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REVIEW

Biogenesis, physiological functions and potential applications of extracellular vesicles in substance use disorders

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Abstract

Substance use disorder (SUD) is a growing health problem that affects several millions of people worldwide, resulting in negative socioeconomic impacts and increased health care costs. Emerging evidence suggests that extracellular vesicles (EVs) play a crucial role in SUD pathogenesis. EVs, including exosomes and microvesicles, are membrane-encapsulated particles that are released into the extracellular space by most types of cells. EVs are important players in mediating cell-to-cell communication through transfer of cargo such as proteins, lipids and nucleic acids. The EV cargo can alter the status of recipient cells, thereby contributing to both physiological and pathological processes; some of these play critical roles in SUD. Although the functions of EVs under several pathological conditions have been extensively reviewed, EV functions and potential applications in SUD remain less studied. In this review, we provide an overview of the current knowledge of the role of EVs in SUD, including alcohol, cocaine, heroin, marijuana, nicotine and opiate abuse. The review will focus on the biogenesis and cargo composition of EVs as well as the potential use of EVs as biomarkers of SUD or therapeutic targets in SUD.

Keywords Microvesicles · Exosomes · Alcohol · Cocaine · Heroin · Marijuana · Nicotine · Opiates

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Introduction

Substance use disorder (SUD) is a chronic, relapsing disease caused by the persistent use of drugs such as alcohol, cocaine, tobacco, and opioids. SUD is now a significant public health problem that results in increased morbidity, mortality, loss of productivity, and increased health care costs [1]. The underlying mechanisms of SUD, however, have yet to be fully explored. While there are several cellular processes linked to causing SUD, emerging evidence suggests that alterations in the quantity and the biological content of extracellular vesicles (EVs) play an essential role in SUD. Therefore, understanding how EVs are involved in the development of SUD could lead to the discovery of novel biomarkers and treatment options for this disease.

Extracellular vesicles are a heterogeneous group of membrane-bound vesicles that are released by various types of cells [2]. EVs carry cargo of nucleic acids, proteins and lipids that can be exchanged between cells [3–5]. As per the International Society of Extracellular Vesicles (ISEV) classification, EVs, which range in diameter from 20 to 1000 nm, consist of several subclasses, including exosomes,

microparticles (also termed ectosomes, microvesicles, shedding vesicles, exosome-like vesicles, nanoparticles), and apoptotic bodies [6–8]. Historically, EVs were identified as early as the 1970s when Aaronson et al. found that *Ochromonas danica* synthesized a variety of large and small intra and extra-cellular membrane-bounded structures derived from membranes associated with the flagella, mitochondria, chloroplasts and plasma membrane [9]. Work in the 1980s identified that the transferrin receptors located within reticulocytes were also linked with 50-nm-sized vesicles that were released into the extracellular space as the reticulocytes matured [10–12]. Since then, EVs have been purified and characterized from several mammalian as well as prokaryotic cells.

The importance of EVs lies in their ability to mediate cell-to-cell communication and their significant roles in various normal physiological processes as well as in pathological conditions such as cardiovascular disease (CVD) [13–15], cancer [16, 17], inflammation [18], and SUD [19–21]. Literature that describes the role of EVs and their cargo in the biogenesis and functional outputs related to drug abuse and addiction is reviewed here. Additionally, we provide a detailed analysis of how EVs could be used as biomarkers and therapeutic targets in SUD.

Biogenesis of EVs in SUD

The biogenesis of EVs occurs either dependent or independent of the endosomal sorting complex required for transport (ESCRT) pathway [22–24]. In the ESCRT dependent pathway, intraluminal vesicles (ILVs) are formed within large multivesicular bodies (MVBs) by invagination of late endosomal membranes that then accumulate proteins and cytosolic components or are trafficked to lysosomes for degradation [24]. The formation of ILVs is regulated by the ESCRT pathway which has been shown to facilitate MVB formation, vesicle budding, and protein cargo sorting [25]. The ESCRT machinery has four functional units known as ESCRT-0, I, II, and III that act together with other proteins to recruit cargo into the ILVs. Evidence also suggests that MVBs and ILVs can form independently of ESCRT function, instead involving proteins of the tetraspanin family (that include CD9, CD63, CD81, CD82, and CD151) [5]. For example, sorting of pre-melanosomal protein (PMEL) to the ILVs of MVBs in melanocytic cells is independent of ESCRT mechanisms [26] but requires the tetraspanin CD63 [27]. Similarly, CD63 can be instrumental in the formation of small (< 40 nm) ILVs in MVBs of HeLa cells, which form independently of the hepatocyte growth factor regulated tyrosine kinase substrate that acts in association with ESCRT-I [28]. The ESCRT-independent pathway has been shown to be mediated via raft-based microdomains that are highly enriched in sphingomyelinases [29]. Two lipid metabolism enzymes (neutral sphingomyelinase and phospholipase D2) have been shown to generate lipids in the limiting membrane of MVBs, which induce inward budding and, thus, formation of ILVs in an ESCRT-independent manner [22, 23]. These studies demonstrate that EVs can be formed by both ESCRT-dependent and independent mechanisms. In this section we will describe how the biogenesis of EVs is modulated by SUD based on the available literature.

Alcohol impairs glial and astrocytic function in the brain, and exposure to alcohol in prenatal stages alters the development of several brain regions such as the cerebellum, cortex, and hippocampus [30, 31]. Additionally, alcohol interferes with communication between nerve cells and suppresses excitatory nerve pathways [32]. Crenshaw B. et al. demonstrated that alcohol, increased heat shock protein-90 (HSP90) and decreased CD18 in the exosomes derived from BV-2 microglial cells [33]. Similarly, increased levels of HSP60, HSP70 and apoptotic proteins FAS and caspase 9 in EVs released from alcohol-stimulated HeLa cells have been observed [34]. In HIV-infected patients, the proteins hemopexin and properdin were decreased in the plasma EVs in HIV^+ smokers and HIV^+ drinkers compared to HIV^+ patients that did not smoke or drink alcohol [35]. These findings indicate that HIV and drug abuse could alter the biogenesis of EVs through the tetraspanins such as CD63.

Cocaine use has also been shown to alter EV characteristics and content. Exposure of human glioblastoma cells to a low concentration of cocaine (150 nM) significantly increased the number of vesicles with 61–80 nm diameter, whereas exposure of these cells to higher concentrations of cocaine (300 nM and 150 μM) resulted in increased release of smaller vesicles (30–40 nm diameter) [36]. In another study, exposure to cocaine increased EV release from neuroblastoma cells through the dissociation of the sigma-1 receptor (Sig-1R) from ADP-ribosylation factor (ARF6), a G-protein regulating EV trafficking, leading to activation of myosin light chain kinase (MLCK) [37]. Trubetckaia et al. showed that cocaine exposure in mice resulted in an increase in EVs release in the serum and the brain [38]. Cocaine-mediated increase of Alix and CD63 in the brain was blocked in α-syn knockout mice, demonstrating the crucial role of α-syn in Alix-mediated formation of MVB ILVs [38]. In line with these findings, a recent study also demonstrated that the use of substances such as cocaine, psychostimulants, marijuana, opiates, and alcohol promoted the secretion of semen EVs in people living with HIV that enhanced actin reorganization, chemotactic migration and adhesion of monocytes [39]. These findings have established that substance abuse alters both the number and composition of EVs in various cells, although the exact underlying mechanisms warrant further investigation.

Composition of EVs in SUD

It is well established that EV cargo can include nucleic acids (messenger RNAs (mRNAs) and microRNAs (miRNA)), cytokines, organelles (mitochondria), bioactive lipids, peptides, ions, growth factors, proteins and transcription factors [3–5]. This diverse and vast cargo can be exchanged between cells, thereby contributing to intercellular communication in a multitude of physiological and pathological conditions, including those seen in SUD. In the context of SUD, several studies have focused on characterizing miRNA cargo; however, much less is known about other EV cargos. Consequently, there is a rapidly growing interest aimed at understanding EV composition and function in the context of SUD. In this section, we discuss the composition of EVs in the context of SUD (Fig. 1).

Table 1 RNA and protein content of EVs and potential for use as biomarkers for SUD

RNA composition of EVs

Seminal studies have demonstrated that EVs contain functional RNA species [40, 41]. Specifically, EVs have been shown to contain mRNAs [40, 42], long non-coding RNAs (lncRNA) [54], miRNAs [40, 50], piwi-interacting RNAs, and ribosomal RNAs (rRNA) [54]. miRNA processing components such as Dicer and AGO1 have also been found within EVs [55–59]. Several miRNAs have been identified as being altered in EVs in animal models or humans affected by SUD (Table 1). For example, miR-27a, *let*-7f, miR-29a, miR-340, miR-122, miR-155, miR-122, miR-192 and miR-30a were found to be elevated in EVs from rodents exposed to alcohol [19, 43–46]. These miRNAs were implicated in alcohol-mediated polarization of monocytes into M2 proinflammatory status, liver injury and inflammation. In HIV-infected and cocaine-treated human monocyte-derived macrophages, Sharma et al., observed a significant increase in miR-130a levels in the EVs derived from these cells. Following the addition of these EVs to primary human pulmonary arterial smooth muscle cells, a decrease in the expression of miR-130a targeted molecules such as phosphatase and tensin homolog and tuberous sclerosis 1 and 2, and concomitant activation of PI3K/protein kinase B signaling was observed [47]. In HIV$^+$ heroin users, Wang et al., showed that the levels of four neuroinflammation-related miRNAs (146a, 126, 21, and *let*-7a) in plasma exosomes were higher in HIV-infected heroin users as compared with the control individuals [48]. Similarly, opiates such as morphine have been shown to enhance HIV transactivator of transcription (Tat)-mediated toxicity in both human neurons and neuroblastoma cells [50]. Morphine and HIV Tat increased the release of miR-29b in EVs from astrocytes and exposure of neuronal SH-SY5Y cells to EVs from morphine-treated astrocytes showed a decrease in the expression of platelet-derived growth factor-B (PDGF-B), with a concomitant decrease in viability of neurons [50]. Interestingly, HIV infection and heroin also upregulated the majority (98%) of a panel of plasma exosomal miRNAs associated with immune regulation and inflammation [48].

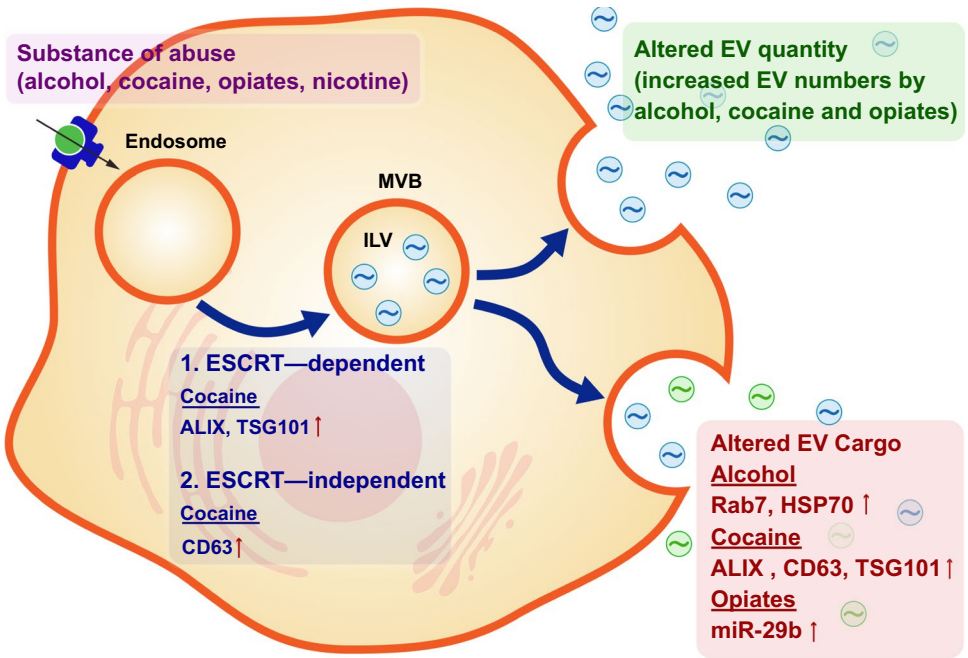

Fig. 1 Substance abuse affects the biogenesis of Extracellular Vesicles (EVs). Drugs of abuse are taken up either by receptor-mediated mechanisms or by diffusion and are encapsulated as endosomes that can fuse with late endosomes to form multivesicular bodies (MVBs) containing intra-luminal vesicles (ILVs). In this process, drugs of abuse alter ESCRT and non-ESCRT components during biogenesis, ultimately resulting in altered EV cargo and/or release

Table 1 RNA and protein content of EVs and potential for use as biomarkers for SUD

Molecule and/or potential biomarker	Drug	Model	Potential source of biomarker	Methods	Change	Function	Ref
RNA species							
miR-27a	Alcohol	Monocytes	Serum	qPCR	Up	M2 monocyte polarization	[20]
Let-7f, miR-29a, and miR-340	Alcohol	Mouse hepatocytes	Serum	RNA-seq and qPCR	Up	Inflammation, liver injury	[43]
miR-122 and miR-155	Alcohol	Mice	Serum	qPCR	Up	Liver damage and inflammation	[44], [44]
miR-122, miR-192 and miR-30a	Alcohol	Mice and humans	Serum	miRNA microarray and qPCR	Up	Liver damage and inflammation	[46]
miR-130a	Cocaine	Macrophages	Serum	RNA-seq and qPCR	Up	Pulmonary smooth muscle proliferation	[47]
miR-146a, miR-126, miR-21 and *let*-7a	Heroin	HIV^+ heroin users	Serum	miRNA microarray and qPCR	Up	Immune regulation and inflammation	[48]
miR-145-3p and miR-181a-5p	Methamphetamine	Rat	Serum	Gene-chip sequencing and qPCR	Up	Neural plasticity and reward circuits	[49]
miR-29b	Morphine	Macaques	CSF, brain	miRNA microarray and qPCR	Up	Regulated PDGF-B and neuronal viability	[50]
Linc00355	Opiates	Human	Urine	qPCR	Up	Cell proliferation	[51]
Malat 1	Opiates	Human	Urine	qPCR	Down	Cell proliferation	[51]
Protein							
CYP2E1	Alcohol	Mice and humans	Serum	Immunoblot	Up	Oxidative hepatocyte injury	[52]
CD40L	Alcohol	Mice hepatocytes	Serum	Chemokine/cytokine array, immunoblot and immunogold EM	Up	Macrophage activation, inflammation	[53]

Increased expression of miR-145-3p and miR-181a-5p has also been reported in serum exosomes from rats exposed to methamphetamine [49]. While reports of lncRNAs in SUD are still scarce, in one study the expression of *LINC00355* and *MALAT1* was found to be significantly lower in urinary exosomes isolated from cigarette smokers and opium-addicted patients with transitional cell carcinoma (TCC) when compared with controls. On the other hand, the expression of *LINC00355* tended to be higher in opium-addicted TCC patients that did not smoke cigarettes compared to opium-addicted smokers [51].

Protein composition of EVs

Proteomic analyses have revealed a set of proteins commonly found in EVs that are routinely used to characterize EVs [3]. Due to their endosomal origin, exosomes contain classical membrane transport and fusion proteins (GTPases, annexins and flotillin), tetraspanins (CD9, CD63, CD81 and CD82), specific stress proteins (Hsc70 and Hsp90), protein members of the ESCRT (Alix and TSG101), and proteins involved in membrane fusion (Rabs and ARF6) [24, 60, 61]. EVs have also been described to contain ADAM10, ACE, EHD4, and major histocompatibility complex [3].

In the context of SUD, Cho et al. reported the increased expression of CYP2E1 in plasma EVs obtained from rats exposed to oral doses of binge ethanol or dextrose controls and also in humans with alcoholism [52]. These EVs from alcohol-exposed rats and patients with alcoholism were shown to be functional and could promote cell death in naïve cells [52]. Verma et al. found that exposure of hepatocytes to alcohol resulted in the release of EVs that contain CD40L in a caspase-dependent manner, which, in turn, led to macrophage activation and inflammation [53]. As of now, the role of EVs containing other types of cargos such as organelles, bioactive lipids, peptides and ions in SUD has not been well studied and deserves attention in the future.

Mechanisms and functions of EVs in SUD

Due to their rich and unique composition and the inherent ability to interact with other cells, EVs play functional roles in many biological processes in the context of SUD, as shown in Fig. 2. EVs from macrophages exposed to alcohol are readily taken up by naïve macrophages leading, in turn, to cellular activation and polarization towards an inflammatory (M2) phenotype [20]. Hepatocytes exposed to alcohol released EVs that contain miRNA cargo that contributes to liver injury and inflammation [43]. In the context of cocaine exposure, macrophage-derived EVs contributed to a significant increase in the proliferation of primary human pulmonary arterial smooth muscle cells (HPASMCs) [47]. Plasma exosomes from HIV-infected heroin users have high levels of neuroinflammation-related miRNAs such as miRs-146a, -126, -21, and -*let*-7a that contribute to immune regulation and inflammation [48]. Plasma EVs released in the context of methamphetamine in rats are involved in the regulation of neural plasticity, reward circuits and the development of addiction [49]. These examples demonstrate the functional roles of EVs in inflammation, immune regulation, cell proliferation, as well as organ injury and damage (Fig. 2). In the following sub-sections, we discuss the current literature on the functional roles of EVs in alcohol, cocaine, marijuana, methamphetamine, nicotine and opioids.

Alcohol

According to the World Health Organization (WHO), alcohol abuse and its related complications contribute to 5% of the global health burden and 6% of total deaths worldwide (WHO 2014) [62]. Alcohol is known to cause liver damage and damage to other organs, including the central and peripheral nervous system, gastrointestinal tract, heart and vascular systems, and endocrine and immune systems [63]. More recently, it has been reported that alcohol intake accelerates several disease conditions such as HIV, tuberculosis and pneumonia (WHO 2014) [62]. Consequently, multiple studies have been carried out to investigate different alcohol-induced hepatic and extrahepatic complications [64, 65]. However, the detailed molecular mechanisms are poorly understood. Interestingly, several reports have identified the effects of alcohol abuse on EV release and altered EV functions, which may be associated with extrahepatic complications [66]. Exposure of human monocytes to alcohol led to increased release of EVs from these cells, which in turn stimulated naive monocytes to polarize into M2

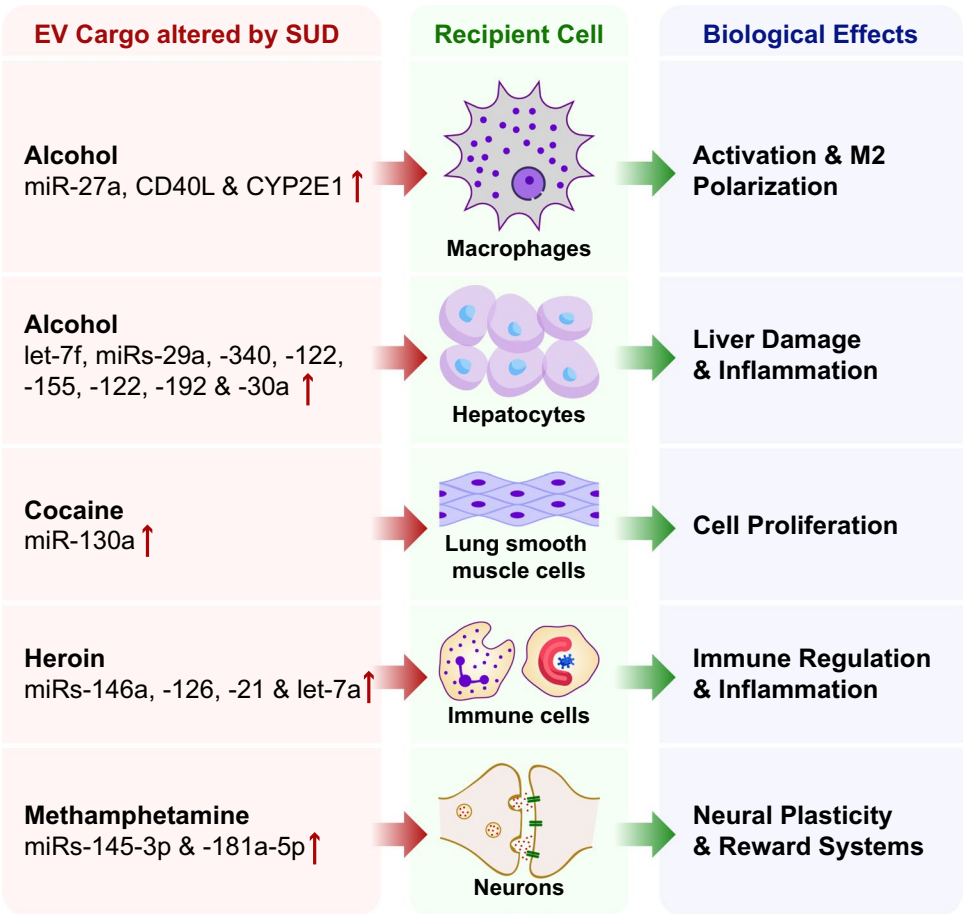

Fig. 2 Extracellular vesicles (EVs) released from cells in the context of substance abuse can exert various biological effects. EVs can activate macrophages and polarize these cells towards an M2 inflammatory phenotype, regulate immune function and inflammation in immune cells, induce liver damage in hepatocytes and modulate neural plasticity and reward circuits in neurons

macrophages [66]. These activated macrophages increased secretion of IL-10, TGF-1β and phagocytic activity. Further studies demonstrated that these effects were mediated by the upregulation of M2-polarizing miR-27a in EVs released from alcohol-exposed monocytes [66]. Another study showed that plasma exosomes that contain substantial amounts of CYP2E1 aggravated alcohol-induced toxicity in both hepatic and monocytic cells [21]. Inhibition of CYP2E1 enzyme activity abrogated the toxic effects in these cells. These authors also validated the induction of plasma exosomal CYP2E1 in a murine alcohol binge drinking model [21].

In a model of alcoholic liver disease (ALD), exposure to alcohol was shown to dysregulate the autophagy pathway and lysosomal function that was accompanied by increased exosome production [67]. In this study, the authors also demonstrated that the release of exosomes in the context of alcohol was regulated by miR-155 [67]. Exposure to alcohol not only affects EV release in peripheral cells but also in the central nervous system (CNS). In line with these findings, ethanol administration to astrocytes increased the number of secreted nanovesicles containing increased amounts of TLR-4, NF-κB-p65, IL-1R, caspase 1, NLRP3, and miR-146a and -182, and reduced amounts of miR-200b [68]. The authors further demonstrated that these EVs were taken-up by neurons, which increased the neuronal levels of the inflammatory protein Cox-2 and miR-146a, compromising the viability of the neuronal cells [68]. A recent report has also shown that exposure of microglia to alcohol resulted in increased exosome biogenesis as well as significantly impacted the morphology, viability and protein content of the microglia [33]. A recent study conducted on humanized mice demonstrated that HIV-infection and ethanol administration increased secretion of human hepatocyte-derived EVs into the serum and the increase in EVs secretion was associated with lysosomal dysfunction [69]. Overall, these studies showed that alcohol abuse impacted several cellular functions by altering the function and content of EVs, which could be considered important targets for abrogating the effects of alcohol abuse.

Cocaine

Cocaine is a naturally occurring and highly addictive stimulant drug. As per the National Survey on Drug Use and Health (NSDUH), there is relatively stable use of cocaine since 2009 [70]. The action of cocaine has been shown to block the functions of the dopamine transporter, thus increasing concentrations of synaptic dopamine in the reward pathways of the brain. In addition, it is well known that exposure to cocaine results in dysregulation of miRNA expression and synaptic plasticity, which, in turn, leads to an increased propensity for the consumption of cocaine. The miRNAs that have been reported to be altered by cocaine include miR-132 [71], miR-181a [72], miR-134 [73], miR-22 [73] and miR-124 [74]. As an example, exposure to cocaine has been shown to decrease the expression of miR-124 in the brain of cocaine-administered rodents [72, 75–77].

Delivery of these miRNAs into the recipient cells could be facilitated by EVs. In this regard, Jarvis et al. demonstrated that cocaine-induced downregulation of astroglial internalization of neuronal CD63-GFP^{+} exosomes resulted in decreased transferred neuron-derived miR-124-3p into astrocytes, which, in turn, lead to decreased GLT1 expression [30]. GLT1 is a protein that regulates synaptic plasticity in the nucleus accumbens (NAc) and is associated with cocaine-seeking behavior [78]. This work thus suggests that EV-miRNA-mediated interaction between neurons and astrocytes could contribute to cocaine addiction. In addition, emerging evidence suggested that cocaine could induce synthesis of the endocannabinoid 2-arachidonoylglycerol (2-AG) in the midbrain, which, in turn, resulted in increased activity of dopaminergic neurons that contribute to cocaine addiction [37]. Nakamura et al. [37] examined a novel pathway by which cocaine induces the release of 2-AG. The authors demonstrated that cocaine increased EV release in a Sig-1R dependent manner. Furthermore, cocaine can also induce the secretion of 2-AG via its interactions with the Sig-1R. This in turn led to the release of 2-AG in EVs, consequently engaging type-1 cannabinoid receptors (CB1) that contribute to cocaine addiction [37]. Another study revealed that cocaine exposure could also increase release of EVs by glioblastoma cells [36]. Sharma et al., demonstrated that HIV–infected and cocaine-treated human monocyte derived macrophages released a higher number of EVs compared to HIV-infected or uninfected cocaine-treated macrophages, with a significant increase in the particle size range to 100–150 nm. These EVs also had increased levels of miR-130a [47]. Overall, these studies showed that exposure to cocaine could increase not only the release of EVs but also the delivery of EV-miRNAs, which, in turn, contributes to cocaine addiction.

Marijuana

A recent study has shown that cannabidiol (CBD) is a potent inhibitor of the release of EVs in cancer cell lines such as prostate cancer (PC3), hepatocellular carcinoma (HEPG2),and breast adenocarcinoma (MDA-MB-231) [79]. It was also shown that cannabinoids sensitize cancer cells to chemotherapy. This study concluded that the anti-cancer effects of CBD are partly due to its effects on EV biogenesis, suggesting that CBD could be considered as a therapeutic agent for targeting EV-mediated pathological events [79]. In another study, exposure of glioblastoma cells to CBD resulted in released EVs containing reduced levels of pro-oncogenic miR-21 and increased levels of anti-oncogenic

miR-126, compared to that of controls [80]. In addition, it was also observed that exposure of glioblastoma cells to CBD resulted in reduced expression of prohibitin, a multifunctional protein with mitochondrial protective properties and chemoresistant functions, suggesting that CBD has implications for the treatment of glioblastoma [80]. Of note, it was observed that EVs released from microglia serve as transporters of endocannabinoids. These endocannabinoids were associated on the surface of the EVs, leading to activation of CB1 and inhibit presynaptic transmission in target GABAergic neurons [81]. Only a few studies have been conducted on the effects of marijuana and its compounds on EVs. However, these reports show a strong effect of CBD on EV biogenesis, which open promising avenues for future research.

Methamphetamine

Methamphetamine is a potent psychostimulant that is among the most commonly used illicit drugs. There are over 35 million users worldwide, thus making methamphetamine abuse a significant global health crisis [82]. Emerging studies have demonstrated that acute and chronic doses of methamphetamine exposure resulted in long-term damage in many brain regions, leading to neurocognitive impairment. However, the mechanisms by which methamphetamine mediates neurotoxicity are still largely unknown.

The effect of methamphetamine on miRNA delivery via EVs has not been examined in detail. In one study, increased expression of miR-145-3p and miR-181a-5p was observed in the serum exosomes from methamphetamine exposed rats [49]. Another study in humans with methamphetamine use disorder demonstrated that the level of miR-9-3p was significantly increased in methamphetamine abusers compared with normal controls [83]. Furthermore, an in vivo study demonstrated that methamphetamine treatment increased the release of endothelial cell-derived EVs with Annexinv^{+}/CD144^{+}/CD41^{-}/CD31^{+} phenotype [84]. These studies support the idea that EVs could serve as an efficient carrier of miRNAs contributing to methamphetamine-mediated neurotoxicity.

Studies have shown that methamphetamine use can exacerbate HIV-1 infection and HIV-associated neuropathogenesis [85–89]. Since methamphetamine exposure can facilitate the release of EVs [84] with HIV-1 components such as Nef proteins [90, 91] and TAR RNA [92, 93], EVs could play an essential role in the development of neuropathogenesis in HIV-1 + methamphetamine users [94].

Nicotine

Smoking of cigarettes is known as a leading cause of preventable disease and premature death all over the world. In the United States, approximately 435,000 people die prematurely from smoking-related diseases each year; overall, there is approximately a 50% chance that a lifelong smoker will die from a complication of smoking [95]. Cytokine profiling analysis revealed that the levels of plasma EV IL-8 and IL-6 expression was significantly upregulated in HIV-positive smokers compared with HIV-positive non-smokers and HIV-negative subjects, respectively [96]. The cytochromes P450 (CYPs)-mediated metabolites of Benzo[a]pyrene (BaP), a major carcinogen in cigarette smoke, have been shown to induce HIV-1 replication [97]. The levels of CYPs 1A1, 1B1, 3A4 were significantly upregulated in EVs derived from HIV-infected U1 cells treated with cigarette smoke condensate (CSC) compared with EVs derived from uninfected U937 cells treated with CSC [98], suggesting upregulated CYPs in EVs could contribute to the enhancement of HIV replication in macrophages. Interestingly, EVs released from CSC-exposed monocytic cells exhibited a protective effect against cytotoxicity [99], indicating a clinical value of EVs as proposed previously [100].

A recent study has demonstrated that nicotine exposure could result in the release of atherogenic exosomes from macrophages. These miRNA-containing exosomes mediate cellular crosstalk which, in turn, leads to proatherogenic phenotypes of vascular smooth muscle cells (VSMCs) [101]. The nicotine-mediated development of atherosclerosis is driven via macrophages-derived miR-21-3p inducing migration and proliferation of VSMC through its target phosphatase and tensin homolog (PTEN) [101]. In addition, nicotine has been shown to increase levels of circulating endothelial cell-derived and platelet-derived EVs, which could be the mechanism by which nicotine induces cardiovascular disease [102]. Although not many studies have been conducted on the effects of nicotine on EVs, the few reports show that EVs may serve as potential carriers of behavior-altering miRNAs that underly the mechanism(s) by which nicotine mediates the pathogenesis of several chronic diseases.

Opiates

Opiates are analgesics extensively used in clinical settings as well as drugs of abuse [103]. Chronic exposure leads to several complications leading to addiction, tolerance and cognitive impairment etc. [104]. EVs derived from morphine-stimulated astrocytes were shown to be taken up by microglial cells which caused activation of the TLR-7-lincRNA-Cox2 axis resulting in impaired microglial phagocytosis [105]. Additionally, intranasal delivery of EVs loaded with lincRNA-Cox2 siRNA restored microglial phagocytic activity in mice administered morphine, suggesting a role for EVs in morphine mediated dysregulation of microglial phagocytosis [105]. In another study, EVs derived from

astrocytes that were exposed to morphine and HIV protein Tat were shown to contain miR-29b. When neuronal SH-SY5Y cells were exposed to these EVs, there was decreased expression of PDGF-B along with decreased viability of neurons. miR-29b was identified to target PDGF-B mRNA resulting in translational repression in SH-SY5Y cells. This study demonstrated the important role of miR-29b in the EVs and its regulation of PDGF-B in HIV-infected opiate addicts [50]. Moreover, morphine has also been shown to induce the expression of miR-138 in morphine-stimulated astrocyte-derived EVs, which can be taken up by microglial cells and, in turn, activates the TLR7-NF-kB axis and ultimately leading to microglial activation [106].

Several miRNAs, namely miR-15b, 181, 125b, and the *let*-7 family, have been implicated in morphine-induced tolerance as well as expression of the μ-opioid receptor. Chronic morphine treatment led to time-dependent increased expression of *let*-7 both in in vitro and in vivo models, which was associated with tolerance [107]. It has also been shown that exosomes loaded with μ-opioid receptor siRNA can effectively be used as treatment for morphine relapse [108]. Detailed studies on EVs from opiate-exposed cells as well as addicts will be necessary for developing strategies to cope with opioid tolerance leading to addiction.

EVs as potential biomarkers for SUD

The literature reviewed here clearly shows that several substances of abuse such as alcohol, cocaine, marijuana, methamphetamine, nicotine and opiates modulate the release of EVs and alter the constituents of these EVs. Besides their roles in cell-to-cell communication, EVs have the potential to serve as potential biomarkers since their counts, content, and origin might provide useful information about pathophysiology. Consequently, several research groups are interested and focused on examining the role of these EVs as potential biomarkers. The potential to use EVs as biomarkers for the diagnosis and prognosis of diseases is supported in part by the stability of exosomal cargo in plasma [109, 110]. In addition, EVs can easily be obtained from blood and urine. In fact, EVs have long been considered as sources of potential molecular biomarkers for the early detection, monitoring and evaluation of drug response in various diseases [111]. In this section, we discuss the potential of EVs as biomarkers of SUD.

Several types of biomarkers can be used in liquid biopsies. Table 1 summarizes potential biomarkers based on the altered composition of EVs in several SUD involving alcohol, cocaine, marijuana, methamphetamine, nicotine and opioids. To our knowledge, there are currently no universal biomarkers associated with SUD; however, the difference in EV composition may serve as potential biomarkers. Given that EVs can cross the blood–brain barrier, brain-derived EVs in the plasma could serve as biomarkers of neuropathogenesis [112–115]. For example, the numbers of neuron-derived EVs in the plasma of neuropsychologically impaired individuals were decreased compared with normal controls [115]. The levels of high-mobility group box 1 (HMGB1), NF-L, and amyloid β proteins were upregulated in the plasma neuron-derived EVs from neuropsychologically impaired individuals were decreased compared with normal controls [115]. Additionally, astrocytic and neuronal-specific proteins—GFAP and L1CAM—are elevated in the plasma EVs from HIV-positive alcohol or tobacco users compared to HIV-positive nonsubstance users [112].

A study on ethanol-fed mice showed that increased CYP2E1 levels in EVs could serve as a general marker of liver injury [52]. Likewise, increased levels of three miRNAs (*let*-7f, miR-29a, and miR-340) in the blood EVs are associated with alcoholic steatohepatitis (ASH) in mice [43]. Four miRNAs (miR-146a, miR-126, miR-21, and *let*-7a) were also found elevated in the plasma of HIV-1 infected heroin users, which make them potential biomarkers for diagnosis and prognosis of the neuroinflammatory disease [48]. High specificity and sensitivity of lncRNAs *UCA1-201, UCA1-203, MALAT1,* and *LINC00355* have been reported previously to have potential for biomarkers in the diagnosis of bladder cancer in opium-addicted and cigarette smokers [51]. Presence of elevated levels of miR-145-3p and miR-181a-5p in serum EVs has been associated with methamphetamine addiction [49]. Three miRNAs, including let-7b-5p, miR-206, and miR-486-5p, were verified to be significantly and steadily increased in heroin abusers [53] and miR-9-3p was significantly increased in methamphetamine abusers compared with normal controls, demonstrating their ability as biomarkers [83]. It is interesting to note that in most of these studies listed in Table 1, not only one miRNA is altered, but also several of them. This suggests the need to develop panels of miRNAs as biomarkers that also need validation in large cohorts of study participants. Although EVs demonstrate promise as potential biomarkers, their clinical applicability is currently limited by lack of well-powered clinical studies investigating the correlation between EV biomarkers and SUD or SUD-related organ injury.

EVs as potential therapeutic vehicles for SUD

Substance abuse has been demonstrated to increase the release of endogenous EVs and alter the composition of the EVs that are released [36], demonstrating a reliance of the host system on EV signaling in response to drug exposure. Several studies have evaluated EVs as therapeutic vehicles because of their ability to carry diverse payloads, their

favorable immunogenic profiles, stability in circulation, biocompatibility, and low toxicity [105, 116]. Though there are many benefits to using EVs as therapies, potential side effects should also be considered. For example, EVs and their cargo have been shown to induce inflammation [117]. Full characterization and evaluation of EV properties such as cargo and source are required to better understand the promise of EV-based therapies. Moreover, optimizing tissue-targeted delivery of EVs remains one of the major challenges in the field. Therapeutic administration of engineered EVs could regulate cellular signals in the brain that perpetuate substance use and addiction as well as decrease the end-organ injury caused by substance use. Despite multiple studies addressing the miRNA and protein signaling involved in the abuse of nicotine [118], alcohol [119], opiates [120], cocaine [121], and cannabinoids [122] (Table 2), there are still minimal data directly demonstrating the EV-mediated shuttling of these molecules. Those that have been published are described below.

Decreasing substance dependence and relapse

Some of the earliest research into EV therapy in substance dependence and relapse focused on alcohol exposure. Chronic alcohol consumption is known to cause neuroinflammation resulting in CNS toxicity. This pro-inflammatory state appears to play a role in propagating additional voluntary alcohol consumption in animals [123]. As such, it has been hypothesized that the anti-inflammatory effects of mesenchymal stem cell (MSC)-derived EVs may decrease chronic alcohol consumption. A study performed in rats that were chronically consuming alcohol demonstrated that intranasal administration of MSC-derived exosomes inhibited alcohol intake by 84%,

Table 2 Selected miRNAs and proteins associated with substance addiction, withdrawal, and relapse that may be targeted by therapeutic EV-mediated delivery

Molecule	Drug	Function	Reference
miRNA			
miR-27a	Alcohol	M2 monocyte polarization	[20]
miR-124	Alcohol	BDNF downregulation	[125]
miR-206	Alcohol	BDNF downregulation	[126]
miR-9	Alcohol	Ca^{2+} and K^{+} channel expression	[127]
miR-431	Cocaine	Arc expression	[128]
miR-212	Cocaine	CREB activation	[129, 130]
miR-101b	Cocaine		[128]
miR-132	Cocaine	CREB and BDNF-mediated synaptic plasticity	[128, 130]
miR-137	Cocaine		[128]
miR-190	Fentanyl	μ opioid receptor expression	[131]
miR-218	Heroin	Gabrb3, GluR2, Ube3a, Nrxn1, Gng3, and Mecp2 expression	[132]
Let-7d	Marijuana	CB_1 receptor signaling	[122]
Let-7a/c/g	Morphine	μ opioid receptor expression	[107]
miR-27a	Morphine	Serpini1 expression	[133, 134]
miR-29b	Morphine		[50]
miR-140-5p	Nicotine	Inhibits Dynamin-1 expression	[135]
miR-504	Nicotine	Upregulates dopamine D1 receptors	[136]
miR-542-3p	Nicotine	Increased nicotinic acetylcholine receptors	[137]
Protein			
GLT-1	Alcohol	Glutamate transport	[138, 139]
mTORC1	Alcohol	Protein synthesis/translation	[140]
LGALS3	Cocaine (+HIV)	Neuronal migration	[141]
GLUL	Cocaine (+HIV)	Glutamate detoxification	[141]
HBB/HBD	Cocaine (+HIV)	Learning and memory	[141]
MCP-5	Methamphetamine	Chemokine	[142]
sTNFR1	Methamphetamine	Chemokine	[142]
NMDAR1	Morphine	Glutamate receptor	[143]
p-CREB	Morphine	Cellular transcription	[144]
Arc/Arg3.1	Morphine	Synaptic plasticity/memory	[145, 146]

decreased relapses, and fully reversed neuroinflammation and hippocampal oxidative stress [124].

Since the study described above, much of the literature assessing the use of EVs as therapeutics for dependence and relapse has focused on opioid use. Exosomes from SH-SY5Y neuroblastoma cells have been pre-treated with sinomenine, an alkaloid used to prevent morphine dependence. When sinomenine pre-treated exosomes are administered to morphine-treated SH-SY5Y cells, the cells demonstrate a decrease in cAMP expression, intracellular Ca^{+}, and expression of p-CREB/CREB compared to exosomes pre-treated with saline [143]. In our previous work, we demonstrated that morphine treatment is associated with an increase in exosomal miR-29b expression from astrocytes. When administering astrocyte-derived exosomes containing miR-29b to Tat protein-treated SH-SY5Y cells, we demonstrated attenuation of PDGF-BB expression and increased neuronal cytotoxicity [50]. Although this study focused on opioid effects in the context of HIV infection, it successfully demonstrated the impact of morphine on the exosomal delivery of miRNAs with a correlation to neuronal protein expression and cell survival.

Extracellular vesicles-mediated therapy may also be able to target substance use relapse. A study by Liu et al. demonstrated that engineering the membrane surface of EVs to express the rabies virus glycoprotein (RVG) peptide effectively delivers μ-opioid receptor siRNA into the brain, leading in turn to downregulation of μ-opioid receptor expression [108]. Importantly, delivery of μ-opioid receptor siRNA loaded EV to the brain prevented relapse in a mouse model of morphine addiction [108].

Additionally, other non-EV carriers have been used for suppression of drug addiction. For instance, the administration of glial cell line-derived neurotrophic factor (GDNF)-conjugated nanoparticles has been shown to decrease the amount of cocaine self-administration in rats [147]. More recently, exosomes have been found to carry pathogen antigens known to evoke immune response, and have, therefore, been examined as carriers for vaccination against various disease processes [148]. Although EVs have not yet been used for vaccination against addiction, nanoparticle-delivered toll-like receptor-based adjuvants have been shown to reduce the level of nicotine entering the brain and may therefore be a promising approach for treating nicotine addiction [149].

Role in repairing end-organ injury induced by SUD

Extracellular vesicles are known to play a major role in the inflammatory response of alcohol-induced liver disease (ALD) through several signaling pathways, including activation of Hsp90, Bax, and caspase-3 [19, 52]. Thus, the administration of exogenous engineered EVs or targeted modulation of endogenous EVs could result in decreased inflammation and fibrosis after ALD. Hepatic stellate cells are liver-specific mesenchymal cells that facilitate repair of the injured liver through deposition of fibrillar collagens. The continued activation of these cells in chronic disease processes such as ALD results in fibrosis, in part due to over-expression of the CCN2 protein [150]. Delivery of miR-214 enriched hepatic stellate cell-derived exosomes to either activated stellate cells or hepatocytes decreases the expression of CCN2 and may protect against fibrosis [151]. Stem cell-mediated recovery of liver injury may be mediated by glutathione peroxidase 1 (GPX1) [152].

Exosomes or exosome-mimetic nanovesicles from hepatocytes can also be used to aid in liver regeneration after ALD. The use of exosome-mimetic nanovesicles generated through serial extrusion of primary hepatocytes through polycarbonate membranes enhanced sphingosine kinase 2 (SK2) after delivery to hepatocytes, resulting in hepatocyte proliferation and liver regeneration [153]. A similar study used exosomes derived from primary murine hepatocytes and also demonstrated transfer of ceramidase and SK2 to injured hepatocytes, resulting in increased cell proliferation and liver regeneration both in vitro and in vivo [154].

Of note, the origin cell for EVs appears to be important in providing the regenerative effects in ALD. The promotion of hepatocyte proliferation was seen with administration of stellate cell- and hepatocyte-derived exosomes but was not demonstrated with exosomes derived from other liver cells such as Kupffer or sinusoidal endothelial cells [154]. Exosomes derived from non-liver stem cells may also provide benefit to the injured liver [155], however, and have the added benefit of potentially providing benefit to other non-liver organs when administered systemically.

Other organs have also been targeted for protection or repair by nanoparticle formulation, including using cerium oxide nanoparticles to inhibit reactive oxygen species production and cell death in cardiomyocytes after cigarette smoke exposure [156].

The future of EV-mediated therapies for SUD

There are no active clinical trials of EV-mediated therapies in drug abuse currently registered in Clinicaltrials.gov, though there are currently more than 20 active NIH-funded projects addressing this question. Most of these studies will provide additional pathophysiological insight into the effects of drug abuse on endogenous EV release and content. Three of the studies specifically focus on using EVs as potential therapeutic avenues. Given the widespread interest in EV signaling, it is likely that the literature in this field will continue to rapidly expand over the next several years.

Conclusions and perspectives

The literature reviewed and summarized here demonstrate the variety of cargo transported by EVs and their effects on biological functions in the context of SUD. The research to date has clearly highlighted the role of EV-mediated transfer of RNA (miRNAs and lncRNAs) and proteins that play important roles in immune regulation, inflammation, cell proliferation and organ injury. Additionally, EV features (number, size distribution, charge, etc.) and cargo (RNAs, DNAs, proteins) could serve as biomarkers and indicators for various human diseases, including SUD. The development of high-sensitivity single EV analysis techniques would significantly advance the potential to use EVs as biomarkers for diseases. Finally, the unique ability of EVs to cross biological barriers, such as the blood–brain barrier, makes EVs ideal for the delivery of therapeutics. Indeed, some studies have demonstrated this possibility, and studies on specific organ and cell type delivery of EVs are underway. All in all, the functional and application roles of EVs in the context of SUD open exciting possibilities for diagnostic and therapeutic advances.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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