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DNA Methylation, Substance Use and Addiction: a Systematic Review of Recent Animal and Human Research from a Developmental Perspective

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Abstract Growing evidence points to the role of epigenetic mechanisms, including DNA methylation, in substance use and addiction. We conducted a systematic review of 47 recent (2012–2015) animal and human studies that investigate DNA methylation and substance use/exposure, spanning preconception to adulthood. The majority of extant studies (i) focused on exposure during adulthood, (ii) examined the effects of alcohol use, (iii) employed a candidate gene approach, and (iv) were cross-sectional. While studies generally support an association between substance use/exposure and DNA methylation and also suggest that developmental context and timing matter, a dearth of longitudinal data and low comparability across studies currently limits the conclusions that can be drawn. Future challenges and directions for the field are discussed.

Keywords Epigenetics · DNA methylation · Addiction · Alcohol · Drug · Review

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Introduction

Addiction to psychoactive substances (e.g., alcohol, illicit drugs) is a debilitating condition characterised by compulsive drug-seeking and repeated harmful use, despite adverse consequences [1]. Like other complex diseases, addiction results from both genetic and environmental factors, which combine to exert additive, evocative, and interactive effects across the lifespan [2•]. How such gene-environment associations operate at a molecular level, however, remains unclear. In recent years, epigenetic mechanisms have been proposed as a potential candidate, as they respond to both genetic and environmental influences [3, 4••] and are thought to mediate vulnerability to disorders, including addiction [3, 4••].

Epigenetic mechanisms, such as DNA methylation (DNAm) regulate when and where genes are expressed without changing the DNA sequence itself [5]. DNAm refers to the addition of a methyl group, primarily in the context of cytosine-guanine (CpG) dinucleotides. The genome contains an excess of 28 million CpG sites, around 10 % of which cluster into CpG 'islands', close to gene promoter regions [6]. Methylated CpG islands impede transcription factors from accessing the DNA sequence. As such, DNAm is typically associated with decreased gene expression, although the functional role of methylation changes within genomic regions other than CpG islands (e.g., intergenic regions) remains unclear [7•]. Importantly, DNAm is dynamic across the lifespan—although patterns are mitotically stable, which can lead to long-term alterations in gene activity, they also show a considerable degree of flexibility over time, enabling cells to respond to changing internal and external inputs [8].

A growing number of studies have begun to clarify the role of DNAm in substance abuse and addiction. Experimental studies in animals have led the way, documenting a number of important findings. First, substance use can alter DNAm—

for example, repeated administration of substances (e.g., alcohol, cocaine) has been found to modify methylation patterns in the reward regions of the brain (e.g., striatum [9]). Second, DNAm contributes to the pathophysiology of addiction. Specifically, drug-induced methylation changes have been shown to influence the expression of genes involved in synaptic plasticity and memory consolidation, which in turn drive long-term neuroadaptations underlying the onset and persistence of addictive behaviours [4••]. Third, animal studies have begun to shed light on the role of developmental context on DNAm and addiction risk. For example, alcohol intake during the first half of pregnancy has been found to alter epigenetic patterns in the developing embryo, leading to reduced fetal growth and congenital abnormalities similar to those observed in human fetal alcohol syndrome, as well as subsequent risk for addiction [10].

So far, studies in humans have provided initial support for animal findings, reporting methylomic differences between substance abusers and drug-free controls across a number of substances and tissue types [9, 11••]. However, unlike animal studies that make it possible to experimentally manipulate the type, extent, and timing of substance exposure, studies in humans have been primarily cross-sectional and correlational, making the causal links between epigenetic changes and subsequent addiction more problematic to draw.

The aim of this systematic review is three-fold: (i) to collate findings from recent animal and human research investigating the link between substance exposure, DNAm, and addiction; (ii) to consider the relevance of timing of substance exposure, beginning in preconception through to adulthood; and (iii) to outline future directions for the field.

Methods

Inclusion Criteria

We included studies that investigated associations between DNAm and substance use/exposure. In line with the journal's focus on current research, we only included articles published during the past 3 years (1 January 2012 to 31 February 2015). No restriction was applied regarding (i) species (e.g., human, mouse), (ii) period of exposure (e.g., prenatal, adulthood), (iii) substance (e.g., alcohol, cocaine), (iv) tissue (e.g., blood, brain), (v) approach (e.g., candidate vs genome-wide), and (vi) design (e.g., cross-sectional vs longitudinal).

Search Strategy

PubMed and PsychInfo were searched for relevant studies written in English. Search terms were applied in MeSH or index terms, as well as text words. Included terms related to either (i) DNA methylation (e.g., methylat*; epigen*), or (ii) substance (e.g., substance use, abuse, dependence; drug; addiction; cocaine; heroin; cannabis; alcohol; opiate*; smoking; tobacco). 'Cancer' and 'medication' were specified as exclusion terms to avoid studies investigating DNAm in relation to clinical drugs.

Study Selection

Our search yielded 621 records, with 381 remaining after filtering out duplicates (see Fig. 1). Titles and abstracts were screened, and studies were excluded if they were not empirical (e.g., reviews), focussed on epigenetic mechanisms other than DNAm (e.g., histone modifications), or examined drugs other than the ones specified above (e.g., clinical drugs). Given that the majority of DNAm studies on tobacco use examined medical diseases (e.g., cancer) as opposed to addiction-relevant phenotypes, studies with tobacco were not included in the review. Sixty-one studies were retained, and their full text articles were assessed for eligibility. Sixteen articles were removed due to the following reasons: (i) six did not include DNAm data; (ii) six did not report direct associations between DNAm and substance use/exposure; (iii) two did not include substance data; (iv) one was published before 2012; and (v) one was based on cell culture data. A total of 45 original reports were therefore included in the systematic review.

Results

Descriptive Summary

Study characteristics are summarised in Table 1 (see also Fig. 2). Twenty-four studies examined animal samples (n_{rat}=14 and n_{mouse}=10) and 21 examined humans. The majority of studies investigated substance exposure during adulthood (n=33), focused on alcohol (n=36), involved peripheral samples (n=25), examined DNAm at a single time point (n=40), and used a candidate gene approach (n=26). The most common peripheral tissue examined was blood (n=18), followed by liver, sperm, pancreas, saliva, placenta, kidney, intestine, and colon. Most commonly examined central tissues were prefrontal cortex (n=5) and hippocampus (n=4), followed by nucleus accumbens, hypothalamus, amygdala, cerebellum, ventral tegmental area, striatum, and neocortex. Below, we describe findings first in animals and then in humans, in order of developmental period of substance exposure.

Animal Studies

Preconception

Three candidate gene studies investigated parental alcohol use prior to conception. In the first, paternal consumption in mice

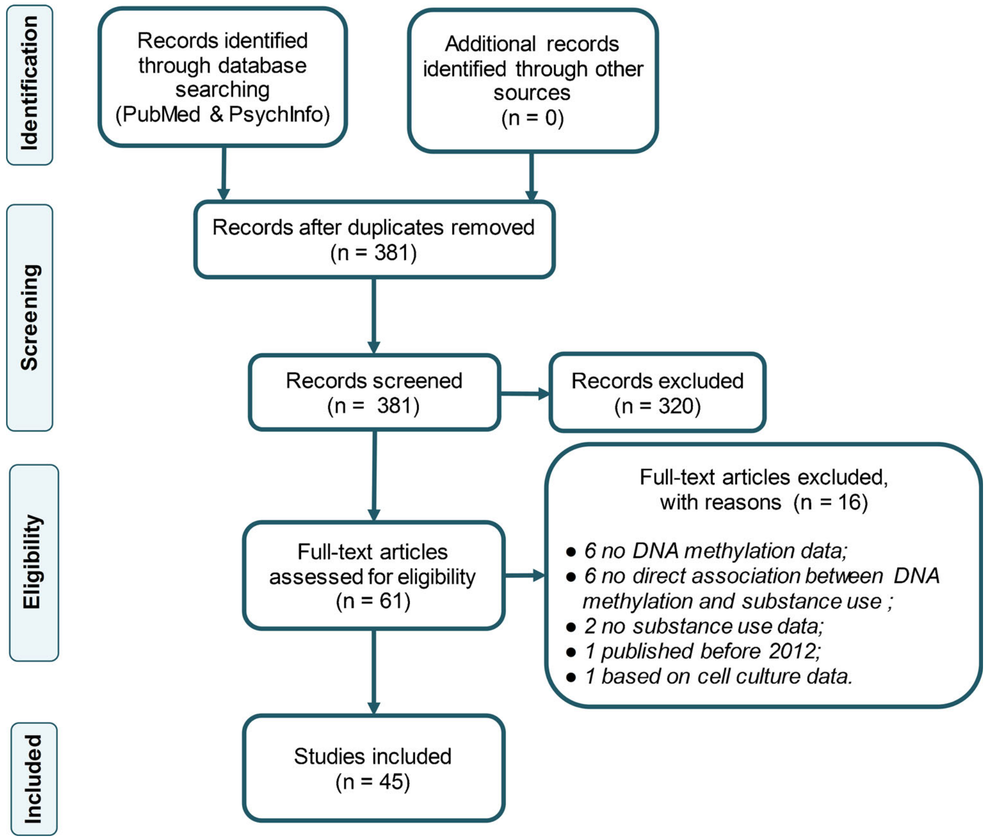

Fig. 1 PRISMA flowchart detailing the filtering steps undertaken to select studies

related to decreased DNAm of *Bdnf*—implicated in stress response and neural development—in paternal sperm cells and offspring ventral tegmental area [12]. In the second, paternal alcohol consumption in rats was associated with increased *Pomc* methylation (another gene relevant in stress response) within both parental sperm and offspring hypothalamus, although findings were specific to the male germline [13]. In contrast, the third study [14] found that DNAm in *H19* CTCF binding sites—involved in imprinting mechanisms—was reduced in offspring tail blood but not in paternal sperm cells.

Prenatal

Three studies from the same working group found that prenatal alcohol exposure associated with increased *Pomc* methylation in the rat hypothalamus, which in turn related to decreased gene expression [13, 15, 16]. These changes were maintained transgenerationally (up to three generations), but could be rescued by gestational choline supplementation. Epigenome-wide associations between prenatal alcohol exposure and DNAm in brain tissue from adult offspring were identified by one study, particularly within genetic pathways related to nervous system development (including the *Cdk5* signalling pathway) and neurological diseases, including the Alzheimer's disease-linked gene *App* [17]. Another study found that in utero, exposure to methamphetamine was associated with aberrant hippocampal DNAm in adolescent mice offspring [18]. Hypermethylated genetic pathways related to cerebral cortex GABAergic interneuron differentiation, while hypomethylated pathways related to embryonic development.

Neonatal

Two mouse studies investigated the effect of neonatal alcohol exposure on global methylation within the hippocampus and neocortex [19, 20]. While the first study reported a *reduction* in global methylation in response to acute alcohol exposure (8 and 24-h postexposure; [20]), the second study observed an *increase* in global methylation in the exposed group across both regions, which could be partially ameliorated by choline treatment [21]. Although of interest, it is important to note that neonatal substance exposure may be less relevant to human studies compared to other developmental periods, as it is relatively uncommon in humans.

Adulthood

This developmental period received by far the greatest research attention (71 % of animal studies, $n=17$) and was primarily examined in relation to alcohol exposure ($n=11$). In global methylation studies, exposed mice were found to have

Table 1 Summary of study characteristics

Summary table (N=45)	
Developmental period	
Preconception	3
Prenatal	6
Neonatal	3
Childhood	0
Adolescence	1
Adulthood	33
Time points (DNAm)	
1 TP	40
2 TP	3
3 TP	2
Approach	
Candidate gene/s	26
Global DNAm	12
EWAS	9
Species	
Animal	24
Human	21
Tissue	
Peripheral	25
Central	21
Substance	
Alcohol	36
Cocaine	6
Cannabis	6
Opiates	3
Methamphetamine	2

N.B. The total number of studies for each characteristic may exceed 45 due to the presence of studies fitting multiple domains. To clarify, global methylation studies examine proxy markers of 'global' DNAm, using repetitive elements such as Alu and Line-1 (comprising of 11–17 % of the genome). Candidate studies focus on DNAm in individual, preselected genes (typically one) based on a priori hypotheses, while epigenome-wide studies (EWAS) are hypothesis-free and investigate thousands of DNAm markers across the genome

EWAS epigenome-wide association studies

lower DNAm in the cerebral cortex [21] but not in liver [22], although reductions were reported in global DNA hydroxymethylation (another type of DNA modification, characterised by the addition of a hydroxymethyl group). Findings from candidate gene studies further indicated that alcohol exposure in adulthood is associated with increased DNAm in multiple genes, including the serotonin receptor *Htr3a* in blood and hippocampal tissue [23], the sodium transporter *Slc5a6* gene in pancreatic tissue [24], and immune function TLR-pathway genes in the liver [25]. Decreased DNAm was instead observed for the glutamate gene *Nr2b* in the prefrontal cortex [26] and *Bdnf* gene in motile sperm [12]. Tissue- and gene-specific DNAm alterations were identified by one study in folate-regulating genes [27]. Finally, no associations were found in opioid-related genes *Pdny* and *Pnoc* in the rat amygdala [28], as well as imprinting-control genes *H19* and *Rasgfl* in mouse sperm [14].

Seven studies examined DNAm in relation to cocaine and/or opiate exposure. No associations with global methylation were reported in the corpus callosum of cocaine-exposed rats after 1 or 30 days of forced abstinence [29], as well as cocaine or heroin-exposed mice [30]—although a specific reduction in hydroxymethylation in the liver following cocaine administration was reported within the same sample [31]. Drug- and tissue-specific effects were also identified in the study by Tian et al. [32] where global DNAm reductions were evident in the prefrontal cortex (but not in the nucleus accumbens) of mice exposed to cocaine (not heroin)—an effect that was reversible through repeated administration of methionine. With regard to candidate genes, increased *Drd2* receptor methylation was observed in the nucleus accumbens of rats exposed to glucocorticoids in utero [33]. This association was specific to morphine administration and reversed by L-dopa treatment. Also in the nucleus accumbens, repeated SAM pretreatment was found to modify cocaine-induced methylation changes in the neuropeptides *Cck* and *Gal*, as well as the glutamate transporter *Slc17a7* [34]. Pol Bodetto et al. [35] reported that methylation of *Pp2cβ*, a gene involved in cellular function, was higher in the brain of cocaine-exposed rats versus controls. Finally, in a study investigating myelin-producing genes, reduced mean DNAm of *Sox10* was identified in the corpus callosum of cocaine-exposed rats, particularly after a period of forced abstinence [29]. None of the studies examined epigenome-wide alterations in response to adult substance exposure.

Human Studies

Prenatal

Two studies examined DNAm in relation to prenatal alcohol exposure. Wilhelm-Benartzi et al. [36] found that maternal alcohol intake positively associated with global LINE-1 (but not with AluYb8) methylation in placental tissue. One candidate gene study found that cord blood methylation of the developmental gene *ZAC1* positively correlated with prenatal maternal alcohol intake as well as associating with reduced fetal and postnatal weight [37].

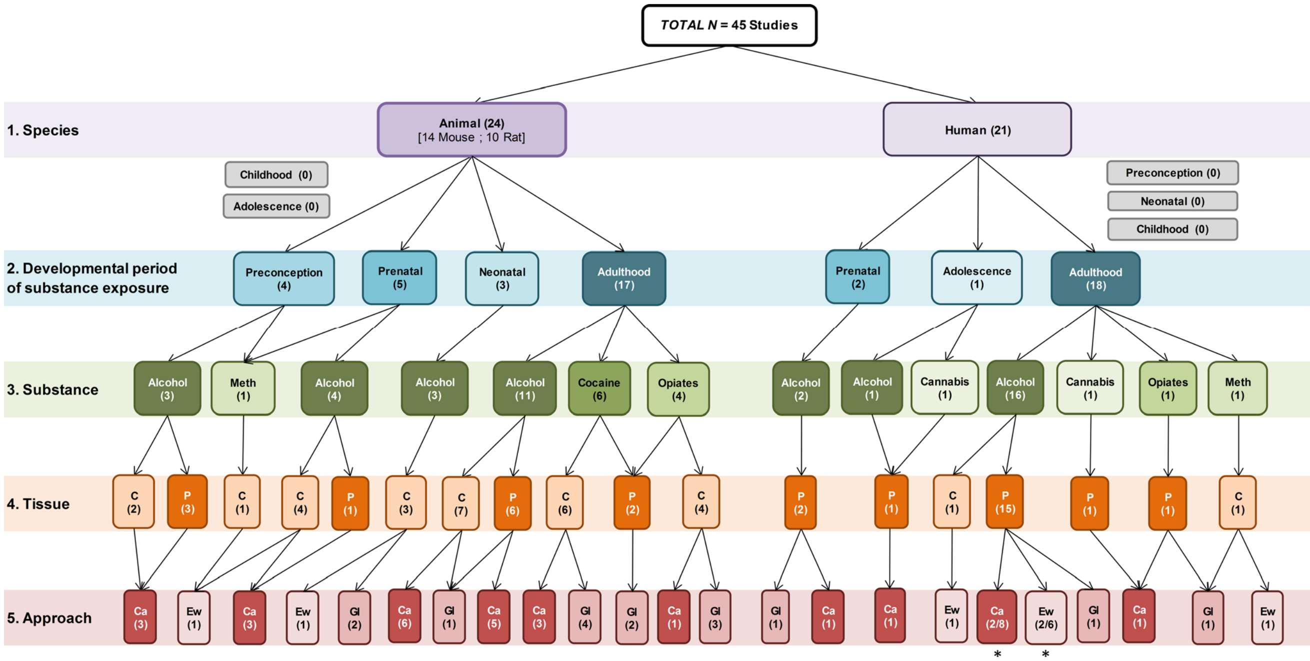

Fig. 2 Flowchart of study characteristics. N.B. The same study may fit into multiple categories. *Grey-shaded boxes* represent developmental periods that have not been investigated. For each level (1–5), *boxes* are shaded according to study frequency (e.g., the least frequently investigated tissue is represented by *lighter shading*, while the most commonly investigated tissue is represented by *darker shading*). *Boxes* marked with an *asterisk* indicate presence of longitudinal studies with repeated measures of DNA methylation (here, *boxes* indicate the number of longitudinal studies out of the total number of studies). *Meth* methamphetamine, *C* central tissue (brain), *P* peripheral tissue, *Ca* candidate gene, *Ew* epigenome-wide, *Gl* global

Adolescence

Only one study examined adolescent substance use. Researching the impact of cannabis smoking on whole blood *COMT* methylation (important for neurotransmitter catalysis), van der Knaap et al. [38] found no main effect of cannabis use. However, a significant methylation by genotype interaction was identified, where Met/Met carriers with higher DNAm were least likely to be frequent cannabis users.

Adulthood

Eighty-five per cent of studies in humans (n=18) examined adult substance use, again focusing primarily on alcohol exposure (n=16). One global methylation study found decreased DNAm in the blood of alcohol drinkers (Alu, not LINE-1 [39]). Results contrast those of increased global methylation identified in the frontal cortex of HIV+ methamphetamine users versus non users [40], as well as in the blood of methadone-substituted former opiate addicts, an effect which was also replicated in independent sample of opioid-treated patients [41].

Candidate gene studies focused mainly on genes involved in neural function, most likely guided by existing neurochemical data regarding addiction on animals and humans. Higher DNAm was observed in the blood of alcohol-dependent individuals within the *HTR3A* serotonin receptor gene [42] and *OPRM1* opioid receptor gene [43]—an association that was also identified in opiate addicts [41]. Lower DNAm of the leptin hormone (*LEP*) gene was instead identified in the blood of patients with stronger alcohol cravings [44]. No significant associations were reported between alcohol use and DNAm in a number of genes, including *PDNY* and *PNOC* opioid-related genes (blood; [42]), the serotonin transporter *5-HTT* in females exposed to trauma (alcohol, cannabis; [45]), the *DAT* dopamine transporter in blood [46], and the drug metabolism gene *UGT1A1* in human liver [47]. Of the candidate gene studies reviewed, two featured repeated measures of DNAm, comparing alcohol-dependent cases versus controls at baseline, day 7 and day 14 posttreatment admission. While the first found significant differences in DNAm of volume-regulating neuropeptides *AVP* and *ANP* both at baseline and between day 7 and 14 of withdrawal [48], the second [49] reported increased nerve growth factor (*NGF*) methylation in cases versus controls, but only between day 7 and 14.

All epigenome-wide investigations focussed on the effect of alcohol in blood. Generally, results were mixed, depending on sample characteristics and methods. In terms of specific genes, two epigenome-wide association studies (EWASs) confirmed associations with alcohol metabolism-related genes, including alcohol and aldehyde dehydrogenases (*ADH1A*, *ADH7*, *ALDH3B2*, *ALDH1A2*) and cytochrome P450 2A13 [50, 51]. In one study [52], the tumour suppressor gene *BLCAP* and *ABR*—involved in vestibular morphogenesis—were hypomethylated in heavy alcohol drinkers versus abstinent controls, suggesting one mechanism by which tumour risk may be higher in alcohol drinkers. In another study, alcohol-dependent discordant siblings showed hypomethylation of *SSTR4*—an important gene for hormonal function—and hypermethylation of the GABA receptor gene *GABRP* [53]. Finally, two EWASs measured DNAm at multiple time points: before and after a 25-day treatment programme [54], or a 12-year interim period [55]. While the former [54] found no significant differences pre-vs-post treatment, the latter [55] observed a general increase in methylation with alcohol consumption over a 12-year period, particularly in *CKM*, *PHOX2A*, and *NPDC1*. With regard to wider biological pathways, EWAS studies indicated that the most common pathways that were hypermethylated in response to alcohol use were those related to G-protein mediated and GTPase signal transduction processes [51, 54, 55], whereas pathways associated with stimulus and stress responses, as well as immune and inflammatory processes, where likely to be hypomethylated [51]. Hypomethylation was also observed in long terminal repeat (LTR) regions of retrotransposons in the superior frontal cortex of post-mortem alcohol users [56]. Other important pathways related to apoptosis [52, 54, 55], metabolism [53], as well as GABA and dopamine systems [40, 53].

Discussion

The aim of the present review was to summarise the latest animal and human research investigating the association between substance use, DNA methylation, and addiction risk, spanning preconception to adulthood. Based on the 45 reports included, we may conclude that there is preliminary support for a link between substance exposure, DNAm, and addiction. However, findings are often mixed and have limited comparability. In this section, we review key similarities and differences across studies, evaluate evidence for the importance of timing of substance exposure, and outline future directions for the field.

Summary of Study Characteristics and Findings

The majority of studies across species focused on substance exposure during adulthood, examined the effects of alcohol, employed a candidate gene approach, and were cross-sectional. One key difference related to tissue, with animal studies most often investigating brain samples and human studies examining DNAm in blood. Global methylation studies were more common in rodents, while epigenome-wide studies were more frequently carried out in humans. Although prospective longitudinal designs were more common in animal studies, the only four reports to include repeated DNAm measures were based on humans.

As a symptom of how young the field is, there are not sufficient data to assess how DNAm of specific genes relates to exposure to specific substances across developmental periods and tissue types. We do, however, highlight five genes that were investigated by multiple studies and, promisingly, showed a consistent direction of associations. In three rodent studies from the same working group [13, 15, 16], increased methylation and decreased expression of *Pomc*—a gene implicated in stress response, metabolism, and immune function—was observed in response to prenatal alcohol exposure across multiple tissues. These results highlight one mechanism through which fetal alcohol programming can occur, contributing to HPA axis dysregulation and increased addiction risk [16]. In two other studies, the opioid receptor mu 1 (*OPRM1*)—a gene important for mediating drug-induced activation of reward pathways—was hypermethylated in the blood of former opiate addicts [41] and alcohol-dependent individuals [43]. It was not possible to establish, however, whether higher methylation was a predisposing factor for addiction and/or a consequence of long-term substance use. Furthermore, hypermethylation of the serotonin receptor 3A (*HTR3A*) was identified in relation to alcohol exposure across both humans [42] and rodents [23]. Finally, a null association between alcohol exposure and methylation in the opioid signaling genes *PDNY* and *PNOC* was reported in human blood [42] and brain tissue in rats [28]. Despite these consistent findings, it is noteworthy that genes investigated by candidate studies did not typically converge with those identified by studies using hypothesis-free, epigenome-wide analyses. Instead, EWAS studies more often reported significant associations with drug metabolizing genes, as well as highlighting wider biological pathways linked to substance exposure, including signal transduction, inflammation, and apoptosis, in addition to stress response and neurotransmission. How these pathways specifically contribute to addiction, however, remains unclear.

Given the limited comparability across studies, we were not able to systematically assess the importance of developmental context in the relationship between substance use, DNAm, and addiction risk. However, the studies reviewed did provide preliminary support for the relevance of timing of substance exposure on DNAm. For example, evidence from animal models demonstrated that substance exposure can influence DNAm even prior to conception, supporting the existence of transgenerational effects [12–14]. Studies also pointed to the prenatal period as a particularly sensitive developmental window. For example, in utero substance exposure influenced DNAm of developmental genes, which in turn affected postnatal outcomes (e.g., reduced postnatal weight [37]), although the relevance of these changes for addiction risk is yet to be characterised. It is important to note that the period between birth and adulthood received very little attention. In fact, none of the studies investigated childhood and only one examined adolescence—a key period of vulnerability for the development of substance use disorders [57].

Current Challenges for the Field

Despite considerable advances in epigenetic research, studies investigating the role of DNAm in substance use and addiction continue to face a number of key challenges [11••, 58••]. Firstly, *our knowledge of the methylome is still limited*. Because we know little about 'typical' methylation patterns in humans, it is difficult to establish when such patterns deviate to contribute to diseased states. This is complicated by the fact that DNAm patterns can vary across multiple factors, including tissue, cell type, sex, and age [59•]. In general, the compilation of reference datasets will be important for providing a 'typical' benchmark against which to compare epigenetic findings. Knowledge is also limited regarding the relative contribution of genetic and environmental influences on observed methylation patterns, which will require the use of genetically informative designs, such as twin studies and studies identifying methylation quantitative trait loci [60, 61]. More work will also be needed to determine the functional significance of identified DNAm changes at transcriptomic, metabolomic, proteomic, and neural biological levels.

A second set of challenges relates to *research methodology*. Methods have varied widely across studies, including differences in preprocessing, quality control, genomic coverage, data analysis, choice of covariates and significance thresholds used for detecting effects. Together, these sources of variability have limited comparability across studies and complicated efforts to replicate findings—a necessary step for weeding out false positives. The increased availability of standardised pipelines will considerably help in this respect [62•]. Furthermore, the integration of discovery and replicate samples will become increasingly important, as was the case for genomic studies. More research will also be needed to determine what sample sizes are required to reach appropriate statistical power, although simulation-based studies are beginning to provide recommendations [63].

A third issue relates to *difficulties in establishing causal relationships* between substance use, DNAm, and addiction. Most of the studies reviewed adopted a cross-sectional approach with DNAm data sampled at only one time point. Human studies, in particular, focussed primarily on adults who had already been exposed to substances. As such, it remains unclear whether DNAm can be a risk factor for, as well as a consequence of substance use, and how substance exposure and DNAm interrelate over time to influence addiction risk.

Below, we propose a number of ways in which future research may strengthen causal inferences and improve understanding of the role of DNAm in substance exposure and addiction.

A Proposed Model for Conducting Research on DNA Methylation, Substance Use, and Addiction

Firstly, it will be important to capitalise on the strengths of animal models to clearly delineate the mechanisms linking substance exposure, DNAm, and addiction. Systematic investigations will need to be conducted within a given substance, across multiple tissues, over developmental periods, and in different strains. Importantly, it will be necessary to collect prospective, repeated measures of DNAm *pre-* and *post*-substance exposure, in order to (i) investigate whether preexposure DNAm predicts individual differences in drug-seeking behaviours, (ii) trace the timing and stability of DNAm changes following exposure, and (iii) clarify whether DNAm *mediates* the effect of substance use on the onset and persistence of addiction. The sampling of multiple tissues over time will also make it possible to establish cross-tissue variability and locate peripheral biomarkers that most closely resemble DNAm changes in neural networks underlying addiction. Furthermore, incorporating additional *omics* data, such as gene expression, serum levels, protein content, and enzymatic activity, will be useful for clarifying the functional relevance of observed DNAm changes at multiple biological levels [e.g., 13, 16]. Importantly, the use of methyl-modifying agents (e.g., methionine, choline [15, 32]) will offer valuable opportunities for testing the reversibility of drug-induced DNAm changes, identifying whether certain developmental periods are more sensitive to intervention, and examining whether normalisation of DNAm patterns parallel changes in addiction-relevant phenotypes. Finally, the availability of methylomic data in relation to different substances will make it possible to disentangle DNAm markers that are common to multiple substances (perhaps reflecting a general liability to addiction) as opposed to substance-specific markers.

The knowledge generated from animal research could then be used to inform the design of human studies and to map out the most promising DNAm markers for further investigation. This will require, however, the use of strategies to maximise cross-species comparability. For example, the use of data from epidemiological birth cohorts that feature repeated measures of DNA [e.g., 64], could allow researchers to examine whether preexposure versus postexposure DNAm changes identified in longitudinal animal studies extend to humans. Furthermore, analytic methods that make it possible to integrate repeated measures of environmental exposure (e.g., substance use), DNAm, and phenotypic outcomes (e.g., addiction)—such as structural equation modelling—will be particularly useful for tracing longitudinal associations and for testing mediation hypotheses [65]. The development of advanced causal inference methods, such as the two-step epigenetic Mendelian

Table 2 Summary of study findings

	Reference	Species	Period of substance exposure	DNAm *N* time points	Substance	Tissue	Approach	Gene	Sample	Findings
1	Knezovich and Ramsay [14]	Animal (mouse)	Preconception (F2); Adulthood (F1)	1	Alcohol	Sperm (F1); tail blood (F2)	Candidate	*H19*, *Rasgfl*	F1: Male mice, 8 alcohol-treated versus 7 controls. F2: offspring from alcohol-treated (n=17) versus control sires (n=29).	No significant difference in sperm methylation between alcohol-treated sires versus controls. *H19* CTCF binding sites were significantly hypomethylated in alcohol-sired offspring compared to controls, which in turn associated with age restricted growth retardation.
2	Finegersh and Homanics [12]	Animal (mouse)	Preconception (F2); Adulthood (F1)	1	Alcohol	Sperm (F1); VTA; PFC (F2)	Candidate	*Bdnf*	F1: Male mice, 25 exposed to ethanol vapour versus 27 controls. F2: offspring from alcohol-treated (n=123; 45 % F) versus control sires (n=104; 50 % F).	Paternal ethanol exposure associates with decreased *Bdnf* promoter methylation in motile sperm as well as VTA of ethanol-sired male and female offspring.
3	Govorko et al. [13]	Animal (rat)	Preconception; prenatal	1	Alcohol	Hypothalamus (ARC, PVN); blood (mononucle-ar cells); sperm	Candidate	*Pomc*	Male and female rats prenatally (GD 7–21) exposed to an alcohol-liquid diet, a control-liquid diet, or rat chow (n=8/group). Also include F2 and F3 generation (n=6–10/group).	Prenatal alcohol exposure associated with increased *Pomc* methylation in POMC neurons and sperm. Suppression of methylation normalised expression levels and function. Alcohol-induced methylation alterations in exposed F1 offspring transmitted to F2 and F3 male (but not to female) germline.
4	Itzhak et al. [18]	Animal (mouse)	Preconception; prenatal	1	Meth	Hippocampus	EWAS	–	60 offspring of methamphetamine-exposed (preconception and postconception) and 62 offspring of control mice. DNAm collected for a subset of female mice (PD40–PD45). DNAm platform: MeDIP; Roche NimbleGen 385k.	62 and 35 promoter regions, in which DNA methylation was elevated and reduced, respectively, in the F1 mice due to in utero methamphetamine exposure. Enriched pathways included 'cerebral cortex GABAergic interneuron differentiation' for hypermethylated sites and 'embryonic development' for hypomethylated sites.
5	Bekdash et al. [15]	Animal (rat)	Prenatal	1	Alcohol	Hypothalamus (mediobasal)	Candidate	*Pomc*	Male rats prenatally (GD 7–21) exposed to an alcohol-liquid diet or a control diet, with or without choline (n=5/group).	Prenatal alcohol exposure associated with increased *Pomc* methylation and decreased expression in the hypothalamus. Gestational choline normalised alcohol-induced effects on *Pomc* methylation, expression, and stress-axis function.
6	Gangisetty et al. [16]	Animal (rat)	Prenatal	1	Alcohol	Hypothalamus	Candidate	*Pomc*	Male rats exposed to alcohol prenatally (GD 7–21) versus controls.	Prenatal alcohol exposure associated with increased *Pomc* methylation and decreased expression in the hypothalamus.
7	Laufer et al. [17]	Animal (mouse)	Prenatal; neonatal	1	Alcohol	Whole brain	EWAS	–	Adult male mice (PD70), 6 alcohol-exposed (cases) and 6 matched controls.	6660 promoter regions differentially methylated. Enriched pathways related to cell death and nervous system development. Network analysis indicated that the 'behaviour, neurological disease, and psychological disorders' network was

Table 2 (continued)

	Reference	Species	Period of substance exposure	DNAm N time points	Substance	Tissue	Approach	Gene	Sample	Findings
										the most significantly affected network, with *App* identified as a hub gene.
8	Nagre et al. [19]	Animal (mouse)	Neonatal	1	Alcohol	Neocortex; hippocampus	Global	–	Neonatal (PD7) mice treated with ethanol (n=10) or saline (n=10); gender-matched.	Global DNA methylation was reduced after ethanol exposure (8 and 24 h) compared to saline treatment (0 h) in the hippocampus and neocortex.
9	Otero et al. [20]	Animal (rat)	Neonatal	1	Alcohol	Hippocampus; PFC	Global	–	28 choline-treated (13 ethanol/7 intubation control/8 non-treated control)+64 non-choline-treated mice (16ET/8IC/8NC); age- and sex-matched.	Ethanol treatment associated with hypermethylation in the prefrontal and hippocampal region, and this was partially ameliorated by choline treatment.
10	Fowler et al. [21]	Animal (mouse)	Adulthood	1	Alcohol	Cerebral cortex	Global	–	10–12-week-old male mice.	Long-term but not short-term ethanol exposure leads to OCM impairment seen from a significant decrease in global DNA methylation in brain.
11	D'Addario et al. [28]	Animal (rat)	Adulthood	1	Alcohol	Amygdala	Candidate	*Pdyn*, *Pnoc*	Male rats exposed to alcohol for 1, 5, or 5 days plus 1 withdrawal day. Each group compared to respective water-administered controls (n=7/group).	No significant association between alcohol exposure and DNA methylation of opioid peptide genes *Pdyn* and *Pnoc* within the amygdala. However, alcohol exposure associated with alterations in specific histone marks.
12	Qiang et al. [26]	Animal (mouse)	Adulthood	1	Alcohol	PFC	Candidate	*Nr2b*	Male 8-week-old mice exposed to ethanol versus controls (n=8 per group).	Chronic intermittent ethanol exposure associated with decreased *Nr2b* methylation (18 CpGs) and increased expression levels in case controls.
13	Rodrigues et al. [33]	Animal (rat)	Adulthood	1	Alcohol/ opiate/ mor-phine	Nucleus accumbens	Candidate	*Drd2*	Male rats exposed to glucocorticoids in utero versus controls. Within these groups, half exposed to repeated morphine administration in adulthood (4 per group; 16 total).	Repeated morphine exposure associated with increased methylation (and decreased expression) of *Drd2* in rats exposed to glucocorticoids. *Drd2* methylation and expression changes in response to morphine reversed by L-dopa treatment.
14	Tammen et al. [22]	Animal (mouse)	Adulthood	1	Alcohol	Liver	Global	–	18 18-month-old male mice and 20 4-month-old mice.	No effect of alcohol consumption on global DNA methylation. Reduced hydroxymethylation observed relative to mice fed with a control diet in young, but not in old mice.
15	Khachatoorian et al. [25]	Animal (rat)	Adulthood	1	Alcohol	Liver	Candidate	*24 genes*	Male rats fed with ethanol or control diet, with or without SAM supplement for 1 month (n=3/group).	Compared to controls, ethanol-fed rats showed higher methylation across genes—this increase was nonsignificant for each individual gene, but significant for average methylation across genes. SAM treatment prevented ethanol-induced methylation changes.

Table 2 (continued)

	Reference	Species	Period of substance exposure	DNAm *N* time points	Substance	Tissue	Approach	Gene	Sample	Findings
16	Wani et al. [27]	Animal (rat)	Adulthood	1	Alcohol	Intestine; colon; kidney; pancreas; liver	Candidate	*Fpgc*, *Ggh*, *Pcft*, *Rft*	Male chronically exposed to ethanol for 3 months versus controls (n=6/group).	Chronic ethanol feeding associated with tissue- and gene-specific methylation alterations in folate regulating and transporting genes.
17	Srinivasan et al. [24]	Animal (mouse)	Adulthood	1	Alcohol	Pancreas	Candidate	*Slc5a6*	Adult transgenic mice carrying the full-length human SLC5A6 promoter, alcohol-fed versus controls.	Chronic alcohol feeding associated with increased *Slc5a6* promoter methylation, negatively influencing pancreatic biotin uptake.
18	Barker et al. [23]	Animal (mouse)	Adulthood	1	Alcohol	Blood; 9 brain regions	Candidate	*Htr3a*	15 male ethanol-drinking mice versus 13 water-drinking controls.	Ethanol-drinking was associated with *Htr3a* hypermethylation in blood and hippocampus, but hypomethylation in the dorsomedial and ventromedial PFC. No significant differences in methylation across the other 6 brain regions investigated.
19	Tian et al. [32]	Animal (mouse)	Adulthood	1	Cocaine/ mor-phine	Nucleus Accumbens; PFC	Global	–	Adult male mice (no sample size or age information specified).	Cocaine but not morphine induced global DNA hypomethylation in the prefrontal cortex (but not in the nucleus accumbens), which was reversible through methionine.
20	Nielsen et al. [29]	Animal (rat)	Adulthood	1	Cocaine	Corpus callosum	Global; candi-date	*Mbp*, *Plp1*, *Sox10*	19 male rats: sham rats with 1 (n=3) or 30 days (n=4) of forced abstinence; cocaine self-administration rats with 1 (n=6) or 30 days (n=6) of forced abstinence.	No difference in global DNA methylation. Candidate genes: reduced mean methylation of *Sox10* relative to controls, especially after a 30-day abstinence compared to a 1-day abstinence. No differences in *Mpb* or *Plp1* sites.
21	Anier et al. [34]	Animal (mouse)	Adulthood	1	Cocaine	Nucleus accumbens; cerebellum	Candidate	*Slc17a7*, *Cck* and *Gal*	Male mice (5–6 months old) randomly assigned to one of four conditions (n=8 per group): (i) saline+saline, (ii) SAM+saline, (iii) saline+cocaine), or (iv) SAM+cocaine.	Repeated SAM pretreatment modifies cocaine-induced methylation changes in *Slc17a7*, *Cck* and *Gal* within the nucleus accumbens but not in the cerebellum.
22	Pol Bodetto et al. [35]	Animal (rat)	Adulthood	1	Cocaine	Caudate putamen	Candidate	*Pp2cβ*	Male 8–9-week-old rats injected repeatedly (1 time daily × 10 days) with either cocaine or saline (n=4/group).	Cocaine-exposed rats showed higher *Pp2cβ* promoter methylation compared to controls.
23	Fragou et al. [30]	Animal (mouse)	Adulthood	1	Cocaine/ heroin	Whole brain; liver	Global	–	8-week-old male mice with either 1) chronic 7-day heroin administration (n=9 vs 9 controls) or 2) chronic 14-day cocaine administration (n=9 vs 9 controls).	No differences in either drug group or tissue versus controls.
24	Chao et al. [31]	Animal (mouse)	Adulthood	1	Cocaine/ heroin	Whole brain; liver	Global	–	8-week-old male mice with either 1) chronic 7-day heroin administration (n=9 vs 9 controls) or 2) chronic 14-day cocaine administration (n=9 vs 9 controls).	No effects of heroin on methylation or hydroxymethylation in brain or liver. Hydroxymethylation (but not methylation) was reduced in the cocaine group in liver (but not brain tissue).

Table 2 (continued)

	Reference	Species	Period of substance exposure	DNAm *N* time points	Substance	Tissue	Approach	Gene	Sample	Findings
25	Wilhelm-Benartzi et al. [36]	Human	Prenatal	1	Alcohol	Placenta	Global	–	Mother-infant pairs (*n*=184, gender and maternal age-matched infants).	Mean LINE-1 (but not AluYb8 or global [mean across 27 k]) levels differed by maternal alcohol use during pregnancy.
26	Azzi et al. [37]	Human	Prenatal	1	Alcohol	Blood (cord)	Candidate	*ZAC1*	254 mother-newborn pairs (subsample from EDEN study; mean age=30).	Pre-pregnancy and prenatal maternal alcohol intake positively correlated with *ZAC1* methylation at birth, which in turn associated with fetal and postnatal weight.
27	van der Knaap et al. [38]	Human	Adolescence	1	Alcohol, canna-bis	Blood	Candidate	*COMT*	463 adolescents (mean age=16, 51 % F).	No association between membrane-bound *COMT* promoter methylation and alcohol or cannabis use; significant interaction with *COMT* Val108/158 Met genotype in predicting high-frequent cannabis use.
28	Ponomarev et al. [56]	Human	Adulthood	1	Alcohol	Superior frontal cortex	EWAS	–	Postmortem tissue from 17 alcoholics and 15 matched controls (6 % females). DNA methylation available for a subsample of 6 cases and 6 controls.	DNA hypomethylation in the LTR region of 3 retrotransposon gene families: *MLT2A1*, *THE1B*, *LTR8.*
29	Zhu et al. [39]	Human	Adulthood	1	Alcohol	Blood	Global	–	Adult alcohol drinkers (*n*=717) versus nondrinkers (*n*=609); mean age=62, 14 % females.	Lower Alu (but not LINE-1) methylation levels in drinkers versus nondrinkers.
30	Zhang et al. [43]	Human	Adulthood	1	Alcohol	Blood	Candidate	*OPRM1*	125 cases with alcohol dependence (mean age=41, 35 % F) versus 69 controls (mean age=39, 51 % F)	Higher overall *OPRM1* methylation in cases versus controls. Associations with individual CpG sites (n=16) did not survive multiple correction.
31	Glahn et al. [48]	Human	Adulthood	3	Alcohol	Blood	Candidate	*ANP*, *AVP*	99 alcohol-dependent patients (mean age=43) versus 101 controls (mean age=36). All male.	Significant difference in *AVP* and *ANP* methylation between cases and controls at baseline (day 1). No difference between day 1 and 7 of alcohol withdrawal. *AVP* and *ANP* methylation (1 CpG each) significantly reduced in cases versus controls between day 7 and 14. DNA methylation not significantly associated with serum levels.
32	Heberlein et al. [49]	Human	Adulthood	3	Alcohol	Blood	Candidate	*NGF*	57 male alcohol-dependent patients during withdrawal.	No significant change in *NGF* methylation and serum levels between day 1 and 7 of alcohol withdrawal. Increase in methylation and decrease in serum levels between day 7 and 14.
33	Beach et al. [45]	Human	Adulthood	1	Alcohol, canna-bis	Blood	Candidate	*5-HTT*	155 adult females from the Iowa adoptees sample (mean age=41).	No significant association between 5-*HTT* methylation and substance use.
34	Nieratschker et al. [46]	Human	Adulthood	2	Alcohol	Blood	Candidate	*DAT*	100 alcoholic patients (mean age=47, 20 % F) versus 100 matched controls. Repeated	No difference in *DAT* methylation between alcoholic patients versus controls; no significant change pre or post withdrawal

Table 2 (continued)

	Reference	Species	Period of substance exposure	DNAm *N* time points	Substance	Tissue	Approach	Gene	Sample	Findings
									DNA methylation available for 85 cases (mean age=47, 18 % F).	in patients; trend association between higher craving and lower methylation.
35	Zhang et al. [42]	Human	Adulthood	1	Alcohol	Blood	Candidate	*82 genes*	285 Alcohol dependent cases (mean age=42, 49 % F) versus 249 controls (mean age=37, 62 % F). Replication sample: 49 cases (mean age=42, 47 % F) and 32 controls (mean age 37, 63 % F).	Of the 384 sites examined (82 genes), one probe in the promoter region of *HTR3A* was significantly associated with European American cases versus controls, after multiple correction (trend association in the replication sample). No significant differences between cases and controls in African Americans.
36	Zhang et al. [51]	Human	Adulthood	1	Alcohol	Liver	Candidate	*UGT1A1*	46 human liver bank samples (mean age=41; 46 % F).	No association between alcohol history and *UGT1A1* methylation (6 CpGs) in liver. However, alcohol history influenced the relationship between DNA methylation and UGT1A1 protein content and enzymatic activity.
37	Yasar et al. [47]	Human	Adulthood	1	Alcohol	Blood	Candidate	*LEP*	164 alcohol-dependent patients (mean age=43; 21 % F).	Lower LEP promoter methylation at baseline associated with higher serum leptin levels during detoxification (day 7) and stronger alcohol craving in alcohol dependent patients.
38	Hillemacher et al. [44]	Human	Adulthood	1	Alcohol	Saliva	EWAS	–	Community sample of 309 hazardous drinkers (age=21–55, 31 % F). Replication sample: 39 individuals from a pharmacological intervention study (mean age=44; 30 % F). DNAm platform: Illumina 27k.	28 sites were significant after FDR correction, where greater methylation levels were associated with poorer control over drinking. Increased *ALDH1A2* methylation also related to faster increase of breath alcohol levels and increased subjective feeling of intoxication.
39	Harlaar et al. [50]	Human	Adulthood	1	Alcohol	Blood	EWAS	–	63 male alcohol-dependent inpatients (mean age=44) and 65 age- and sex-matched healthy controls. DNAm platform: Illumina 27k.	1710 sites were differentially methylated in cases versus controls with $p<0.005$. Top-ranked sites included genes previously shown to be related to alcohol metabolism (e.g., *ADH1A*, *ALDH3B2*, *CYP2A13*). Enriched biological pathways for hypomethylated sites included defence response, response to external stimulus, and immune system process, while hypermethylated sites were enriched for mitosis and signal transduction.
40	Philibert et al. [52]	Human	Adulthood	1	Alcohol	Blood	EWAS	–	165 age-matched females: (i) 40 abstinent (mean age=47), (ii) 47 mild, (iii) 50 moderate, and (iv) 28 heavy alcohol users. DNAm platform: Illumina 450k.	No differences in pretreatment versus posttreatment. *ABR* differentially methylated in heavy/moderate alcohol users versus abstinent patients.

Table 2 (continued)

	Reference	Species	Period of substance exposure	DNAm *N* time points	Substance	Tissue	Approach	Gene	Sample	Findings
41	Philibert et al. [54]	Human	Adulthood	1	Alcohol	Blood	EWAS	–	10 alcohol-dependent patients (mean age=45) and their discordant siblings as controls (mean age=44). Sex not specified. DNAm platform: Illumina 450k.	865 hypomethylated and 716 hypermethylated sites in alcohol dependent patients versus controls (*SSTR4* most hypomethylated; *GABRP* most hypermethylated). Enriched pathways related to metabolic function.
42	Zhao et al. [53]	Human	Adulthood	2	Alcohol	Blood	EWAS	–	Two time points within a 12-year period; cases (n=10; mean age=40; males) differ from age- and sex- matched control (n=10) in alcohol consumption at time point 2, but not 1. DNAm platform: Illumina 27k.	200 differentially methylated sites in cases between time points with a majority of sites increasing in methylation over time; sites correlated with alcohol consumption over a 12-year period related to genes such as *CKM*, *PHOX2A*, *NPDC1*, *ADCY9*.Enriched pathways included signal transduction, Notch signalling, and p53 network.
43	Weng et al. [55]	Human	Adulthood	2	Alcohol	Blood	EWAS	–	33 subjects with heavy alcohol consumption (mean age=45; 24 % F) and 33 age- and sex-matched controls; repeated measures for 25 cases before and after a 25-day treatment programme. DNAm platform: Illumina 450k.	8636 FDR-corrected differentially methylated sites (56 Bonferroni-corrected). Age, gender, and ethnicity not included as covariates. Enriched pathways included apoptosis and GTPase signalling. No observed differences before versus after treatment.
44	Doehring et al. [41]	Human	Adulthood	1	Opiate	Blood	Global; candi-date	*OPRM1*	85 methadone-substituted former opiate addicts (mean age=35; 30 % F) versus matched healthy controls. Replication sample: 63 opioid-treated pain patients (age >50; 43 % F) versus matched non-opioid treated controls.	Higher *OPRM1* methylation (1 site) and global (LINE-1) methylation in former opiate addicts versus controls. Association with global methylation reproduced in an independent sample of opiate-treated pain patients versus controls. Within this sample, higher global methylation was also positively associated with average daily opioid dose and pain scores.
45	Desplats et al. [40]	Human	Adulthood	1	Meth	Frontal cortex	Global; EWAS	–	HIV-seropositive cases with (n=13) and without (n=14) methamphetamine-abuse (age- and gender-matched). DNAm platform: Illumina 450k.	Increased global methylation in methamphetamine HIV+ users compared to HIV+ non-users; EWAS: enriched pathways included L-dopa degradation, ERK/MAPK signalling and Dopamine-DARPP32 feedback in cAMP signalling.

randomisation [66, 67], may also show promise for testing causal pathways documented in animals. As discussed above, the inclusion of additional biological markers (e.g., serum levels) will be necessary for establishing the functional relevance of identified DNAm markers. Furthermore, given the scarce availability of central tissues in human research (i.e., postmortem), it will be important in the future to investigate whether peripheral DNAm markers can be related to in vivo structural and functional brain data (e.g., striatal activity when viewing addiction-related cues). Finally, increased use of approaches that capitalise on co-methylation patterns between CpG sites, such as regional or network-based approaches, will be important for reducing multiple testing and increasing power to detect effects in humans, enabling to move beyond individual methylation sites toward wider biological systems [68].

Conclusions

DNA methylation is emerging as an important molecular mechanism mediating substance use response and addiction risk. However, the limited understanding of the epigenome, heterogeneity across studies, a reliance on cross-sectional designs, and lack of replications make it difficult to interpret the relevance of the extant data for mechanisms of addiction. Rapid developments in knowledge, methodology, and research designs will offer exciting opportunities for delineating the role of DNAm in the pathophysiology of addiction, as well as testing its potential clinical utility as an exposure indicator, disease biomarker, and therapeutic target (Table 2).

Compliance with Ethics Guidelines

Conflict of Interest Charlotte A.M. Cecil, Esther Walton, and Essi Viding declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- •• Of major importance

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