of studies to date have directly linked drug-induced transcription factors and effector proteins to the changes in cytoskeleton dynamics underlying synaptic plasticity (reviewed in [20] and [21]). Several drugs of abuse are known to trigger transcriptional regulators like ΔFosB, Creb, Mef2, and Nfκ B, which induce either transient or enduring structural plasticity in brain regions associated with reward and motivation, such as the nucleus accumbens (NAc), ventral tegmental area (VTA), prefrontal cortex (PFC), hippocampus (HPC) and amygdala (AMY) [22]. For instance, converging evidence from several studies indicates that psychostimulant-induced ΔFosB increases spine density in the dopamine receptor type 1 expressing medium spiny neurons (Drd1-MSNs) of the striatum through selective modulation of cytoskeletal associated proteins like cofilin, actin-related protein 4 (Arp4), and activity-regulated cytoskeletal protein (Arc) [23–26]. Below we highlight studies that reveal these drug-induced influences on the cytoskeleton for some common drugs of abuse. Notably, the nature of these influences are substance, brain region, and cell-type specific. Based on these findings several studies have investigated whether drug-induced changes in the cytoskeleton and its regulators in turn influence drug-related behaviors such as drug sensitivity, seeking, intake and withdrawal. We discuss these studies and others that have utilized pharmacological or genetic tools to manipulate the cytoskeleton dynamics as a means to potentially curb or reverse behaviors associated with SUD.

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2.1. Actin dynamics associated with cocaine

A study in rats by Toda and colleagues provided some of the earliest evidence for modulation of actin cytoskeleton dynamics by psychostimulants [27]. They reported that acute exposure to cocaine resulted in elevated filamentous (F-) actin in the NAc, but not in the PFC or dorsal striatum, less than an hour after drug administration. This effect was transient, as F-actin levels normalized within two hours. The pattern was similar following three weeks of withdrawal from a seven-day chronic cocaine exposure protocol, with F-actin still elevated in the NAc and unchanged in the striatum, but reduced in the PFC. Thus, chronic cocaine resulted in an enduring upregulation of F-actin, which lasted at least three weeks from the last exposure. Interestingly, acute cocaine challenge after three weeks of withdrawal from chronic cocaine did not affect actin remodeling beyond what was observed without the challenge. Concurrent with F-actin changes, ABPs that promote actin polymerization or depolymerization were also altered in the NAc, albeit differentially in acute versus chronic cocaine. Acute cocaine resulted in elevation of phosphorylated cofilin (p-cofilin) and adducin, which reduce actin depolymerization by reducing actin severing and capping of actin barbed ends, respectively. Withdrawal from chronic cocaine, on the other hand, was associated with an overall promotion of actin polymerization through elevated Mena, p-Vasp and p-cortactin (p-Cttn), despite reduced p-cofilin. These findings demonstrate the differential involvement of ABPs in regulating variable plasticity associated with acute versus chronic cocaine exposure.

To understand the effects of chronic cocaine-induced cytoskeleton plasticity on future drug-related behavior, the authors suppressed actin polymerization in the NAc core using infusions of a Lim kinase (Limk) inhibitor, which inhibits cofilin phosphorylation, or Latrunculin A (LatA), which depolymerizes cycling actin by sequestering actin monomers. The animals first underwent repeated cocaine exposure, followed by three weeks of forced abstinence. Infusions were then delivered shortly before a cocaine challenge and subsequent reinstatement testing. These manipulations, which disrupt actin cycling, enhanced reinstatement lever pressing, without altering cocaine-induced locomotor activity. Furthermore, both the Limk inhibitor and LatA also disrupted the cocaine-induced increase in actin cycling and co-sedimentation of F-actin with filopodia-associated ABPs. These results suggest that the elevated actin cycling associated with chronic cocaine withdrawal allows for controlled reward seeking in a non-reinforced reinstatement test. In other words, such plasticity is essential for adaptive reward pursuit and may be disrupted in compulsive reward seeking observed in cocaine use disorder. In a related study, the group determined that acute cocaine injection after withdrawal from repeated cocaine increased spine head diameter in the NAc core and sensitized the locomotor response; both changes were blocked by intra-NAc LatA administration [28]. The contrasting effects of LatA treatment on locomotor sensitization in these two studies may be due to the use of DMSO versus aCSF-based vehicles. The DMSO vehicle used in the 2006 study appeared to block the expression of a sensitized locomotor response in the control group, which, in turn, may have masked the LatA effects. Notably, DMSO effects may be related to alterations in microtubule dynamics as this relationship has been previously identified [29–31]. Regardless, together these studies point to Limk/cofilin as important regulators of cocaine-induced actin dynamics and subsequent cocaine-related behaviors.

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In a follow-up study from the same laboratory, Shen and colleagues sought to examine whether chronic cocaine-induced actin remodeling and synaptic plasticity influence the effects of subsequent cocaine challenge [32]. Rats withdrawn for three weeks after seven days of cocaine exposure had larger spine head diameter and elevated F-actin in the MSNs of the NAc core, consistent with the results from 2006 study. On the other hand, acute cocaine challenge in animals with the same history of chronic cocaine and withdrawal led to increased spine density and biphasically changed spine head diameter over the first two hours (increase in larger spine heads followed by increase in thinner spine heads). A concurrent increase in different ABPs (Arp3 at 45 min and cofilin at 120 min) indicated that these proteins may subserve the morphological observations. Interestingly, acute cocaine challenge in animals without any prior history of cocaine led to elevated spine density only six hours after cocaine administration. This morphological plasticity was underscored by a corresponding increase in levels of F-actin and decrease in levels of Arp3 and p-cofilin. Together, these findings suggest that a history of chronic cocaine exposure and forced withdrawal results in neuronal plasticity that augments the sensitivity of the actin cytoskeleton to subsequent cocaine exposure. To understand the functional implication of these molecular changes, NAc postsynaptic density (Psd) proteins and NMDA receptors were examined in the same study. The authors found that acute cocaine in animals without cocaine history elevated the levels of NMDA scaffolding protein Psd-95 and the NMDA receptor subunits, NMDAR1 and NMDAR2a. However, acute cocaine in animals *with* a history of cocaine exposure and withdrawal did not affect these proteins. Finally, to understand impacts on neurotransmission, local field potential (LFP) was measured in NAc MSNs in response to PFC stimulation. Regardless of prior cocaine history (with or without), acute cocaine reduced LFP amplitude during the first two hours. However, LFP amplitude returned to and exceeded baseline levels by four hours in the group without cocaine history, while it failed to return to baseline in the group with a history of cocaine. These findings demonstrate long-term potentiation (LTP)-like functional plasticity with acute cocaine and long-term depression (LTD)-like plasticity in animals with a history of cocaine and withdrawal. Taken together, these results point to a potential mechanism by which chronic cocaine may influence the motivation during withdrawal to continue cocaine seeking and use.

Evidence suggests that the effects of cocaine on neuronal plasticity depend on the specific cell type. For instance, Kim and colleagues reported that following repeated cocaine treatment, D1R-MSNs in NAc exhibit decreased membrane excitability and frequency of miniature inhibitory postsynaptic currents (mIPSCs), but increased frequency of miniature excitatory postsynaptic currents (mEPSCs) [33]. Conversely, D2R-MSNs exhibit a decrease in mEPSC frequency with no change in excitability or mIPSC frequency. Morphological analyses also revealed a selective increase in spine density in D1R-MSNs after chronic cocaine exposure. Several other studies have described cell-type specific molecular, structural, and functional plasticity in the NAc and other brain areas in relation to cocaine exposure [34–37]. It is beyond the scope of this review to discuss these studies, but we note that any indication of cell type specificity in cocaine-induced cytoskeletal plasticity should lead to more targeted therapeutics for cocaine use disorder.

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A few studies have demonstrated that cocaine regulates GTPases that interact with actin downstream. Marie-Claire and colleagues investigated the three members of the Rnd family of RhoGTPases, which promote spine maturation (Rnd1), stimulate dendritic branching (Rnd2), and regulate actin organization (Rnd3) [38]. Following acute cocaine administration in mice, the authors identified different temporal and spatial gene expression patterns in the PFC, HPC, and striatum. Rnd1 and 2 were elevated in the PFC four hours after cocaine administration. Rnd3, on the other hand, was elevated in the striatum two hours later and in the HPC after six hours. In a related study, Kiraly and colleagues demonstrated that Kalirin7 (Kal7), a RhoGEF that activates Rac1 GTPase in the kal7/Rac1/Pak pathway upstream of actin, is essential for morphological and behavioral responses to cocaine [39]. The authors reported that one week of chronic cocaine exposure increased Kalirin7 mRNA and protein levels in the NAc in adult mice and rats. Further, following four or eight days of repeated cocaine treatment, wildtype mice exhibited a robust increase in dendritic spine density in the NAc, with the eight-day treatment also resulting in larger spines on the NAc neurons. These morphological changes were absent in the *Kal7* knockout mice, suggesting that cocaine-induced elevation of Kal7 is an essential component of dendritic spine plasticity. The authors also reported that the knockout mice exhibited reduced cocaine CPP despite displaying a normal place preference for food. Together these findings demonstrate the role of NAc Kal7 in chronic cocaine-induced plasticity necessary for retention and expression of cocaine-associated learning. Dynamic actin following cocaine learning is implied, although a direct assessment of those dynamics is warranted.

Dietz and colleagues examined the influence of Rac1 (Rac/Pak/Limk pathway) on cocaine CPP in mice [40]. First the authors reported that cocaine challenge after repeated cocaine treatment transiently reduced active Rac1 levels in the NAc, starting fifteen minutes after cocaine challenge and returning to baseline within forty-five minutes. On the other hand, cocaine challenge reduced inactive cofilin after both fifteen minutes and four hours. These results are consistent with the antagonistic effect of Rac1 activity on cofilin activity. Further, since cofilin severs actin, these findings suggest increased F-actin levels. Indeed, cocaine challenge also increased spine density in the NAc MSNs. Based on these findings, the authors used genetic tools to up or downregulate the levels of Rac1 or cofilin in the NAc MSNs and measured the effects on cocaine CPP, reward sensitivity, and spine density. Bilateral injection of the dominant negative mutant of Rac1 in the NAc shell promoted CPP at a cocaine dose that did not induce a preference in control mice or alter acute locomotor responses to the drug. It also mimicked the drug-induced increase in spine density in control mice. Conversely, the constitutively active mutant of Rac1 blocked cocaine CPP, the locomotor activating effects of the drug, and the drug-induced increase in spine density. In contrast, overexpression of a constitutively active mutant of cofilin increased the rewarding effects of cocaine, did not alter locomotor responses to the drug, and increased spine density. Together these results suggest that chronic cocaine-induced reduction in Rac1 and increase in cofilin activity elevate actin turnover, dendritic spine plasticity and reward sensitivity. Further, they are necessary for the expression of cocaine seeking, highlighting the therapeutic potential of targeting Rac1/cofilin to regulate behavioral responses to cocaine.

Pulipparacharuvil and colleagues targeted Mef2, a transcription factor for the actin regulators N-Wasp, Wave3, and Profilin1 [41]. They found that repeated cocaine exposure

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over seven days suppressed Mef2 activity in the NAc four to twenty-four hours after the last exposure, whereas a single administration of cocaine suppressed Mef2 activity at only twenty-four hours post-injection. Viral-mediated suppression of Mef2 activity enhanced dendritic spine density in the NAc and reduced sensitivity to cocaine, whereas increasing Mef2 activity blocked both the cocaine-induced increase in spine density and the enhanced sensitivity to cocaine (locomotion and place preference). This effect of Mef2 on cocaine sensitivity may contribute to drug tolerance and escalation of intake, occurring more rapidly following chronic exposure. Thus, Mef2 and its actin regulators may be potential targets to prevent use escalation and transition to severe cocaine use disorder.

Our group has sought to target a direct regulator of actin polymerization in dendritic spines, NmII [42]. We trained mice in a four-day cocaine CPP paradigm and two days later administered blebbistatin (Blebb), a small molecule inhibitor of NMII [43]. We found that systemic Blebb treatment thirty minutes prior to a drug-free retrieval test had no effect on CPP expression at the first test but blocked it the following day in a manner consistent with a blockade of contextual memory reconsolidation. These results suggest that actin can be dynamically influenced by drug-associated cues and contexts allowing for its targeting in a drug-free state. And taken together, these studies on cocaine suggest that actin cycling may be targeted via upstream mediators to reduce or disrupt behaviors associated with the motivation for cocaine.

2.2. Actin dynamics associated with methamphetamine

There is surprisingly little literature on the direct effects of methamphetamine on the neuronal cytoskeleton. However, several studies have identified changes in the expression of cytoskeleton associated proteins in the brain in response to methamphetamine administration, indirectly implicating the cytoskeleton. In a study using MRI and transcriptomics with nonhuman primates, Choi and colleagues reported reduced HPC volume and alterations in the expression of several cytoskeleton-related genes in the HPC after acute (single injection, 2 mg/kg) or chronic methamphetamine exposure (8 weeks of injection at increasing doses, 0.1–0.75 mg/kg) [44]. Curiously, some actin-associated genes, including MYH10 (NMIIB), ELMO1, EPB41L1, PPP1R9A and DBN1 were downregulated following both acute and chronic methamphetamine treatment. Further analysis using qRT-PCR and immunohistochemistry showed significant reduction in mRNA and protein levels of cofilin2 in both acute and chronic methamphetamine exposure, suggesting increased actin depolymerization. It is worth noting that the imaging and molecular analyses performed on the acute methamphetamine group were conducted at least four weeks post-drug treatment, suggesting that methamphetamine-induced cytoskeleton changes persist well beyond the period of drug exposure.

In a proteomic study, Bosch and colleagues discovered several differentially expressed cytoskeleton-associated proteins in the dorsal striatum synaptosomes of rats with thirty days history of methamphetamine self-administration, followed by two weeks of forced abstinence [45]. The altered proteins included actin cytoplasmic1 (Actb), tubulin alpha (Tub1a, 1b, 4a), Cttn, and tubulin polymerization-promoting protein (Tppp). In another proteomic study, Yang and colleagues also identified several differentially expressed

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cytoskeleton proteins, including profilin-2 in the striatum, profilin-2, septin2 and vimentin in the NAc, neurofilament, Gfapδ, and collagenα1 in the HPC, neogenin1 precursor in the cingulate cortex and Gfapδ in the PFC in rats with a history of methamphetamine CPP and extinction [46]. Staszewski and Yamamoto reported elevated proteolysis of the cytoskeleton protein spectrin, which interacts with actin, along with elevated extracellular glutamate in the striatum following a binge methamphetamine treatment (four injections every two hours, 10 mg/kg) [47]. In another study, using a lower dose of methamphetamine (7.5 mg/kg), Tata and Yamamoto also reported elevated striatal glutamate levels, but not spectrin proteolysis [48]. However, when animals were exposed to ten days of chronic unpredictable stress prior to binge methamphetamine treatment, this lower dose enhanced extracellular glutamate and resulted in spectrin proteolysis in the striatum. These findings point to a cytoskeleton basis for binge methamphetamine-induced glutamate excitotoxicity and stress-potentiated neuroplastic changes that may contribute to drug- or stress-induced methamphetamine relapse.

These findings implicate various cytoskeleton-associated proteins in the plasticity associated with various regimen of acute and chronic methamphetamine exposure. These proteins are involved in different stages of drug experiences including learning, extinction, forced abstinence and relapse, and in different brain regions. Together, these studies provide several targets that may be investigated as potential therapeutics for managing specific aspects of methamphetamine use disorder.

In our laboratory we have investigated actin cytoskeleton dynamics following methamphetamine exposure in a mouse CPP paradigm [49]. Two days after the final methamphetamine exposure, mice were briefly re-exposed to the methamphetamine context and their brains were processed the next day for spine density analysis. Neuronal spine density was increased in the basolateral AMY (BLA) following re-exposure to a methamphetamine-associated context. Surprisingly, this structural plasticity was blocked when context re-exposure was preceded by LatA administration. In another experiment using the same methamphetamine CPP model, we reported an active actin cycling state in mouse BLA, represented by a higher ratio of monomeric (G-) to filamentous (F-) actin, two days after the last methamphetamine treatment and *without* context re-exposure [50]. These findings were some of the first to suggest that methamphetamine may induce a state of sustained actin cycling in dendritic spines of the BLA long after methamphetamine exposure, independent of memory retrieval. To understand whether methamphetamine-induced actin cycling in the BLA influences future reward related-behaviors, we utilized the highly specific inhibitor of cycling actin, LatA. Infusion of LatA into the BLA thirty minutes before drug-free testing selectively disrupted the expression of methamphetamine-associated memory, but the same treatment had no effect on a BLA-dependent auditory fear memory [49]. The disruption of methamphetamine-associated memory was long-lasting, persisting across at least five days of repeated testing after the single LatA infusion delivered on the first day of testing. We further determined that the effect was independent of the strength of methamphetamine conditioning, as CPP expression after extended training was also disrupted by actin depolymerization. Even more intriguing, the same intra-BLA infusion of LatA delivered an entire day before expression testing, when mice were sitting in their home cage, similarly disrupted methamphetamine-associated memory. This is a typical

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control experiment in reconsolidation studies, where memory would be expected to remain intact. The disruption that occurred when depolymerization and memory reactivation were uncoupled by twenty-four hours indicates a retrieval-independent mechanism where memory storage is likely being targeted and further supports the possibility that methamphetamine induces a sustained state of actin cycling in the BLA. Finally, similar results were obtained using a self-administration model. Intra-BLA LatA infusions thirty minutes before testing also disrupted context-induced reinstatement of extinguished methamphetamine seeking, which persisted at least until the following day [49]. These results point to the significance of a dynamic actin state induced in the BLA by methamphetamine as an Achilles Heel for the maintenance of methamphetamine-associated memory and the motivation to seek the drug that can be exploited for a treatment strategy.

Consistent with these results from our group, Shibasaki and colleagues also reported actin involvement in the expression of methamphetamine CPP in mice [51]. They observed disrupted methamphetamine CPP expression in mice with mutant actin depolymerizing factor (ADF) and in wildtype mice with the actin stabilizer phalloidin administered intracerebroventricularly 30 min prior to methamphetamine treatment. These findings suggest that dynamic actin is essential for methamphetamine learning and memory. Indeed, they reported significantly increased protein levels of ADF, cofilin, G-actin and F-actin in the limbic forebrain following methamphetamine conditioning suggesting a dynamic actin state. These effects were observed in both wildtype and ADF mutant mice with G- and F-actin changes occurring to a lesser degree in the ADF mutant mice.

Together, these studies provide strong support for targeting the actin dynamics to influence methamphetamine seeking behavior. However, because actin is critical to a host of processes throughout the body, direct actin depolymerization is not a viable therapeutic approach. Therefore, we targeted its upstream regulator NmII with the pan-myosin II inhibitor Blebb, which we discovered to be highly CNS penetrant. Using this molecular motor inhibitor through both intra-BLA infusion and, systemic administration, we found recapitulation of all the behavior results obtained with LatA, using CPP and self-administration models [52]. This included demonstrating that a single administration of Blebb could prevent methamphetamine seeking for at least a month. Using RNAseq analysis, we discovered that the BLA in mice contains mRNA for five different myosin genes, of which the NmIIb (*Myh10*) isoform was most highly expressed. Thus, we sought to specifically target this gene to test its role in Blebb-mediated disruption of methamphetamine seeking. Knockdown of NmIIb alone in the BLA using shRNA or siRNA before or after methamphetamine conditioning was sufficient to block methamphetamine-associated learning and memory. These results strongly suggest that NmIIb plays a key role in methamphetamine-induced actomyosin dynamics to influence future methamphetamine-related behaviors. Notably, the effect of NmII inhibition on methamphetamine CPP was present in male and female adult and adolescent animals, with no impact on other behaviors [52,53].

The observed Blebb effects were specific to methamphetamine and amphetamine, as it produced an immediate and lasting disruption of associations for both of these drugs, but not for food reward, cocaine, morphine, nicotine or another amphetamine class drug, mephedrone [43,52,54]. However, reconsolidation effects were observed for all drugs

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tested, except morphine. Regional specificity was also found, as intra-BLA infusions of Blebb disrupted methamphetamine-associated memory, but infusions into other regions of the neural circuit supporting this behavior, such as the NAc, dorsal HPC, and orbital frontal cortex, had no impact [43]. More recent work from our group has implicated the corticotropin-releasing hormone receptor 2 (CRF2) as at least partially responsible for the specificity of NmII inhibition for methamphetamine and BLA [55].

With regards to the effects of Blebb on structural plasticity, we found that systemic Blebb reduces spine density in the BLA following methamphetamine conditioning, but not auditory fear conditioning [52]. More recently, using time lapse serial two photon imaging to measure spine motility, we demonstrated that methamphetamine conditioning does indeed produce persistent actin dynamics in spines of the BLA, but not dorsal HPC [56]. Further, these sustained actin dynamics are mediated by NmII, as administering Blebb to methamphetamine-conditioned mice prior to slice preparation resulted in an arrest of the aberrant spine motility.

Taken together, these data provided significant support for pursuing NmII as a therapeutic target and we are actively developing a single administration NmII-based small molecule medication for methamphetamine use reduction.

2.3. Actin dynamics associated with alcohol

Some of the earliest indications of drug effects on the cytoskeleton came from studies identifying microtubule depolymerization and intermediate filament rearrangement in the hepatocytes of alcohol fed rats and patients with alcoholic liver disease [57–60]. These changes were hypothesized to compromise liver function by disrupting protein secretion or cellular structural integrity. The effects of ethanol on the cellular cytoskeleton of the central nervous system was reported in a study by Allansson and colleagues showing reversible conformational changes in filamentous actin in primary cortical astrocytes [61]. Further studies on cultures of cerebellar granule cells, hippocampal cells, or PC12 cells showed F-actin destabilizing effects of acute ethanol (100 mM for 30 s) and F-actin stabilizing effect of chronic ethanol (50–100 mM for 4 days) [62–64]. Chronic alcohol also led to increased F-actin apposition to Psd-95, suggesting increased synaptic contact which is hypothesized to be a compensatory homeostatic response to alcohol-induced inhibition of NMDAR [64]. Notably, a lower concentration, but longer duration of ethanol exposure (30 mM for 14 days) resulted in decreased levels of F-actin and its GTPases Rac, Cdc42, and RhoA in primary hippocampal cells [65]. This differential effect of dose and length of chronic alcohol exposure on cellular cytoskeleton may represent the dynamic plasticity underlying differential behavioral responses throughout the alcohol use trajectory.

The *in vivo* effects of ethanol on actin cytoskeleton and the role of actin regulating proteins on ethanol-related behavior were demonstrated in two separate, cross-species studies published concurrently. One of these studies by Offenhauser and colleagues found that Eps8, a protein that regulates actin through activation of Rac or by capping the actin barbed ends, is highly expressed in mouse cerebellar granule neurons (CGNs) which are implicated in the sedative and motor incoordination effects of acute ethanol [66–68]. Acute ethanol treatment delocalized Eps8 and reduced Eps8 clusters in post-synaptic structures of

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the CGNs, with parallel loss of F-actin. This ethanol-induced actin remodeling was absent in Eps8 knockout neurons, suggesting a direct interaction between Eps8 and actin. Using acute cerebellar slices, the study showed that a higher dose of acute ethanol (400 mM vs 100 mM) led to reduced NMDA currents and pronounced actin depolymerization. However, this was absent in Eps8 knockout mice, suggesting a role for Eps8 in ethanol-induced actin dynamics, functional plasticity in the CGNs, and perhaps acute responses to ethanol. Finally, the authors investigated whether Eps8 influenced future alcohol-related behaviors. Mice lacking Eps8 were resistant to the sedating effects of ethanol, resulting in increased consumption and preference. In Drosophila, Rothenfluh and colleagues found that mutations in the RhoGAP18B gene or expression of constitutively active forms of Rho1 or Rac1 (RhoGAP18B/Rac1/Arfaptin/Arf6/actin pathway) result in resistance to the sedating effects of high dose ethanol, similar to that observed in WHIR mutants with disrupted RhoGAP18B [69]. Together these findings suggest that Eps8 and Rho or Rac, the upstream regulators of cofilin and actin, are critical regulators of the behavioral response to ethanol and may be targeted to influence aberrant alcohol seeking and consumption.

Several other studies in mice have investigated the influence of alcohol on actin and its upstream mediators. In an intermittent access voluntary drinking paradigm, Laguesse and colleagues found that excessive ethanol drinking in mice led to increased translation and protein levels of Prosapip1, an upstream regulator of actin, in the NAc but not dorsolateral or dorsomedial striatum [70]. This effect on Prosapip1 expression was specific to excessive ethanol drinking, as moderate ethanol and saccharin or sucrose did not affect protein levels. Excessive ethanol drinking also led to elevated F-actin levels in the NAc. Using genetic overexpression and knockdown of Prosapip1 in the NAc of alcohol naïve mice, the study showed corresponding changes in the levels of F-actin, directly linking this protein to actin dynamics. The knockdown of Prosapip1 in the NAc produced several effects. It blocked alcohol-induced elevation of F-actin, dendritic spine area, and rectification index in the shell MSNs. When knocked down in NAc of mice with a history of voluntary alcohol consumption and alcohol self-administration, alcohol, but not sucrose, seeking was reduced during reinstatement. It also reduced alcohol CPP, but not lithium conditioned place aversion (CPA). Together these findings demonstrate an important role for Prosapip1 in alcohol-induced changes in actin dynamics, spine morphology, and neurotransmission, as well as future alcohol seeking. Prosapip1 thus presents as an important target to curb alcohol-related behaviors.

Laguesse and colleagues targeted another regulator of actin polymerization, mTorc2, whose activity is enhanced in the mice dorsomedial striatum (DMS) by excessive alcohol consumption [71]. Reduction of mTorc2 activity by genetic knockdown of its regulator, Rictor, which recruits Tiam1, attenuated DMS actin polymerization and binge-like alcohol consumption, whereas an mTorc2 activator enhanced consumption. These findings support targeting the actin cytoskeleton via the mTorc2/Tiam1 pathway or other mTorc2 pathways like mTorc2/Akt and mTorc2/Sgk1 to reduce alcohol consumption.

Using an ethanol vapor model of physical dependence, Shibasaki and colleagues investigated the role of actin depolymerizing factor (ADF) in ethanol withdrawal-induced behavioral sensitization to ethanol [72]. Ethanol dependent mice showing signs of ethanol

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withdrawal demonstrated heightened locomotor sensitivity to ethanol ten hours after withdrawal, as well as enhanced ethanol CPP three days after withdrawal. Concurrently, they observed continually elevated ADF and G-actin expression but fluctuating F-actin expression in the VTA at various points of the three-day withdrawal period, suggesting a dynamically cycling actin during withdrawal. Daily administration of the F-actin stabilizing agent phalloidin during this period significantly suppressed the heightened ethanol-induced place preference and locomotor activity.

Together, these studies provide a wide range of molecular factors that can be targeted to influence actin dynamics regulating alcohol-related behaviors. Interestingly, they also demonstrate unique molecular influences on actin in several key brain regions of the reward circuitry. Future studies could investigate whether combining two or more of these targets more effectively influences alcohol-related behaviors.

In addition to the study by Rothenfluh and colleagues described above, several studies in Drosophila demonstrate actin-mediated modulation of alcohol effects. In one study, Butts and colleagues manipulated the expression of either Rac1, a regulator of cofilin, or cofilin itself, an actin severing protein, in the mushroom bodies of flies. They reported bidirectional regulation of the acquisition of alcohol preference, demonstrating the essential role of actin turnover for alcohol learning [73]. Ojelade and colleagues targeted Ras suppressor 1 (Rsu1) of the integrin/Rsu1/Rac1/actin pathway [74]. Whereas flies normally acquire ethanol preference over repeated exposure, the authors discovered that flies with global loss of Rsu1 showed heightened ethanol preference already at the first exposure and maintained that preference over successive exposures, indicating attenuated alcohol sensitivity. Interestingly, flies lacking Rsu1 only in the mushroom bodies showed normal levels of preference on the first day but did not increase their preference over time indicating heightened reward sensitivity. The failure of Rsu1 deficient flies to escalate their preference over time, which wildtype flies demonstrate, points to the role of this gene in regulating ethanol reward sensitivity. The differences in global versus localized Rsu1 effects point to the significance of developing targeted therapeutics. The study also investigated whether RSU1 contributes to human alcohol-related behavior. Analysis of three different human genetic datasets revealed an association between a specific single nucleotide polymorphism (SNP) in the RSU1 gene and ventral striatum activation during reward anticipation. Other SNPs were linked to alcohol consumption in adolescence and adulthood. These results demonstrate an evolutionarily conserved function of actin in alcohol reward learning, memory, and consumption.

2.4. Actin dynamics associated with opioids

The positive rewarding experiences of drug use, as well as the negative experiences of drug withdrawal are supported by drug-induced synaptic plasticity [75–77]. The cytoskeleton dynamics underlying this plasticity have been extensively studied with opioids. Martin and colleagues reported a decrease in the F/G-actin ratio and spine density in the NAc of rats following heroin self-administration [78]. Additionally, the mRNA and protein levels of drebrin, an actin bundling protein, were also reduced in the NAc, but not caudate putamen, following heroin, but not sucrose, self-administration. Viral overexpression of drebrin in

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the NAc rescued the heroin-induced reduction of F/G-actin ratio and spine density, and attenuated heroin-primed reinstatement. Viral knockdown of drebrin, on the other hand, resulted in opposite effects. In both cases, dendritic spine density was negatively correlated with total active responses during reinstatement, suggesting that heroin-induced dendritic plasticity may be influencing future heroin-seeking behavior. Heroin self-administration also decreased the surface expression of AMPA and NMDA receptor subunits and corresponding EPSCs in the NAc and drebrin expression rescued these changes. These results indicate that heroin impacts drebrin expression, which leads to alterations in actin-mediated spine density and signal conduction in the NAc. Finally, the authors reported that following heroin self-administration in D1- and D2-cre transgenic rats or repeated administration of morphine in D1- and D2-ribotag mice, drebrin expression decreased exclusively in D1-type MSNs in the NAc. Drebrin overexpression in D1, but not D2 MSNs attenuated drug-induced reinstatement. These data demonstrate that cell type-specific function of drebrin is conserved across species, type of opioid, and mode of drug administration. Together, these results present drebrin as an important therapeutic target for opioid use disorder. The cell-type specific plasticity associated with opioid exposure have been reported by several studies, warranting further investigation of cell-type specific cytoskeletal dynamics and drug-related behaviors for targeted therapeutics [79–81].

In another study using heroin self-administration in rats, Chen and colleagues reported an elevation in F/G-actin ratio in the dorsal HPC [82]. This actin remodeling was blocked by bilateral intra-HPC infusion of LatA, the Camkk inhibitor STO-609 or the Rac1 inhibitor NSC23766 prior to each behavioral training session. These manipulations also inhibited heroin seeking and intake under a progressive ratio schedule. These results demonstrate the involvement of Camkk1/CamkIα/Rac1 signaling cascade leading to actin polymerization in heroin-related plasticity that perpetuates drug seeking and consumption [82]. Points along the pathway, therefore, may be targeted to curb drug seeking and intake. Interestingly, in a study by Wang and colleagues, infusion of the Rac1 inhibitor NSC23766 into the dorsal HPC prior to naloxone-induced morphine withdrawal did not affect conditioned place aversion [83]. Although the two studies differ in their design and goals, they allude to an interesting possibility that Rac1-mediated actin rearrangement may be particularly critical to the rewarding aspects of opioid use.

Studies that investigate potential molecular targets both in the context of rewarding and aversive states of drug use may be important in identifying manipulation targets and protocols that effectively curb the full spectrum of drug-associated behaviors [84,85]. To this end, a recent study investigated spatiotemporal dynamics of actin and its associated proteins during acquisition and extinction of morphine-associated memory [86]. Mice were trained in a morphine CPP paradigm and brain tissue was collected immediately following a retrieval test conducted at one of three time points – one day after the last training session, after ten days of extinction, or after ten days of forced abstinence in the home cage. Western blot analysis showed differential expression of p-actin, Arc/Arg3.1, and p-Erk1/2 protein in the NAc and dorsal HPC in these groups when compared to a control saline CPP group. Whereas morphine CPP increased pErk/Erk ratio in the NAc and pErk/Erk ratio and Arc levels in the dorsal HPC, extinction or forced abstinence increased the levels of all three measures in both regions. Notably, the elevated p-actin/actin ratio in

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the abstinence group was lower than that in the extinction group in both brain regions. These alterations were regionally specific, as no major changes were observed in the medial PFC or caudate putamen. These findings suggest that actin dynamics differ across brain regions and are influenced by recency of drug exposure, abstinence, and extinction learning. This discovery points to the significance of selectively targeting various elements of actin dynamics, depending on the specific drug use or abstinence history.

The negative affective state following withdrawal from opioid use is established as a prime driver of continued drug seeking and use and has been a focus of several studies. Conditioned place aversion (CPA) is a widely used model to study aversive states induced by naloxone which precipitates withdrawal in animals that have had a single prior exposure to morphine. Hou and colleagues found that CPA precipitated by naloxone induces actin rearrangements (change in the F/G-actin ratio) in the AMY and dorsal HPC, but not the NAc [87]. In addition, the cytoskeletal associated protein Arc was upregulated in the AMY. Interestingly, bilateral excitotoxic lesions of the AMY or intra-AMY administration of propranolol, a β-adrenoceptor antagonist, led to significant reductions in actin rearrangement in the dorsal HPC. This demonstrates an intriguing communication between the AMY and dorsal HPC in modulating actin dynamics that support naloxone-precipitated morphine withdrawal. The authors next tested whether the naloxone-induced CPA is influenced by actin dynamics around the time of naloxone pairing. Rats infused with LatA in the AMY ten minutes before naloxone pairing, but not thirty minutes after, displayed attenuated CPA. Interestingly, intra-HPC infusion of LatA attenuated CPA when administered at the post-naloxone time point. In a follow-up study from the same laboratory, Wang and colleagues found that inhibition of the small GTPase RhoA, an upstream regulator of actin, in the dorsal HPC prior to naloxone pairing attenuated CPA and actin polymerization [83]. These findings indicate that different regulators of actin may be involved in different brain regions for acquisition, consolidation, and maintenance of the naloxone precipitated aversive state.

Using the same model of morphine withdrawal, Wang and colleagues applied proteomics and immunoblotting of AMY extracts from animals following CPA and discovered a significant downregulation of a highly conserved ABP, twinfilin1 (Twf1) [88]. Further, analysis of synaptosomal preparations from the AMY one hour after CPA revealed an increased F/G-actin ratio, indicating actin polymerization. Viral-mediated overexpression of Twf1 in the AMY prior to morphine withdrawal protocol decreased the F/G-actin ratio, suggesting actin depolymerization or preparation for upcoming actin polymerization, and reversed the withdrawal-induced spine density and CPA. Together, these studies directly implicate actin dynamics in opioid withdrawal-induced aversive states and point to different upstream targets in different brain regions to interfere with different stages of this state. It will be important to determine if similar changes occur in naloxone-independent opioid withdrawal that is precipitated after chronic opioid exposure.

Several studies have examined the role of actin in opioid reward and withdrawal by direct targeting. In a mouse model of morphine CPP, Li and colleagues targeted actin during a brief memory reactivation period when actin is known to be dynamic [89]. Mice trained to associate a context with morphine reward in the CPP paradigm were infused with LatA in the NAc shell or core immediately after a brief exposure to the morphine-paired context

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and tested the following day for CPP expression. LatA infused into the NAc shell, but not core, reduced morphine CPP expression. This effect persisted for more than two weeks and, importantly, resisted reversal with morphine priming. Furthermore, LatA infused six hours after context exposure did not affect the place preference. These findings provide evidence that reactivation of morphine associations through a brief re-exposure to the morphine context can open a brief reconsolidation window for disruption by targeting actin polymerization. In line with this idea and based on our findings that actomyosin inhibition disrupts cocaine and nicotine-associated memory through reconsolidation, our group has investigated if morphine reward memory is similarly vulnerable [43]. Interestingly, NmII inhibition prior to the first test for CPP expression had no immediate or reconsolidation-based impact on morphine-associated memory. Together with the findings from Li et al. [89], these results suggest a dissociation between the role of actin and myosin in the reconsolidation of morphine-associated memory. When considered in light of the cocaine and nicotine results, it also indicates, perhaps not surprisingly, that there are mechanistic differences in how the actin cytoskeleton is regulated in response to different classes of drugs of abuse.

2.5. Actin dynamics associated with nicotine

The literature investigating the influence of nicotine on the neuronal cytoskeleton is sparse. Kovacs and colleagues provided some of the earliest evidence of nicotine-induced effects on neuronal actin [90]. In mice treated with daily nicotine (0.4 mg/kg) for two weeks, actin protein level was reduced in the tissue homogenate fraction of striatum and cortex but elevated in the synaptoneurosome preparation of HPC and cortex. Natarajan and colleagues assessed the protein levels of the actin binding protein Cttn in the HPC and PFC after nicotine exposure on each day of the five days of CPP training [91]. Compared to the control saline CPP group, nicotine exposure only decreased Cttn in the HPC on day 5, suggesting a delayed impact of repeated nicotine exposure. In a series of experiments, nicotine was found to regulate LTP in an actin dependent manner [92–94]. The LTP of synapses is an essential component of memory consolidation and thus of learned drug associations, which can contribute to drug seeking. Consolidated LTP, which occurs approximately thirty minutes after LTP induction, is generally resistant to disruption by LTD stimulations or other depotentiation protocols. However, in the presence of nicotine, low frequency stimulation reversed consolidated LTP in dorsal HPC [92,93]. This occurred in both young and adult rats and the effect was mediated through desensitization of alpha7 nicotinic acetylcholine receptors (nAChRs) in an NR2A-NMDAR dependent manner. Galvez and colleagues further demonstrated that nicotine mediated depotentiation of LTP in the dorsal HPC was prevented by jasplakinolide, an actin stabilizer [94]. Together, these studies suggest that acute nicotine has an actin destabilizing effect. Curiously, the dorsal HPC LTP was resistant to reversal by acute nicotine in rats with a history of chronic nicotine exposure (0.5–1 mg/kg twice daily for 7–17 days) suggesting that chronic nicotine exposure may influence plasticity mechanisms, including actin dynamics, in a manner that robustly stabilizes LTP [93]. Indeed, chronic nicotine has been previously shown to lower the threshold for LTP induction in the dorsal HPC [95]. This may render nicotine associations and conditioned behaviors resistant to disruption, perpetuating use.

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Few, if any, studies have targeted neuronal actin dynamics to test the effects on nicotine withdrawal, seeking, or intake. In a study from our laboratory examining the effects of NmII inhibition on nicotine CPP, we found that Blebb administered thirty minutes prior to testing did not have an immediate effect, but blocked expression of the nicotine-associated memory the following day [54]. This result was akin to what we observed with cocaine CPP under the same manipulation protocol, consistent with a disruption of reconsolidation. Interestingly, methamphetamine exposure rendered nicotine-associated contextual memory vulnerable to disruption by NMII inhibition, such that nicotine CPP was disrupted on the first day of testing (more on actin dynamics in polysubstance cases in Box 2).

3. Beyond actin, beyond neurons

Most studies have identified neuronal actin as the primary mediator of the structural and functional plasticity induced by drugs of abuse. However, microtubules and intermediate filaments, which also comprise the cellular cytoskeleton, in both neurons and glia, also play a significant role in dendritic spine morphology and synaptic plasticity and are known to be modulated by drugs of abuse [96–98]. Below we briefly discuss some of these studies for each of the drug categories.

3.1. Association of cocaine with cytoskeleton beyond neuronal actin

Beitner-Johnson and colleagues discovered that chronic, but not acute, administration of either cocaine or morphine in rats decreases levels of the three neurofilament (the neuronal intermediate filament) proteins, NF-200 (NF-H), NF-160 (NF-M), and NF-68 (NF-L) in the VTA, but not in substantia nigra, locus coeruleus, midbrain or spinal cord [99]. These changes were specific, as the drug treatments did not affect other cytoskeleton or associated proteins, and neurofilament levels were not altered by two non-reinforcing psychoactive compounds, imipramine and haloperidol, or by chronic stress. Using equivalent daily dose of cocaine (25 mg/kg) in mice, Kovacs and colleagues reported a significant decrease in protein levels of NF-M and NF-L in HPC, increases in these protein levels in the cortex, and a decrease in NF-M levels in the striatum [90]. These results suggest chronic cocaine-induced effects on NF are conserved across species but vary by brain region. Fattore and colleagues reported a two-fold or higher increase in the expression of Gfap, a cytoskeletal intermediate filament protein exclusively expressed in astrocytes, in adult mouse dentate gyrus one day after acute or chronic cocaine treatment [100]. Scofield and colleagues reported reduced Gfap levels in the NAc core, but not in dorsomedial PFC, following extinction of cocaine self-administration [101]. A concurrent reduction in the number of Gfap labelled astrocytes was also observed. Furthermore, the NAc core astrocytes exhibited reduced surface area, volume, and colocalization with the synaptic marker synapsin1 – changes consistent with attenuated neuroglial interaction. These studies suggest that astrocytic cytoskeleton is differentially involved in synaptic plasticity in a brain-region specific manner at various stages of drug use. These findings warrant studies targeting both astrocytic and neuronal cytoskeleton to comprehensively determine the therapeutic strategies for drug use problems.

Several studies have implicated microtubule and associated proteins in psychostimulant related plasticity. Krasnova and colleagues assessed gene expression in the HPC and

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frontal cortices of rats following cocaine or saline CPP [102]. In a microarray analysis of the tissues obtained twenty-four hours after the last conditioning session, several genes were differentially expressed, including upregulation of neuronal and glial actin-associated proteins and different tubulin proteins, in the two brain regions. Using RT-PCR approach they confirmed upregulation of tubulin α6, tubulin β2, tubulin α ubiquitous, and ezrin, an astrocyte selective ABPs, in the HPC. Bouvrais-Veret and colleagues reported hypersensitivity to acute and subchronic locomotor effects of cocaine in mice without Stop, a microtubule associated protein [103]. This finding suggested that microtubule interaction with its associated proteins modulates behavioral response to drugs of abuse. Extending these findings, Calipari and colleagues demonstrated that inhibition of microtubule polymerization using podophyllotoxin in the NAc of mice on the first day of cocaine CPP conditioning prevents development of locomotor sensitization and contextual reward association [104]. In a rat cocaine self-administration model, they further showed that levels of synaptosomal microtubule associated protein Eb3 was elevated during extended withdrawal (30 days), but Src activity (which regulates actin dynamics) was elevated during both acute (1 day) and extended withdrawal. Using HSV-mediated mRNA overexpression, withdrawal related Eb3 overexpression was implicated in cocaine seeking and motivation, but not consumption, whereas Src activity was implicated in withdrawal-induced incubation of cocaine craving. These experiments demonstrate the bidirectional interaction between drugs of abuse like cocaine and microtubules and their associated proteins. It is apparent from these findings that microtubule dynamics contribute to initial drug learning and drug-induced alteration of this dynamics influences future reward-related behaviors, such as craving and seeking. Furthermore, these results point to the possibility of targeting proteins like Srcin1, which interacts with Eb3 and Src to regulate drug-related microtubule and actin dynamics [97]. These studies point to microtubule as an important regulator of psychostimulant-related cellular and behavioral changes and, thus, a viable target for psychostimulant use disorder.

3.2. Association of methamphetamine with cytoskeleton beyond neuronal actin

As described in Section 2.2. proteomics studies in rats have identified changes in intermediate filament and microtubule related proteins, in addition to actin related proteins, following repeated methamphetamine exposure [45,46]. These include changes in tubulin alpha (Tub1a, 1b, 4a), and tubulin polymerization-promoting protein (Tppp) in the dorsal striatum during extended withdrawal from methamphetamine self-administration, and changes in neurofilament (NF-L) and Gfapδ in the HPC and PFC after methamphetamine CPP extinction. It is unclear how each of these proteins interact to influence these drug states, but these findings highlight the significance of examining substance related effects on all three cytoskeleton types.

In one of the few studies examining the effects of psychostimulants on axonal transport, Killinger and Moszczynska reported a loss of stable long-lived microtubules and dopamine markers in the striatum, but not in the substantia nigra pars compacta, following binge methamphetamine exposure (four sessions of 10 mg/kg every two hours) in male rats [105]. These deficits were prevented by co-treatment with a low dose of the microtubule stabilizing agent epothilone D (EpoD) and exacerbated by a higher dose of EpoD. Additionally, the

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low dose EpoD prevented the methamphetamine-induced increase in striatal dopamine turnover and increased behavioral stereotypy during methamphetamine treatment. These findings suggest that different phases of methamphetamine use may dynamically influence dopamine signaling in the nigrostriatal pathway by altering microtubule stability and axonal transport of dopamine markers. In another study, MDMA, an amphetamine-class drug, disrupted mitochondria trafficking in axons of primary hippocampal neurons incubated in pharmacologically relevant dose of MDMA for 90 min [106]. This effect was dependent on Tau, a microtubule associated protein, and suggests a microtubule mediated regulation of energy supply and neurotransmission during drug exposure. These results warrant further *in vivo* investigation of microtubule associated proteins as therapeutic targets for substance use disorders.

3.3. Association of alcohol with cytoskeleton beyond neuronal actin

In vitro studies using rat astroglial primary cultures from the cortex or HPC have shown that acute alcohol exposure induces transient transformation of the actin cytoskeleton [61], whereas chronic exposure leads to actin cytoskeleton rearrangement and alterations in the levels, assembly, and cellular organization of the actin cytoskeleton and microtubules [65,107]. Acute or chronic alcohol exposure has also been shown to affect axonal transport in an ex-vivo setup. McLane reported reduced transport of radiolabeled protein in sciatic nerve preparation treated acutely with ethanol or one from rats fed 6.7% ethanol chronically for 16–28 weeks [108]. Whether similar alcohol-induced disruption in axonal transport also occurs in the brain and by which cytoskeletal mechanism are topics of investigation worth pursuing for better understanding of drug influence on neuronal function. In a study in rats, Liu and colleagues found that excessive voluntary consumption of 20% ethanol in an intermittent access paradigm resulted in elevated polysomal mRNA and synaptosomal or total protein levels of Crmp-2, a microtubule associated protein, in the NAc but not in the control regions like the cerebellum or the motor cortex [109]. These changes in gene and protein expression were observed immediately after a binge-like drinking episode and after one day of withdrawal. However, they returned to baseline after one week of withdrawal. Using an immunoassay, they further demonstrated that a session of excessive alcohol intake also enhances Crmp-2 binding on microtubules and microtubule assembly through activation of the AKT/GSK-3β/Crmp-2 pathway. Finally, systemic administration of the Crmp-2 inhibitor lacosamide in rats and mice after one day of withdrawal from excessive alcohol consumption reduced binge-like alcohol drinking, without affecting sucrose consumption, locomotor activity or anxiety. Furthermore, shRNA-mediated knockdown of Crmp-2 in the NAc of mice prior to alcohol training also reduced excessive alcohol intake and preference throughout training. These findings demonstrate the significance of the microtubule regulating protein Crmp-2 in regulating and maintaining the drive for excessive alcohol at different stages of alcohol consumption trajectory. It is worth noting that lacosamide is an approved anticonvulsant prescription medication which also modulates voltage-gated sodium channels, making it unclear whether the observed effects in this study are solely mediated by Crmp-2 [110]. Nonetheless, these findings provide strong support for testing lacosamide or for developing other small molecule inhibitors of Crmp-2 as a therapeutic for alcohol and other substance use disorders.

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3.4. Association of opioids with cytoskeleton beyond neuronal actin

Administration of both acute and chronic morphine in animals has been shown to alter gene and protein expression of microtubule-related proteins and neurofilaments. For instance, using cDNA microarray analysis, Loguinov and colleagues found that mice treated with a single injection of morphine showed downregulation of α-tubulin and stathmin mRNA in the medial striatum, which was prevented by coadministration of the opioid antagonist naloxone [111]. On the other hand, using RT-PCR and immunoblotting assay, Marie-Claire and colleagues reported downregulation of striatal α-tubulin, stathmin, and Tau mRNA and protein in rats with a history of chronic morphine administration [112]. They also reported elevated protein levels of Gfap. Studies investigating influences of these gene expression changes on neuronal function and behavior are lacking. As discussed above for cocaine, Beitner-Johnson and colleagues discovered that chronic, but not acute, administration of morphine in rats decreases levels of all three types of NF in the VTA [99]. Given the role of NF in slow axonal transport, the group further studied the effects of chronic morphine on axonal transport in the VTA to NAc pathway. They reported a 50% reduction in slow axonal transport, as measured by the ratio of radiolabeled methionine in the two regions [113]. A few early studies have shown compromised axonal transport of proteins in the nigrostriatal pathway during morphine exposure, tolerance, abstinence and withdrawal, implicating microtubule or other cytoskeletal function [114,115]. More recently, a study by Luo and colleagues found compromised axonal transport and mitochondrial function, along with reduced levels of neurofilament NF-H in rats after six rounds of chronic heroin exposure and withdrawal [116]. Together, these findings demonstrate non-actin neuronal and glial cytoskeleton responses to both acute and chronic opioid exposure. However, their influence on future drug-related behaviors remains to be determined.

Kruyer and colleagues studied the interaction between astrocytes and afferent neurons in the NAc to better understand cue-induced relapse of heroin seeking [117]. In male rats trained to self-administer heroin, extinction of heroin seeking behavior resulted in an enduring reduction in synaptic proximity of astroglia within the NAc. This was restored during 15 minutes of cue-induced heroin seeking but returned to extinction levels within two hours. Such responses were absent with self-administration of sucrose. Further, knockdown of ezrin, an astrocyte-selective ABP, using morpholino antisense approach in the NAc for three days during extinction training reduced astroglial association with synapses and potentiated cue-induced heroin seeking. These findings demonstrate the dynamic role of the astrocytic actin cytoskeleton in extinction learning and cued renewal of heroin administration. In a follow up study, the group reported opposite findings in the ventral pallidum (VP), a region with dense projections from the NAc [118]. In this second study, which used male and female rats, they found a selective increase in VP astrocyte proximity with D1-expressing NAc terminals during extinction of heroin self-administration. Reducing this proximity using antisense knockdown of ezrin after three consecutive extinction sessions disrupted extinction of heroin seeking. These findings point to the important roles of astrocyte cytoskeleton dynamics in synaptic plasticity associated with adaptive drug-related learning, memory, and motivation.

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3.5. Association of nicotine with cytoskeleton beyond neuronal actin

Although studies on nicotine are rare, Bunnemann and colleagues reported decreases in NF-H and NF-M in the VTA, but not in substantia nigra, following chronic nicotine exposure in rats [119]. In mice, with an equivalent daily dose (0.4 mg/kg) of chronic nicotine treatment, Kovacs and colleagues reported a significant decrease in protein levels of NF-L and NF-M in HPC, decrease in NF-L in the striatum, and increase in NF-H levels in the cortex [90]. These results suggest nicotine-induced effects on NF are conserved across species but vary by brain region.

Taken together, these studies suggest that drug-induced cellular plasticity in the CNS goes well beyond actin dynamics in neurons, warranting deeper investigation of the relationship between substances of abuse and the neuro-glial cytoskeleton as a whole.

4. Conclusion

The studies discussed in this review demonstrate a bidirectional relationship between substances of abuse and the brain cytoskeleton, whereby exposure to substances alter cytoskeleton dynamics, which, in turn, influence behavioral expressions of substance withdrawal, seeking, and intake. These studies identify substance-induced alterations of several proteins within the signaling cascades leading up to the cytoskeleton in several different brain areas (summarized in Table 1). Some studies utilize pharmacological and genetic tools to investigate the contribution of these proteins to future drug-related behaviors. Key among these proteins are Limk, cofilin, Arp3 and kalirin7 for cocaine, spectrin and NmII for methamphetamine, prosapip1, Eps8, and Rsu1 for alcohol, and drebrin and twinfilin1 for opioids. Some proteins are implicated in more than one substance, i.e. ADF for methamphetamine and alcohol, Rac1 for cocaine, heroin, and alcohol, and NmII for cocaine, methamphetamine, heroin, alcohol, and nicotine. These common regulators may be particularly effective therapeutic targets for polydrug use cases. Indeed, work from our laboratory has demonstrated the utility of NmII inhibition in disrupting drug seeking and intake for different substances. Several studies suggest that substance-induced alterations in cytoskeleton dynamics are brain region, and cell-type specific and can vary depending on the specific phase of the substance exposure history. Similarly, although a majority of the studies implicate the neuronal actin cytoskeleton, evidence points to the significance of other cytoskeleton proteins and non-neuronal cells in substance-related behaviors.

Based on these observations we propose a few directions for future research. First, the lack of research on the relationship between nicotine and the brain cytoskeleton is striking. Research should focus on investigating the targets identified by existing studies on other drugs of abuse, as well as identifying novel targets. In this vein, research on the interaction between cannabinoids and hallucinogens and brain cytoskeleton is also lacking, but highly relevant in the context of a recent rise in their use [120–126]. Second, given the identification of multiple targets within the same or different brain areas for the same substance, studies should explore combinatorial therapeutics targeting multiple proteins of interest. Likewise, a target identified for a particular drug may be investigated for other drugs in the same or different drug class. It is also relevant to apply combination therapies

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in relation to non-actin cytoskeleton and non-neuronal cell types. Third, therapeutic development projects would benefit from investigating targets throughout the full spectrum of drug experience including initial intake, transition to excessive intake, acute withdrawal, abstinence, and relapse. This would allow for targeted therapies that may be beneficial for specific phase/s in the substance use trajectory. Finally, it is important to study the therapeutic benefits of these targets in models of severe forms of SUD involving dependence and variable access (e.g. intermittent), where cytoskeleton dynamics may unfold differently compared to models of less severe form of SUD. We conclude that cytoskeleton targeting is a promising avenue for the development of novel therapeutics for substance use disorders, focused on the goal of restoring the brain's plasticity to a pre-drug state.

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Box 1

Cytoskeleton and polysubstance exposure

It is extremely common for individuals to use multiple drugs from the same and different drug classes. For instance, individuals that drink alcohol commonly also ingest nicotine (e.g. smoke cigarettes), and opioids are often used to curb the stimulating effects of methamphetamine. In this context, it is imperative to understand how cytoskeleton dynamics are influenced by polysubstance use, especially when, in isolation, these substances often exert opposing effects on neuronal plasticity. For instance, in studies using rodent models of self or experimenter-administered drugs, psychostimulants (cocaine, amphetamine) and nicotine increase spine density and dendritic branching in NAc and mPFC, whereas morphine and alcohol decrease these key markers of structural plasticity (reviewed in [75]).

A relatively few studies have investigated the cytoskeleton in the context of polysubstance use. In an *in vitro* study using photoactivated localization microscopy, Tobin and colleagues determined the potential for ethanol to interact with opioid receptors and induce rearrangement of membrane components and cytoskeletal proteins [138]. They found that brief exposure (20 minutes) to pharmacologically relevant levels of ethanol (20- and 40-mM) led to a rearrangement of the lipid raft marker glycosylphosphatidylinositol (GPI) in a mu opioid receptor dependent manner. Additionally, ethanol induced pronounced actin polymerization leading to partial co-localization with GPI. This study alludes to actin as a common denominator subserving interaction between alcohol and opioid and warrants further studies of actin dynamics in alcohol and opioid polysubstance use.

In our laboratory we tested whether nicotine or morphine CPP alone or in combination with methamphetamine CPP are susceptible to disruption by NmII inhibition, similar to the disruption of methamphetamine CPP [54]. We discovered that nicotine or morphine CPP alone were unaffected by NMII inhibition by Blebb treatment on the first day of testing, with disruption only observed on the second day in a reconsolidation-like effect. On the other hand, when nicotine conditioning was combined with methamphetamine conditioning, administered on alternating days of CPP training, Blebb produced an immediate disruption of CPP expression, which carried over into the second day. Interestingly, simultaneous nicotine and methamphetamine conditioning produced a conditioned place aversion instead of a preference in the control group, and this aversion was blocked in the group receiving Blebb treatment. These results suggest that similar actin dynamics may be at play for both rewarding and aversive experiences that can result from nicotine and methamphetamine administration. For morphine, polydrug conditioning with methamphetamine either on separate days or simultaneously resulted in preference for the drug-paired context, which was blocked by Blebb treatment thirty minutes prior. These findings begin to provide insight into the feasibility of interfering with maladaptive polysubstance experiences.

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Box 2

Substances, cytoskeleton, and blood brain barrier

It is now well established that drugs of abuse affect the integrity of the blood brain barrier (BBB), which in turn disrupts cellular homeostasis and communication by inducing neurotoxicity and neuroinflammation [127,128]. The cytoskeleton and tight junction proteins are prime mediators of endothelial cell function at the heart of the BBB. A comprehensive discussion of the relationship between various drugs of abuse and cytoskeleton-mediated BBB dysfunction is beyond the scope of this review and we point readers to some recent reviews on this topic [127,129,130]. Here we briefly discuss some studies investigating the impact of methamphetamine on brain endothelial cytoskeleton and BBB integrity.

Several studies have shown that methamphetamine disrupts BBB permeability [131–133]. Xue and colleagues discovered enhanced BBB permeability following repeated methamphetamine treatment in rats [134]. Western blot analysis of HPC tissue from these animals showed enhanced levels of proteins in the RhoA/ROCK pathway including RhoA, ROCK, myosin light chain (MLC), p-MLC, cofilin, p-cofilin and matrix metalloproteinase (MMP)-9, as well as reduced levels of tight junction proteins like occludin, claudin-5 and ZO-1. The study also reported increased levels and rearrangement of F-actin. Although these changes suggest the role of actin cytoskeleton and its upstream regulators in methamphetamine-induced disruption of BBB integrity, it is unclear if these changes occur in the brain parenchyma or vasculature. Thus, the group used isolated rat brain microvascular endothelial cells (RBMECs) and demonstrated that methamphetamine treatment of these cells mimicked the protein expression changes observed *in vivo*. Finally, inhibitors of RhoA and ROCK were found to reverse the methamphetamine-induced changes in protein expression, actin rearrangement and permeability. Whereas this study identified some key players of methamphetamine-induced BBB disruption, including actin and tight junction proteins, a study by Park and colleagues had previously demonstrated a direct link between methamphetamine-induced rearrangement of actin and changes in tight junction proteins [135]. In both cultured endothelial cells and brain microvessels isolated from adult rats, methamphetamine treatment activated Arp2/3 and enhanced actin polymerization and occludin endocytosis. Arp2/3 is involved in actin nucleation and, accordingly, its inhibition with CK-666 reversed actin polymerization, increased occludin levels and restored disrupted BBB in the brain endothelial cells and microvessels, suggesting a role for actin dynamics in occludin reorganization and BBB disruption. In other studies, immortalized bEnd.3 cells treated with increasing doses of methamphetamine showed dose-dependent disruption of the actin filaments, claudin-5 translocation to the cytoplasm and α-tubulin deacetylation [136,137]. These findings highlight the significance of considering cytoskeletal changes in the brain vasculature when determining the relationship between substances of abuse and cytoskeleton dynamics in the CNS.

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Table 1

Cytoskeleton Related Therapeutic Targets for Substance Use Disorders.

Substance	Cytoskeleton and associated proteins	Brain regions	References
Cocaine	**Microfilament:** Actin, Cofilin, Adducin, Mena, Vasp, Cttn, LimK, Arp3, Rac1, Kal7, Mef2, N-Wasp, Wave3, Profilin1, Rnd1, Rnd2, Rnd3, NMIIb, Ezrin **Intermediate filament:** NF-H, NF-M, NF-L, Gfap **Microtubule:** Tubulin α6, Tubulin β2, Tubulin α ubiquitous, Stop, Eb3, Src, Srcn1	NAc, PFC, Striatum, VTA, HPC, cortex,	[27,28,32, 38–41,43, 99–104]
Methamphetamine	**Microfilament:** Actin, NmIIb, Elmo1, Epb41L1, Ppp1R9A, Dbn1, Cofilin2, Actb, Cttn, Spectrin, Profilin2, Septin2, Vimentin, Collagenα1, Neogenin1 precursor, ADF, **Cofilin Intermediate filament**: NF-L, Gfapδ **Microtubule:** Tub1a, Tub1b, Tub4a, Tppp	NAc, PFC, Striatum, HPC	[43–54,105]
Alcohol	**Microfilament:** Actin, Eps8, Prosapip1, mTorc2, ADF, Camkk, Rac1, Rsu1, Cofilin, Rictor, Tiam1 **Microtubule:** Crmp-2, Akt, Gsk-3β	NAc, DM Striatum, VTA, mushroom body	[68–74,109]
Opioids	**Microfilament:** Actin, NMIIb, Drebrin, Erk, Arc, RhoA, Twf1, Ezrin **Intermediate filament**: NF-H, NF-M, NF-L, Gfap **Microtubule**: α-Tubulin, Stathmin, Tau	NAc, HPC, AMY, Striatum, VP,	[43,78,82,83, 86,87–89,99, 111,112,116, 117,118]
Nicotine	**Microfilament:** Actin, NMIIb, Cttn **Neurofilament:** NF-H, NF-M, NF-L	Striatum, cortex, HPC, VTA	[54,90–94,119]