Caspani Giorgia (Orcid ID: 0000-0003-1697-1437)
Bailey Alexis (Orcid ID: 0000-0003-1541-1964)

Metabolic phenotyping of opioid and psychostimulant addiction: A novel approach for biomarker discovery and biochemical understanding of the disorder

Giorgia Caspani[1†], Viktoria Sebők[2†], Nowshin Sultana[2], Jonathan R. Swann[1,3*] and Alexis Bailey[2*]

[1] Department of Metabolism, Digestion and Reproduction, Faculty of Medicine, Imperial College London, UK

[2] Pharmacology Section, Institute of Medical and Biomedical Education, St George's University of London, London, UK

[3] School of Human Development and Health, Faculty of Medicine, University of Southampton, UK

† and *Equal contribution

Correspondence:

Alexis Bailey, e-mail: abailey@sgul.ac.uk, +44 20 8725 0075

Jonathan Swann, e-mail: j.swann@imperial.ac.uk, +44 20 7594 0728

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Author Contributions

GC and VS planned and drafted the manuscript. NS verified and cross-referenced the information presented in the review with the original primary papers. JRS and AB provided support throughout the writing process by sharing their expertise in the field of metabolomics and addiction, respectively. All authors contributed to the final manuscript.

Abstract

Despite the progress in characterizing the pharmacological profile of drugs of abuse, their precise biochemical impact remains unclear. The metabolome reflects the multi-faceted biochemical processes occurring within a biological system. This includes those encoded in the genome but also those arising from environmental/exogenous exposures and interactions between the two. Using metabolomics, the biochemical derangements associated with substance abuse can be determined as the individual transitions from recreational drug to chronic use (dependence). By understanding the biomolecular perturbations along this time course and how they vary across individuals, metabolomics can elucidate biochemical mechanisms of the addiction cycle (dependence/withdrawal/relapse) and predict prognosis (recovery/relapse). In this review, we summarize human and animal metabolomic studies in the field of opioid and psychostimulant addiction. We highlight the importance of metabolomics as a powerful approach for biomarker discovery and its potential to guide personalised pharmacotherapeutic strategies for addiction targeted towards the individual's metabolome.

Keywords

Addiction, Cocaine, Heroin, Metabolism, Metabolomics, Methamphetamine, Morphine

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Abbreviations:

3-HB – 3-hydroxybutyric acid

5-HIAA – 5-hydroxyindoleacetic acid

ASI – addiction severity index

BLA – basolateral amygdala

Cer – Cerebellum

CPP – conditioned-place preference

DSM-V – Diagnostic and Statistical Manual of Mental Disorders fifth edition

DOPAC – 3,4- dihydroxyphenylacetic acid

Gln-Glu-GABA – glutamine-glutamate-GABA

GPC – Glycerol phosphocholine

Hi – Hippocampus

LC-MS/MS – liquid chromatography with tandem mass spectrometry

NAA – N-acetylaspartate

NAc – Nucleus accumbens

NAD – nicotinamide adenine dinucleotide

NAG – N-acetyl-glycoprotein

NAMPT – nicotinamide phosphoribosyltransferase

NMN – nicotinamide mononucleotide

OAG – O-acetyl-glycoprotein

Pc – phosphocholine

PDGF – platelet-derived growth factor

PFC – Prefrontal Cortex

SIRT1 – Sirtuin 1

Str – Striatum

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TCA – Tricarboxylic acid

Th – Thalamus

Trx-1 – thioredoxin-1

VTA – Ventral tegmental area

Key definitions

Metabolomics: an analytical technique used to identify and quantify large sets of metabolites present within an organism, cell, or tissue.

Metabolome: the set of small molecules, or metabolites, that interact within a biological system.

Metabolic phenotyping: the comprehensive analysis of metabolites present in a given sample.

Metabolic phenotype: the "biochemical fingerprint" or "metabolic signature", the metabolic state of an individual, influenced by both intrinsic (e.g. metabolic processes) and extrinsic (e.g. drug intake) factors.

Pharmacometabolomics: a branch of metabolomics that deals with relating the baseline metabolic signature of an individual with their response to pharmacotherapy.

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Metabolomics as a tool to understand drug addiction

Drug addiction is a chronic relapsing brain disorder characterised by compulsive drug seeking, loss of control over drug intake and the emergence of physical and emotional withdrawal symptoms when the drug is absent (Koob and Le Moal, 2001; Koob and Kreek, 2007; Wee and Koob, 2010). It is thought to arise, at least partly, as a result of the long-term compensatory mechanisms that occur in response to the increase in striatal dopaminergic transmission along the mesolimbic pathway elicited by drug use. However, a detailed and refined understanding of the biochemical processes underlying addiction and the nature of the profound interpersonal variability in drug responses and progression of the disorder are still unclear.

The observation that addicted individuals present a range of metabolic abnormalities led to the notion of drug addiction as a "metabolic disease" (Dole and Nyswander, 1967). Thus, a global investigation of the biochemical perturbations characteristic of the disease may be successful in providing mechanistic insights into disease states and progression. Metabolomics uses high-resolution analytical chemistry techniques to simultaneously measure a large number of low molecular weight molecules in a biological sample. This results in large datasets where the variables (*i.e.* metabolites) largely outnumber the observations (*i.e.* mice or human participants). As such, appropriate dimensionality reduction techniques are necessary to analyse the entire metabolic profile in relation to an outcome of interest (Worley and Powers, 2013). Multivariate models can be constructed to predict class membership (*e.g.* disease state) or a continuous response variable (*e.g.* behavioural data) from linear combinations of the original variables (Saccenti et al., 2014). The predictive ability and significance of the model can then be assessed through cross-validation and permutation testing, respectively. While acknowledging the interrelation of metabolites, multivariate methods allow for assessing the "weight" or contribution of each metabolite to the overall predictive model (Saccenti et al.,

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2014), aiding the identification of potential biomarkers. This unique biochemical fingerprint, referred to as the metabolome, reflects the metabolic processes occurring in the biological system at the time of analysis and its overall metabolic status (Kosmides et al., 2013). In addition, the metabolome contains exogenous molecules entering the system (*e.g.* dietary factors and xenobiotics) and products resulting from their breakdown. Drug addiction is a unique disorder in that it arises from the combination of genetic risk factors and exposure to an exogenous substance (*i.e.* drug of abuse). By capturing the "metabolic phenotype" originating from both endogenous processes as well as from the interaction with exogenous molecules (Figure 1), metabolomics provides a unique technique to investigate the biochemical basis of addiction.

Metabolomic approaches are also a powerful tool for biomarker discovery. As the fourth most costly mental disorder in the European Union (Gustavsson et al., 2011), addiction is a major public health issue with serious socioeconomic implications, and efforts continue to be made to improve the diagnosis and management of this disorder. The diagnosis of drug addiction is based solely on identifying characteristic symptoms and behaviours in accordance with the Diagnostic and Statistical Manual of Mental Disorders fifth edition (DSM-V) (American Psychiatric Association, 2000). No reliable diagnostic test currently exists for primordially predicting drug addiction vulnerability and for identifying individuals at risk of relapse or at risk of co-morbidity. When pharmacological options for managing addiction symptomology are available (*e.g.* methadone/buprenorphine for opioid withdrawal and naloxone for craving), efficacy is often limited and responses are highly variable. It also remains impossible to predict the efficacy and potential side effects of pharmacotherapy on an individual basis. These challenges demonstrate the need for quantitative biomarkers to predict an individual's addiction risk, disease progression, relapse vulnerability, and response to interventional strategies. Pharmacometabolomics is a branch of metabolomics whereby an individual's

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baseline metabolic phenotype is used to predict their handling and response to a pharmacological intervention (Kaddurah-Daouk and Weinshilboum, 2014). As our understanding in this area grows, a personalised approach to care delivery and clinical decision-making in the management of substance abuse disorders may become possible.

This review summarizes the findings of published studies in humans and rodent models investigating the biomolecular perturbations elicited by opioids and psychostimulants on the brain (target organ) and peripheral tissues/biofluids with translational value (*e.g.* blood, urine, and hair). The nomenclature of receptors and enzymes mentioned in this review aligns with the Concise Guide to PHARMACOLOGY 2019-2020 (Alexander et al., 2019). As our understanding in this area grows, a personalised approach to care delivery and clinical decision-making in the management of substance abuse disorders may become possible.

Metabolic phenotyping of opioid addiction

Opioids, such as morphine and heroin, are highly addictive substances. Their rewarding effects are mediated by their ability to induce dopamine transmission in the nucleus accumbens (NAc) by relieving the inhibition of GABAergic interneurons on mesolimbic dopamine-releasing neurons in the ventral tegmental area (VTA), (Spanagel and Weiss, 1999). The following section provides an overview of animal and human studies investigating the metabolic changes associated with the distinct stages of opioid (morphine and heroin) addiction. While the analysis of brain samples can help unravel biochemical pathways affected by repeated opioid administration (Gao et al., 2007; Deng et al., 2012; Hu et al., 2012; Li et al., 2017) or involved in the reinforcing effects of the drugs (Meng et al., 2012), sequential sampling of plasma and urine samples allows for the identification of biomarkers of the different addiction states (*i.e.* euphoria, tolerance, abstinence, withdrawal, (Zaitsu et al., 2014; Liu et al., 2015)) and

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predictors of treatment outcome (*i.e.* response and relapse, (Zheng et al., 2013; Ning et al., 2018)). Human studies have also been conducted to understand the perturbations in the hair metabolome driven by heroin (Xie et al., 2016) and to investigate the metabolic changes induced by withdrawal from opioids (Mannelli et al., 2009). These studies are summarised in Table 1.

Metabolic signature of morphine addiction

^{1}H NMR spectroscopy-based studies of the metabolic abnormalities induced by repeated morphine administration on brain samples were conducted in rhesus monkeys (Deng et al., 2012) and rodents (Gao et al., 2007; Hu et al., 2012). Significant disturbances in the glutamine-glutamate-GABA (Gln-Glu-GABA) axis, which are markers of oxidative stress and involved in neurotransmission, were a common finding. The specific changes reported varied depending on the species and the brain region considered and are reviewed in Table 2. Some discrepancies regarding the direction of change of these metabolites may also be underpinned by differences in experimental design (*e.g.* dose and length of drug administration, see Table 1). Disturbances in the equilibrium state between GABA, glutamate and glutamine, with a general increase of GABA and decrease in glutamate, were consistent with microdialysis studies showing increased GABA and decreased glutamate within the medial prefrontal cortex (mPFC) (Ramshini et al., 2019), NAc (Sun et al., 2011a) and hippocampus (Kang et al., 2006) in response to morphine. Such changes were ascribed to alterations in tricarboxylic acid (TCA) cycle activity and enhanced conversion of glutamate into GABA by GAD. A decrease in GABA degradation has also been suggested (Gao et al., 2007). The shift in the Gln-Glu-GABA equilibrium state observed in these studies may also be the result of neuroadaptations caused by the effect of morphine on GABAergic neurotransmission, which is known to mediate the rewarding properties of opioids. This is supported by evidence suggesting that GABA and

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activation of the GABAergic system attenuates the reinforcing effects of drugs of abuse via its modulatory effect on the mesolimbic dopaminergic pathway (Tsuji et al., 1996; Westerink et al., 1996) and that its disruption is involved in the development of tolerance and dependence to opioids (Sepúlveda et al., 2004; Hu et al., 2012). These metabolic changes were not evident until day 10 of morphine administration, suggesting that long-term adaptive mechanisms underlie these alterations (Gao et al., 2007). The membrane constituent phosphocholine and the phosphoinositol precursor *myo*-inositol were dysregulated in response to morphine exposure, indicating an effect of morphine on membrane integrity. *Myo*-inositol is also an osmolyte highly expressed in glial cells. Changes in the abundance of *myo*-inositol and the neuronal marker *N*-acetylaspartate (NAA) may reflect glial hypertrophy and altered neuronal morphology and activity. Glial cells are emerging as an important player in addiction pathophysiology due to their role in supporting neurotransmission and brain energy metabolism (Miguel-Hidalgo, 2009). Morphine has been shown to affect neuronal maturation *in vitro* by modulating astrocytic proliferation (Stiene-Martin et al., 1991). Irrespective of species and brain regions considered, an increase in lactic acid was observed in all studies (Deng et al., 2012; Hu et al., 2012). Lactic acid is the end-product of anaerobic cellular metabolism and is produced when energy demand exceeds the rate of oxidative metabolism. Elevated lactic acid indicates tissue damage and impaired pyruvate oxidation (Veech, 1991). Given that lactate can only be completely oxidised in mitochondria, increased lactic acid observed in these studies is likely to be an indication of mitochondrial dysfunction, energy metabolism impairment, oxidative stress and/or upregulation of the enzyme lactate dehydrogenase. Consistently, metabolites related to oxidative stress such as the antioxidants glutathione, taurine and creatine also showed a large deviation from controls following repeated morphine administration. Feng et al. (2013) suggested hippocampal mitochondrial damage and decreased mitochondrial DNA copy number as a hallmark of addiction. Using cultured rat pheochromocytoma cells and

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mouse neurons treated with morphine, the authors showed that oxidative stress caused by morphine administration led to mitochondrial damage and autophagy. Given the involvement of mitochondria in synaptic remodelling, mitochondrial dysfunction is likely to have downstream effects on synaptic plasticity and neurotransmission, with an inevitable downstream impact on the addiction cycle (Figure 2). In support of this hypothesis, the upregulation of ROS in the hippocampus, as a result of morphine administration, was shown to elicit increased inhibitory and decreased excitatory synapses, while the antioxidant compound platelet-derived growth factor (PDGF) reverses the synaptic effects of morphine (Cai et al., 2016). Similarly, the antioxidative compound thioredoxin-1 (Trx-1) inhibits morphine-induced conditioned-place preference (CPP) in transgenic mice by modulating $GABA_B$ and dopamine D_1 receptor expression (Li et al., 2018), clearly suggesting a key role for oxidative stress in modulating, at least partly, the reinforcing effects of morphine.

The central (Meng et al., 2012) and peripheral (Zaitsu et al., 2014) biochemical mechanisms underlying the context-dependent learning associated with the rewarding effects of drugs of abuse were investigated in rodents undergoing morphine-induced CPP in two independent GC-MS studies. A total of 21 metabolites were observed to significantly differ in the central metabolic profiles of morphine-treated and control mice (Meng et al., 2012). Of these, the elevation of *myo*-inositol and its derivative *myo*-inositol phosphate support the disrupting action of morphine on membrane integrity and astrocytic morphology and proliferation. The increase in nicotinamide, a product of nicotinamide adenine dinucleotide (NAD) metabolism that is also involved in reward memory consolidation, suggests a role of this metabolite in drug-associated learning. The nucleotide uracil and the amino acids proline and valine were also upregulated. While indicative of protein breakdown, both proline and valine contribute to glutamate and glutamine metabolism, further supporting a dysregulation in the Gln-Glu-GABA

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axis (Meng et al., 2012). The effect of morphine on the plasma and urinary metabolomes was investigated by Zaitsu *et al.* (2014) in a CPP paradigm. Twelve urinary metabolites and four plasma metabolites were strongly predictive of high CPP scores (Zaitsu et al., 2014), highlighting the exciting potential for metabolomics to elucidate novel predictive biomarkers for addiction severity. Morphine-CPP rats exhibited an increase in *N*-propylamine, but a decrease in 3-hydroxybutyric acid (3-HB), L-tryptophan and cysteine in the plasma compared to control rats, and an increase in 2-oxoglutarate, fumaric acid, malic acid, L-threonine and a decrease in glutamic acid, isoleucine, L-valine, L-aspartic acid, oxamic acid, 2-aminoethanol, indoxyl sulfate and creatinine in the urine. Given that L-tryptophan is elevated in the brain following morphine administration (Messing et al., 1978), a decrease in its circulatory levels suggests that morphine administration results in the uptake of L-tryptophan from the systemic circulation into the brain. Importantly, the increase in TCA intermediates such as 2-oxoglutarate, fumaric acid and malic acid, and the reduction in glutamic acid and 3-HB support the upregulation of the TCA cycle and the inhibition of β-oxidation of fatty acids and ketone body metabolism for energy production. Similarly, a separate GC-MS-based metabolomic study investigating the metabolic changes in the plasma of rats chronically exposed to an escalating dose of morphine (Liu et al., 2015) reported alterations in TCA cycle intermediates, such as oxalic acid and the malonic acid derivative aminomalonate, alterations in the Gln-Glu-GABA axis, as demonstrated by decreased glutamine and increased β-D-glucose, and in membrane components, such as cholesterol, linoleic acid and stearic acid. In line with Zaitsu *et al.* (2014), a disruption in the metabolism of amino acids and related compounds was also observed by Liu *et al.* (2015), with decreases in alanine, proline, glycine, serine, threonine. As alanine and glycine are by-products of GABA degradation by the enzyme GABA-transaminase, their reduction may be indicative of decreased GABA breakdown. Conversely, proline is associated with both the TCA cycle and the pentose phosphate pathway and is

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involved in arginine and glutamate metabolism. Once again, these biochemical disruptions point towards dysfunctional energy metabolism.

Several studies have assessed the biochemical effects of common pharmacological interventions used to assist opioid-dependent individuals overcome their dependence. The drug naloxone is an antagonist at the μ-opioid receptor used to suppress craving in opioid dependent individuals undergoing abstinence. The effect of naloxone on the plasma metabolic profile of morphine-treated rats was evaluated by Liu *et al.* (2015). Rats receiving naloxone after being treated with morphine for 5 days had higher circulating amounts of lactic acid, oxalic acid, aminomalonate, 2-oxoglutarate, β-D-glucose, linoleic acid, cholesterol and palmitic acid and reduced alanine, valine, proline, glycine, serine, threonine, pyroglutamic acid, hydroxyproline, erythronic acid, glutamine, arabinose, rhamnose, isoleucine, succinate, 4-hydroxybutanoic acid and leucine compared to rats receiving naloxone after saline (Liu et al., 2015). Lower threonine, glycine, and serine and higher glucose and oxalic acid was noted in all states (including "euphoria", 30 minutes post-morphine), implying that disturbances in amino acids (and potentially neurotransmission) and oxidative stress were not recoverable by naloxone and may reflect "life-time exposure" to morphine. This demonstrates the potential of metabolomics to elucidate the biochemical processes underlying each stage of the disorder progression.

NMR-based metabolic profiling was used to study various brain regions from morphine-treated rhesus monkeys and rats undergoing detoxification with the long acting opioid methadone or the α2 adrenergic agonist clonidine (Deng et al., 2012; Hu et al., 2012). Upon detoxification, the majority of morphine-induced metabolic variation was normalised to baseline (Deng et al., 2012; Hu et al., 2012), although several metabolites remained altered in specific brain regions (Table 3). While clonidine was generally more effective than methadone in reversing the biochemical effects of morphine in both species (Deng et al., 2012; Hu et al., 2012), rats

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showed a more profound reduction in withdrawal symptoms in response to methadone compared to clonidine (Deng et al., 2012). Advanced correlation analysis is warranted to investigate causality between the behavioural effect of pharmacotherapeutic interventions and their effectiveness in restoring pre-dose biochemical profiles.

The effect of six days of methadone-aided detoxification on the plasma metabolome was investigated in a human study (Mannelli et al., 2009). A liquid chromatography electrochemical array platform was used to measure purine, monoaminergic and redox metabolites in 14 opioid-dependent individuals undergoing methadone detoxification and 10 non-drug users. Opioid-dependent participants were given methadone orally at 9-10 am daily, and blood samples were collected on day 2 and 3 at 10-11 am. The ratio of glutathione/oxidised glutathione and the antioxidants α- and γ-tocopherol were significantly higher in opioid-dependent participants, indicating significantly higher oxidative capacity provided for by methadone therapy. Changes in α-tocopherol antioxidant activity are consistent with previous studies evaluating antioxidant serum levels in heroin users undergoing methadone detoxification (Rodríguez-Delgado et al., 2002; Díaz-Flores Estévez et al., 2004). This may indicate a protective effect of methadone in reducing physical opioid withdrawal symptoms by a mechanism involving ROS removal. The increase in plasma guanine and xanthosine reported in the methadone group may represent alterations in energy production through the conversion of guanine and xanthosine into their corresponding nucleotides via salvage pathways. The combination of high guanine and low guanosine in the plasma has been shown to be associated with brain toxic insult and increased dopamine turnover (Loeffler et al., 1998; Ciccarelli et al., 1999), while guanine-based purines also participate in GABAergic and glutamatergic transmission (Majumder et al., 2007; Schmidt et al., 2007). Significant differences in purine metabolites were found between control and drug-dependent subjects undergoing methadone detoxification (Mannelli et al., 2009), but

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further investigation is required to assess if any of these metabolites can serve as biological markers of addiction or of response to methadone treatment. No significant differences in phenylalanine, tryptophan and tyrosine metabolites and monoamines (dopamine, serotonin, noradrenaline) were found between the groups. However, *N*-methylserotonin was present in greater amounts in the plasma of opioid dependent patients. Interestingly, *N*-methylserotonin and its derivative bufotenine have also been shown to be elevated in other psychiatric disorders and have been associated with hallucinogenic effects (Takeda et al., 1995), further supporting a common metabolic dysregulation in a range of psychiatric conditions. However, the authors reported that *N*-methylserotonin, but none of the other metabolites, was higher in the eight of the 14 drug-dependent participants also tested positive for both cocaine and cannabis (Mannelli et al., 2009). These results should be viewed as preliminary because of the presence of confounding biological differences between opioid-dependent patients and non-drug users and in future studies caution should be taken to control for smoking pattern, age, ethnic background, lifestyle and the diet. Although beyond the scope of this review, the potential of metabolomics to evaluate the interindividual pharmacokinetic properties (thus the ideal, tailored dose) of methadone have been demonstrated and can have implications in the personalised treatment of addiction (Dinis-Oliveira, 2016).

Metabolic signature of heroin addiction

Zheng *et al.* (2013) monitored serum and urinary metabolic changes in response to a 10-day escalating dose of heroin administration in rats, followed by four days of abstinence ("withdrawal") and four days of re-administration of the opioid ("relapse") . Initial heroin administration caused abnormalities in pathways associated with energy metabolism. The concomitant reduction in serum free fatty acids (palmitic acid, linoleic acid, oleic acid, non-esterified cholesterol, docosahexaenoic acid, octadecanoic acid) and increase in the TCA cycle

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intermediate citrate in both serum and urine suggest a depletion of fatty acids for energy production and the upregulation of the TCA cycle as a source of energy. Changes in the concentrations of aspartate, hydroxyproline, tryptophan, leucine, valine, phenylalanine, threonine and thymine imply that amino acid metabolism was also perturbed. Elevations in aspartate, the serotonin precursors 5-hydroxyindoleacetic acid (5-HIAA) and serotonin indicate changes in neurotransmission. An effect of heroin administration on membrane integrity is indicated by increased *myo*-inositol-1-phosphate and *myo*-inositol. An increase in circulating *myo*-inositol was detectable early (by day 5), while the increase in aspartate occurred later (between day 5 and 10), indicating time-dependent changes in the effect of heroin on membrane integrity. These results indicate that membrane disruption precedes (and may be responsible for) changes in amino acid metabolism. After 4 days of abstinence, the urinary and serum metabolomes were comparable with those of controls indicating a rapid return to homeostasis. However, variation was observed in their recovery. While none of the urinary metabolic abnormalities elicited by drug exposure persisted at withdrawal, serum *myo*-inositol-1-phosphate and threonate remained increased and decreased, respectively. This suggests that membrane damage and long-term abnormalities in synaptic density are a long-term consequence of heroin exposure persisting well into withdrawal (Figure 2). Similar observations to the chronic heroin group were noted in the relapse group, showing deviations from the control group and overlap with the animals that had 10 days of heroin exposure. This indicates that the metabolic state of relapse is comparable to that of long-term heroin exposure (Zheng et al., 2013).

Ning *et al.* (2018) also compared the serum metabolic profile of rats undergoing heroin self-administration re-exposure after a period of abstinence (used to model relapse) to drug-naïve rats. Consistent with previous observations, disruptions were seen in pathways related to

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energy homeostasis (TCA intermediates, keto bodies), phospholipid cycling (cell membrane components: choline, phosphocholine and glycerol phosphocholine) and neurotransmission (amino acids and related molecules: choline, phenylalanine and glutamine). However, in contrast to the previous study by Zheng *et al.* (2013), these authors (2018) observed an inhibition of the TCA cycle, demonstrated by an increase in glucose and decrease in pyruvate and fumarate (and a decrease in lactate). In addition, the ketone bodies 3-HB and acetoacetate were increased with heroin indicating a shift from oxidative phosphorylation to ketogenesis as an alternative source of energy. These differences may result from the variation in the sampling time points between the studies reflecting the short- and long-term adaptive mechanisms in energy metabolism to heroin administration.

A similar mouse study showed equivalent results in brain samples analysed by ultra-performance liquid chromatography time-of-flight mass spectrometry (Li et al., 2017). Chronic heroin administration disturbed central energy metabolism, demonstrated by an elevation of citrate and nucleotide monophosphates. Increases in the excitatory neurotransmitter glutamate, the catecholamines dopamine and adrenaline, and in the neuromodulators histamine and melatonin also point towards abnormalities in neurotransmission elicited by chronic heroin use. The elevation of histamine may also link chronic heroin use with the reported activation of mast cells and other components of the immune system in addicted individuals (Galli et al., 1993). A two-day withdrawal from heroin was sufficient for catecholamines to return to baseline levels, suggestive of an autonomic re-adjustment during abstinence. In contrast, histidine was observed to decrease upon withdrawal while phenylalanine, tryptophan and *N*-acetylserotonin increased, pointing to adaptive mechanisms involving the serotonergic system.

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These findings are supported by the results of a human study that investigated the hair metabolome of heroin abusers (Xie et al., 2016). The heroin group showed increased concentrations of sorbitol and cortisol and decreased concentrations of arachidonic acid, glutathione, linoleic acid, and myristic acid (Xie et al., 2016). The impact of heroin on the HPA axis via opioid signalling may underlie the variation noted in cortisol. A decrease in the free fatty acids arachidonic acid, linoleic acid, and myristic acid is consistent with increased energy production, as seen in the mice exposed to heroin (Zheng et al., 2013).

Metabolic phenotyping of psychostimulant addiction

Psychostimulants increase striatal dopamine concentrations by increasing dopamine levels in the NAc. Cocaine inhibits the reuptake of dopamine in mesolimbic dopaminergic neurons projecting from the VTA to the NAc by blocking dopamine transporters located presynaptically, while amphetamine and methamphetamine facilitate presynaptic dopamine release. Psychostimulants also stimulate the release of other monoamines such as serotonin and noradrenaline (Kim et al., 2019). Rodent metabolomic studies using brain tissue have explored the abnormalities in central metabolic processes driven by acute (Li et al., 2012; Kaplan et al., 2013; Olesti et al., 2019) and repeated psychostimulant administration (Li et al., 2012, 2014; Adkins et al., 2013; Bu et al., 2013; McClay et al., 2013; Kong et al., 2018; Lin et al., 2019). Biofluids including urine, blood and hair samples have also been studied to understand the metabolic consequences of exposure (Yao et al., 2013; Goodwin et al., 2014; Choi et al., 2017; Sánchez-López et al., 2017; Olesti et al., 2019), abstinence (Shima et al., 2011; Zheng et al., 2014; Zhang et al., 2016a; Kim et al., 2019) and the establishment of drug reinforcement (Zaitsu et al., 2014). Human studies investigating the impact of psychostimulants on hair (Kim et al., 2020) and serum/plasma metabolome (Costa et al., 2019;

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Lin et al., 2019), and the effect of abstinence from cocaine (Patkar et al., 2009) will also be reviewed. These studies are summarised in Table 1.

Metabolic signature of cocaine addiction

The central metabolic signature of rats undergoing both acute and repeated cocaine administration was investigated using NMR spectroscopy-based metabolomics (Li et al., 2012). 7-day cocaine-CPP increased glutamate and GABA in the NAc and enhanced GAD activity, implying altered neurotransmission along the Gln-Glu-GABA axis. The reported ability of cocaine to elicit glutamine production by glial cells may explain these observations (Sá Santos et al., 2011). These alterations were evident after repeated administration but not after a single dose. Such changes are consistent with what observed with morphine (Gao et al., 2007) indicating a common adaptive, long-term response to chronic drug exposure (Figure 2). Indeed, adaptive functional changes are known to occur at glutamatergic synapses in the NAc in response to repeated cocaine administration (Maze et al., 2010). As these changes mirror behavioural sensitization (Ungless et al., 2001; Thomas and Malenka, 2003; Russo et al., 2009), they represent a key molecular component of the addictive properties of cocaine. Lactate, which is produced via anaerobic metabolism and can be metabolised through the TCA cycle, was decreased after a single dose but increased after chronic administration, indicating a long-term compensatory change in energy metabolism in response to cocaine exposure. An NMR-based study by Kong *et al.* (2018) suggested that disturbances in energy metabolism may be explained by epigenetics mechanisms. Cocaine-conditioned mice exhibited significantly higher concentrations of nicotinamide mononucleotide (NMN) and nicotinamide adenine dinucleotide (NAD) in VTA and NAc. NMN and NAD are produced from nicotinamide by the enzyme nicotinamide phosphoribosyltransferase (NAMPT) and play a role in energy

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metabolism. NAMPT was shown to be upregulated in cocaine-conditioned mice via an epigenetic mechanism involving NAD-dependent histone deacetylase Sirtuin 1 (SIRT1), thus pointing towards a role for SIRT1 in epigenetic regulation of genes, such as NAMPT, that control energy metabolism (Kong et al., 2018). Altered creatine levels may also be indicative of a shift in normal energy metabolism (Li et al., 2012). The observed dysregulation of creatine (increased in NAc and decreased in striatum) and taurine (increased in both areas) have been proposed to reflect oxidative damage. While an increase in taurine and decrease in its metabolic precursor cysteine could indicate the induction of a brain protective mechanism following cocaine administration, a single dose of cocaine induced a reduction in taurine in NAc, suggesting a short-term depletion of its antioxidant capacity before the long-term adaptive increase. The concentrations of NAA, a marker of neuronal density synthesised in mitochondria, was increased in NAc and striatum after both acute and chronic cocaine administration, pointing to an immediate effect of the drug on mitochondrial dysfunction. Finally, membrane damage is indicated by alterations in *myo*-inositol, glycine and choline concentration, which were affected by a single dose of cocaine (Li et al., 2012).

A quantitative evaluation of the global neurobiochemical profile of cocaine-treated rats was achieved by ion mobility mass spectrometry (Kaplan et al., 2013). Acute cocaine administration significantly reduced thalamic and striatal glucose, with the greatest decrease seen in the thalami. In the frontal cortex, cocaine exposure increased glucose content, indicating region-specific shifts in glucose metabolism following cocaine treatment. The availability of serotonin, noradrenaline, glucose, dopamine, DOPAC and 5-HIAA in the thalamus, striatum and PFC was also altered as a result of cocaine exposure (Kaplan et al., 2013), indicating abnormalities in neurotransmission induced by the drug. This is consistent with the reported ability of psychostimulants to promote the release of other monoamines such

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as serotonin and dopamine (Kim et al., 2019), which is thought to be part of the underlying mechanism of cocaine reward (Sora et al., 2001).

Abnormalities in neurotransmitter metabolism were also reported in brain and blood samples of rats administered a single dose of cocaine (Olesti et al., 2019), and in the serum of rats undergoing a cocaine self-administration protocol (Goodwin et al., 2014). Using a targeted liquid chromatography with tandem mass spectrometry (LC-MS/MS) approach, significant elevations were noted in acetylcholine in the PFC; valine, leucine, GABA, glutamate, choline, acetylcholine, carnitine, acetylcarnitine, creatine, creatinine, adenosine in the hippocampus, and choline and adenosine in the striatum. In the cerebellum, glutamate, choline, acetylcholine, carnitine, creatinine were increased. In plasma, choline and creatine were increased, while creatinine was decreased. Some of these alterations are likely to reflect pharmacological effects of cocaine on the muscle and the brain. For example, cocaine-induced rhabdomyolysis (muscle injury) can lead to altered creatine and creatinine metabolism, with downstream consequences on the brain high-energy phosphate system (Lyoo et al., 2003), while the reported inhibition of choline acetyltransferase by cocaine (Wilson et al., 1994) could explain the increase in hippocampal acetylcholine. A separate study also reported an increase in (3-methoxy-4-hydroxyphenyl)ethylene glycol and kynurenic acid, metabolites of noradrenaline and tryptophan, respectively. Both metabolites were previously implicated in cocaine addiction (Rockhold et al., 1991; McDougle et al., 1994) and may be related to disturbances in catecholamine (*i.e.* noradrenaline and dopamine) and serotonin metabolism. Significantly alterations in amino acid metabolism were also reported by an LC-MS metabolomics study (Sánchez-López et al., 2017), where mice were infused daily for 21 days with cocaine, either alone or administered with ethanol. Cocaine alone elevated plasma methionine and decreased argininosuccinic acid and *N*-ε-acetyl-l-lysine, while co-infusion of cocaine and alcohol resulted

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in elevated carnosine, spermidine and serotonin, and decreased methionine, argininosuccinic acid and N- ε-acetyl-l-lysine (Sánchez-López et al., 2017). The derangements in methionine and argininosuccinic acid suggest an overall increase in ROS and nitric oxide production, two factors that contribute to liver injury in cocaine-dependent individuals (Aoki et al., 1997). In contrast, N-ε-acetyl-l-lysine could provide acetylated lysine residues for the epigenetic changes underlying cocaine-based reinforcement.

Biochemical modulations in plasma and urine were assessed in rats undergoing cocaine-CPP (Zaitsu et al., 2014). While significant metabolic changes were found in plasma of cocaine-treated rats (higher L-threonine and n-propylamine; lower cysteine and spermidine), no metabolic variation was identified in urine following treatment relative to controls. In a separate rat study by Yao *et al.* (2013), clear differences were observed in the urine between cocaine and a control group. Several factors may account for these differences, including differences in animal strain, cocaine dose, length and means of drug administration as well as in chromatographic techniques (LC-MS in Yao *et al.* (2013) vs GC-MS in Zaitsu et al. (2014)). Additionally, there is ample evidence that cocaine metabolism differs profoundly between mice and rats, possibly explaining the more marked behavioural and hepatotoxic phenotype in response to cocaine in mice relative to rats (Thompson et al., 1979).

The persistence of biochemical modulations following acute withdrawal from cocaine was studied in the NAc of rats 2h, 24h, and 48 h following a single exposure (Li et al., 2012). The metabolic profiles of the treated animals were distinct from the control group at all time points, indicating that biomolecular alterations persist in the NAc up to 48 hours after exposure, with only a subset of metabolites returning towards baseline concentrations. A single exposure was not sufficient to cause changes in GABA. However, taurine was significantly decreased and

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NAA was increased as soon as 2 hours after a single dose, suggesting that oxidative stress and membrane disruption occur early during the addiction cycle. Interestingly, lactate and phosphocholine remained elevated 48 hours post-dosing. The involvement of lactate in synaptic plasticity hints at a direct role in consolidating drug-related memories (Hillard, 2005; Wang et al., 2019). Astrocyte-neuron lactate transport in the basolateral amygdala (BLA) is critical for the reconsolidation of cocaine memory and strategies reducing lactate concentrations (by blocking glycogenolysis in astrocytes) in the BLA of mice undergoing a CPP paradigm have been shown to prevent the establishment of cocaine-induced CPP and to decrease cocaine-self administration (Zhang et al., 2016b). Importantly, while GABA was not dysregulated after a single cocaine exposure nor after repeated administration, it was significantly altered in mice exposed to cocaine-CPP, highlighting that contextual conditioning of a drug is able to induce metabolite changes in the brain which are independent of the effect of the drug administration *per se*. Therefore, the inclusion of a group of animals treated with the drug but not undergoing CPP should be considered when investigating cocaine-CPP effects on the metabolome. In a separate study, the metabolic perturbations in energy supply (creatine, creatinine, adenosine), oxidative stress (glutathione, spermidine), neurotransmission (pyroglutamic acid, glutamine, glutamate, GABA), mitochondrial function (carnosine) and membrane integrity (choline) induced by cocaine self-administration were still evident after one day abstinence in PFC, striatum and NAc, but normalised at week 3 in all brain areas except the striatum (Zhang et al., 2016a). The finding that cocaine use leads to long-term metabolic abnormalities in the striatum may explain the presence of drug craving long after withdrawal from the drug (Volkow et al., 2006).

Understanding the metabolic changes occurring upon drug withdrawal is important to shine light on the biochemical mechanisms underlying recovery from addiction. A human study

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involving 18 cocaine-dependent individuals investigated the effect of two weeks of abstinence on their plasma metabolic profile (Patkar et al., 2009). This study found significant alterations in purine and tryptophan metabolism, as reported in opioid-dependent individuals upon detoxification (Mannelli et al., 2009), but no changes in oxidative stress related metabolites. It is generally believed that oxidative stress might be applicable for acute rather than more prolonged intoxication, which may explain these findings. Plasma metabolic profiles from cocaine-dependent individuals were correlated with their addiction severity index (ASI) drug scores. Specifically, *N*-methylserotonin accounted for 62% of variance in severity of drug abuse based on ASI drug score and combined with xanthine it accounted for 73%. These findings implicate plasma *N*-methylserotonin and xanthine as good candidate biomarkers for assessing and predicting addiction severity. In accordance with Mannelli *et al*. (2009), no significant changes in serotonin metabolism were observed, indicating that the biosynthesis of serotonin from tryptophan was unaffected by cocaine. Instead, the increase in *N*-methylserotonin suggests a dysregulation of the enzyme that metabolizes serotonin to *N*-methylserotonin following chronic cocaine exposure. This hypothesis requires further investigation and future studies with larger sample sizes and more appropriate controls are warranted.

Metabolic signature of methamphetamine addiction

The animal studies investigating the metabolic effects of methamphetamines have reported contradicting findings. Similar to what was observed for cocaine, several studies reported disrupted energy metabolism as a consequence of chronic methamphetamines intake (Shima et al., 2011; Zheng et al., 2014; Kim et al., 2019). A significant depletion of TCA cycle intermediates (Shima et al., 2011; Zheng et al., 2014) and branched-chain amino acids (Zheng et al., 2014; Kim et al., 2019) was observed in the blood and urine of rats repeatedly exposed

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to methamphetamines. On the other hand, no change in TCA intermediates was detected in plasma and urine after methamphetamine-CPP training (Zaitsu et al., 2014). As glycolysis is downregulated upon drug deprivation (Muneer et al., 2011), it can be speculated that the metabolic effect of methamphetamine on the TCA cycle is due to acute withdrawal and cannot be elicited by a chronic CPP paradigm. Collectively, these results suggest that different drug administration protocols have distinct effects on plasma and urine metabolic signatures, possibly as a result of adaptive mechanisms to repeated drug use and/or to the presence or absence of contextual learning (*i.e.* CPP). A common finding to these studies was altered lipid metabolism. Reduced plasma lauric acid and increased urinary stearic acid were induced by methamphetamine-CPP (Zaitsu et al., 2014). In the absence of contextual learning, methamphetamine elicited changes in the β-oxidation of free fatty acids and the formation of 3-HB, indicative of altered lipid turnover, as well as changes in glycerophospholipids and sphingolipids, suggestive of membrane breakdown, in both urine and blood, although the direction of change is unclear (Shima et al., 2011; Zheng et al., 2014). Changes to lipid metabolism may represent a compensatory mechanism to meet the increased energy demand induced by methamphetamine exposure. Methamphetamine was seen to elicit different effects on some lipids compared to heroin. For example, serum *myo*-inositol and *myo*-inositol-1-phosphate was increased in response to heroin (Zheng et al., 2013) but reduced in response to methamphetamine (Zheng et al., 2014). Further evidence of altered energy metabolism in response to methamphetamine comes from a study performed on *Drosophila melanogaster* (Sun et al., 2011b). Flies fed on a methamphetamine-supplemented diet had lower circulating trehalose, the major blood sugar in the *Drosophila*, indicating higher metabolic rates and/or increased glycolysis. Interestingly, trehalose supplementation increased the flies' lifespan, indicating that methamphetamine toxicity is linked to a depletion of energy co-factors and that replenishing these co-factors may attenuate the negative effects of the drug (Sun et al., 2011b).

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The change in the concentrations of neuroactive compounds in the blood of methamphetamine-treated animals additionally suggests altered excitability at the CNS level following methamphetamine intake. Zheng *et al.* (2014) observed increased aspartate and glutamate but lower alanine and glycine in the serum of rats chronically exposed to methamphetamine. Kim *et al.* (2019) reported a reduction in plasma phenylalanine, tyrosine, and tryptophan biosynthesis pathways with methamphetamines, indicative of a potential downstream effect on catecholaminergic and monoaminergic neurotransmission. In support of this hypothesis, increases in tryptophan and tyrosine and decreases in serotonin were reported in the whole brain of mice treated with a single methamphetamine dose (McClay et al., 2013). This study investigated the different mechanisms underlying acute and chronic methamphetamine exposure. While acute methamphetamine treatment elicited alterations in energy related metabolites (*e.g.* reduced fructose, increased lactate, malate, 2-hydroxyglutarate, succinate, fumarate), chronic methamphetamine-administered mice had higher amounts of the sugars fructose and sorbitol, the antioxidants putrescine and ergothioneine and the membrane component phosphocholine compared to the control animals (Adkins et al., 2013; McClay et al., 2013). These results suggest that acute administration is characterised by disrupted energy metabolism and alterations in neurotransmitters and fatty acid metabolism, while the chronic effects of methamphetamine are further associated with oxidative stress and membrane damage.

Bu *et al.* (2013) and Lin *et al.* (2019) also demonstrated impaired energy metabolism, along with changes in neurotransmitter and oxidative stress metabolites in the brain of chronically methamphetamine-treated rats. These metabolites were predominantly related to GABA, monoamine and oxidative stress metabolites, as well as TCA cycle intermediates and indicators of neuronal membrane disruption. Bu *et al.* (2013) reported decreases in dopamine, glutamine

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and glutamate in the hippocampus, NAc and PFC, consistent with previous models of chronic methamphetamine administration (Moszczynska et al., 2004; Thomas et al., 2008). The decrease of glutamine and glutamate mirrored the decrease of GABA and 2-oxoglutarate, which is partly due to decreased TCA cycle activity and, possibly, increased glutamate uptake. Succinic acid semialdehyde levels increased, consistent with its role as an intermediate of GABA catabolism. A decrease in glutamate and glutamine was also observed in the NAc and dorsal hippocampus by Lin *et al.* (2019), although these metabolites, along with the amino acid and excitatory neurotransmitter aspartate, were downregulated in the ventral hippocampus. Together, these findings suggest that the disturbance to Gln-Glu-GABA axis in the brain may be involved in the behavioural sensitization to methamphetamine. Together with a general increase in nucleotides like ADP, GMP, AMP in NAc and dorsal hippocampus (but a decrease in the ventral hippocampus), these findings point towards an alteration in energy homeostasis in a brain region-dependent manner. A reduction in the antioxidant glutathione was reported in both studies (Bu et al., 2013; Lin et al., 2019). Moreover, a reduction in NAA and an increase of phosphocholine was observed in brain regions of chronically treated rats, indicating that oxidative damage was present alongside neuronal and mitochondrial dysfunction (Bu et al., 2013). The increase in homocysteine, an amino acid and precursor of methionine, could be regarded as an indicator of apoptosis and neuronal hypersensitivity to excitation as well as DNA damage (Kruman et al., 2000). Moreover, the increase in homocysteine may be caused by the inhibition of methionine synthesis by methamphetamine (Chandra et al., 2006), leading to changes in DNA methylation. Increased *myo*-inositol and phosphocholine are consistent with membrane disruption (Bu et al., 2013) and may reflect cell death due to the severely neurotoxic properties of methamphetamine (Zheng et al., 2014). Phospholipids were generally downregulated in NAc and dorsal hippocampus but upregulated in the ventral hippocampus (Lin et al., 2019), suggesting region-specific effects of methamphetamine treatment. Finally,

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Bu and colleagues (2013) found no significant correlation between metabolic disruptions and locomotor sensitization behaviour. Given that locomotor sensitization is linked to increased craving and vulnerability to relapse (Robinson & Berridge 1993; Vanderschuren & Pierce 2010), this finding suggests that these metabolites may have poor predictive potential. Future studies are needed to determine whether a CPP design and the resulting contextual learning are needed in order to find a significant correlation with locomotor sensitization.

Mass spectrometric analysis of the metabolic effects of repeated methamphetamine self-administration in rat urine and hair revealed abnormalities in the metabolism of mineralocorticoid, fatty acid amides, and in mitochondrial fatty acid oxidation (Choi et al., 2017). In the urine, ion features corresponding to potential urine markers of methamphetamine addiction were detected but only putatively assigned. In the hair, a decrease in deoxycorticosterone suggests altered central production of neurosteroids (Mellon and Griffin, 2002), while an increase in carnitine and acylcarnitines are indicative of elevated metabolic capacity by mitochondrial oxidation of fatty acids. The reduction observed in the fatty acid amides oleamide and stearamide, known to interact with endocannabinoid, glutamatergic and GABAergic signalling, may indicate a modulation of central neurotransmission. The authors concluded that methamphetamine evoked more dramatic metabolic changes in the hair than in the urine. This may reflect the greater stability of hair and longer accrual of biochemical information related to drug-induced metabolic perturbations compared to urine samples. As such, hair metabolomics should be considered as a non-invasive way to profile the addiction status of an individual.

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A study conducted on the hair metabolome of methamphetamine users revealed additional abnormalities in the abundance of amino acids and lipids (Kim et al., 2020). The amino acids arginine and methionine, both known ROS scavengers (Luo and Levine, 2009; Liang et al., 2018), were downregulated in the hair of drug abusers, which may indicate higher susceptibility to oxidative stress. Lower phosphatidylcholines, but higher lysophosphatidylcholines and sphingomyelin suggested a dramatic dysregulation of lipid biosynthesis/metabolism pathways, which is consistent with what reported from animal models of methamphetamine exposure (Kim et al., 2019; Lin et al., 2019) as well as human studies on cocaine and methamphetamine abusers (Ross et al., 2002). These observations point to the possibility that chronic drug use activates phospholipase A2, the enzyme that cleaves phosphatidylcholine into lysophosphatidylcholine. The elevation of acylcarnitines is consistent with findings from animal studies (Kim et al., 2019) and suggests abnormal β-oxidation of fatty acids and mitochondrial dysfunction. A reduction in carnitines (both free and acetylated forms) and lower long-chain fatty acids was also reported in the fasting serum metabolic profile of crack-cocaine users (Costa et al., 2019). The circulating levels of lactate, histidine and tyrosine were also elevated in the crack group. Thus, increased energy needs induced by crack abuse may result in disrupt the mitochondria (indicated by carnitine/fatty acid metabolism), resulting in a shift to alternative energy sources (elevation of lactate) and the altered biosynthesis of neurotransmitters (histamine and catecholamines from histidine and tyrosine respectively), (Figure 2). In the serum, metabolites that were dysregulated in methamphetamine-addicted individuals included TCA intermediates, amino acids, and other biomolecules (Lin et al., 2019). An increase in excitatory amino acids (glutamine, asparagine, glutamate) and a decrease in inhibitory amino acids (glycine, alanine) point to an increase in brain activity induced by the drug. Acetylcholine was also elevated (while choline was reduced) in the plasma of methamphetamine users, collected at fasting (Lin et al., 2019). Similar findings associated with

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altered energy metabolism, steroid biosynthesis, amino acid and fatty acid metabolism were reported in response to other types of psychostimulants, such as MDMA, amphetamine and mephedrone in human plasma (Nielsen et al., 2016; Steuer et al., 2020), suggesting a common mechanism of action. the metabolic phenotype. Shima *et al.* (2011) reported that the methamphetamine-induced plasma and urinary alterations (mainly associated with altered TCA intermediates, amino acids and fatty acids) persisted 24 hours after the last drug administration but were not evident at 96 hours. In a separate study, two days of detoxification was generally sufficient to restore the serum and urinary metabolic profile of methamphetamine-treated rats to pre-treatment levels (Zheng et al., 2014). In the serum, several amino acids including glutamine, glutamate and aspartate (involved in neurotransmission and energy metabolism) were completely restored, while fatty acids like arachidonic acid, decanedioic acid, stearic acid and glycerol-3-phosphate (involved in membrane stability and energy metabolism) were only partially recovered. Isoleucine, palmitic acid, creatinine, citrate and 2-oxoglutarate did not return to pre-treatment values. In urine, only lactate was persistently altered after withdrawal (Zheng et al., 2014). Similarly, Kim *et al.* (2019) reported that twelve- or 24-hour abstinence resulted in the reversal of the metabolic abnormalities in glycerophospholipids, sphingolipids and most amino acids elicited by methamphetamine self-administration. However, the concentrations of aspartate, glutamate and glycine remained significantly lower than controls 12 and 24 hours after the last methamphetamine administration. Acetylcarnitines and biogenic amines were further altered during abstinence, suggesting that the dynamic response of the metabolome to methamphetamine exposure spans at least 24 hours following the last administration. Moreover, serotonin exhibited a short-term increase (immediately after self-administration), but a long-term decrease after methamphetamine exposure (12 hr and 24 hr after the last exposure). Similarly, pathway analysis demonstrated a decrease in the phenylalanine, tyrosine,

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and tryptophan biosynthesis and in the valine, leucine, and isoleucine biosynthesis pathways immediately after self-administration, but an increase after 12 and 24 hours relative to controls. These results highlight the importance of investigating the time profile of metabolic responses to drugs of abuse, which may provide biomarkers relevant for discriminating addiction states. Moreover, the time when the metabolic change occurs may inform on whether the change is the result of a short-term molecular mechanism or of a longer-term compensatory mechanism (*e.g.* transcriptional/epigenetics). Exploring these molecular mechanisms may help to guide the development of interventional strategies for clinical practice.

Future perspectives

In recent years, findings from metabolomic studies have provided a metabolic perspective to the addiction cycle that complements our neurophysiological and neuropharmacological knowledge (Figure 2). These studies have shown that opioid and psychostimulant addiction results in largely overlapping metabolic derangements. The increase in energy demand observed in such studies as a short-term consequence of drug exposure reflects the pharmacological profile of those drugs on the brain and other organs. As the individual transitions towards a state of dependence, metabolomic studies reveal biochemical changes indicative of mitochondrial dysfunction (*e.g.* NAA), cell death and oxidative stress (*e.g.* lactate, choline, taurine) and energy depletion. Such alterations are reflective of known adaptations induced by long-term administration of drugs of abuse on the brain and other organs. Energy depletion, for instance, may be reflective of the presence of a homeostatic compensatory mechanism which opposes the chronic pharmacological effect of the drug. In relation to the brain this includes a) the broadly characterised suppression of the mesolimbic reward pathway (decline of basal dopamine levels and D_2 receptor levels in the striatum) which is thought to

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underline some of the emotional withdrawal symptoms and craving induction commonly experienced in dependence and b) the hypofunctioning of the frontal cortex which is associated with impaired decision making and loss of top-down control over drug administration, rendering the behaviour "automatic". Impairment of interrelated cellular processes of mitochondria metabolism, oxidative stress and apoptosis assessed by membrane potential and respiratory chain activity, by elevation of indictors of oxidative stress (*e.g.* superoxide dismutase and glutathione) and by TUNEL staining, has consistently been reported in brain, heart and liver, following long term administration of substances of abuse, although diversity exists in relation to the severity of those effects. Nonetheless, these changes have been associated with addictive behaviour pathology, neuro-, cardio-toxicity and cognitive decline among other pathological consequences of long-term drug abuse. Alterations in neurotransmitters and their precursors balance (*e.g.* dopamine, serotonin, Gln-Glu-GABA axis) following chronic drug use identified from metabolomic studies are consistent with neurochemical adaptations reported in the brain via other experimental approaches. These are reflective of compensatory homeostatic neuroadaptations which underline the behavioural manifestations of addiction (*e.g.* tolerance, physical and emotional withdrawal symptoms, relapse).

With the emergence of metabolomics, biomarker research has shifted from the hypothesis-driven study of single molecules to the simultaneous measurement of thousands of compounds with no *a priori* knowledge. This hypothesis-generating approach facilitates the identification of biochemical pathways involved in pathological processes and the discovery of new pharmacological targets. Understanding the biochemical changes occurring at each stage of the addiction cycle can facilitate the identification of diagnostic and prognostic biomarker panels to provide objective measures of addiction and rehabilitation. Despite the success of metabolomics in clinical research, its translation to clinical practice has been undermined by

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issues of reproducibility and cost. Clinical metabolomics relies on the comparison of the metabolic profile of a single individual with "healthy" reference ranges. Thus, the existence of standardised protocols of sample collection, preparation, data analysis and metabolite identification, along with the selection of an appropriate control population and validated quality control pipelines need to be in place before metabolomics can be routinely applied in the clinic. Targeted analysis of a small panel of diagnostic/prognostic markers measured in easily accessible samples, such as urine, blood or hair, could be routinely run to objectively monitor an individual's progress. By leveraging individual differences in the metabolome, metabolomics provides an objective measure that can inform personalised approaches to addiction to maximise the chances of recovery. Well-characterised longitudinal studies where the metabolic profile of an individual is measured at baseline, before the initiation of pharmacological detoxification therapies, will allow to identify early predictive markers and personalised treatment strategies tailored to the metabolome of the individual patient. The prediction of addiction outcomes based on neuroimaging data has already been successful (Reske and Paulus, 2008). The use of metabolic markers quantified from non-invasive samples (*e.g.* urine and hair) could offer a more feasible and cost-effective method to bring precision medicine to clinical practice.

Conclusion

In recent years, the misuse of prescription opioids in the US has led to what is currently known as the "opioid epidemic", a public health crisis costing $26 billion to the US healthcare system and 16000 deaths in 2013 alone (Florence et al., 2016). Identifying markers of addiction can help identify those at risk and lower the rate of fatalities. Drug abuse affects 35 million people

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worldwide (United Nations, 2020), and with a relapse rate of 40-60% (NIDA, 2020), achieving personalised and effective treatment options is a pressing issue. Metabolomic techniques performed on accessible samples offers great promise to facilitate the implementation of precision medicine interventions to achieve faster diagnosis and better treatment efficacy based upon an individual's metabolic phenotype.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Data availability statement

Data sharing is not applicable to this article because no new data were created or analysed in this study.

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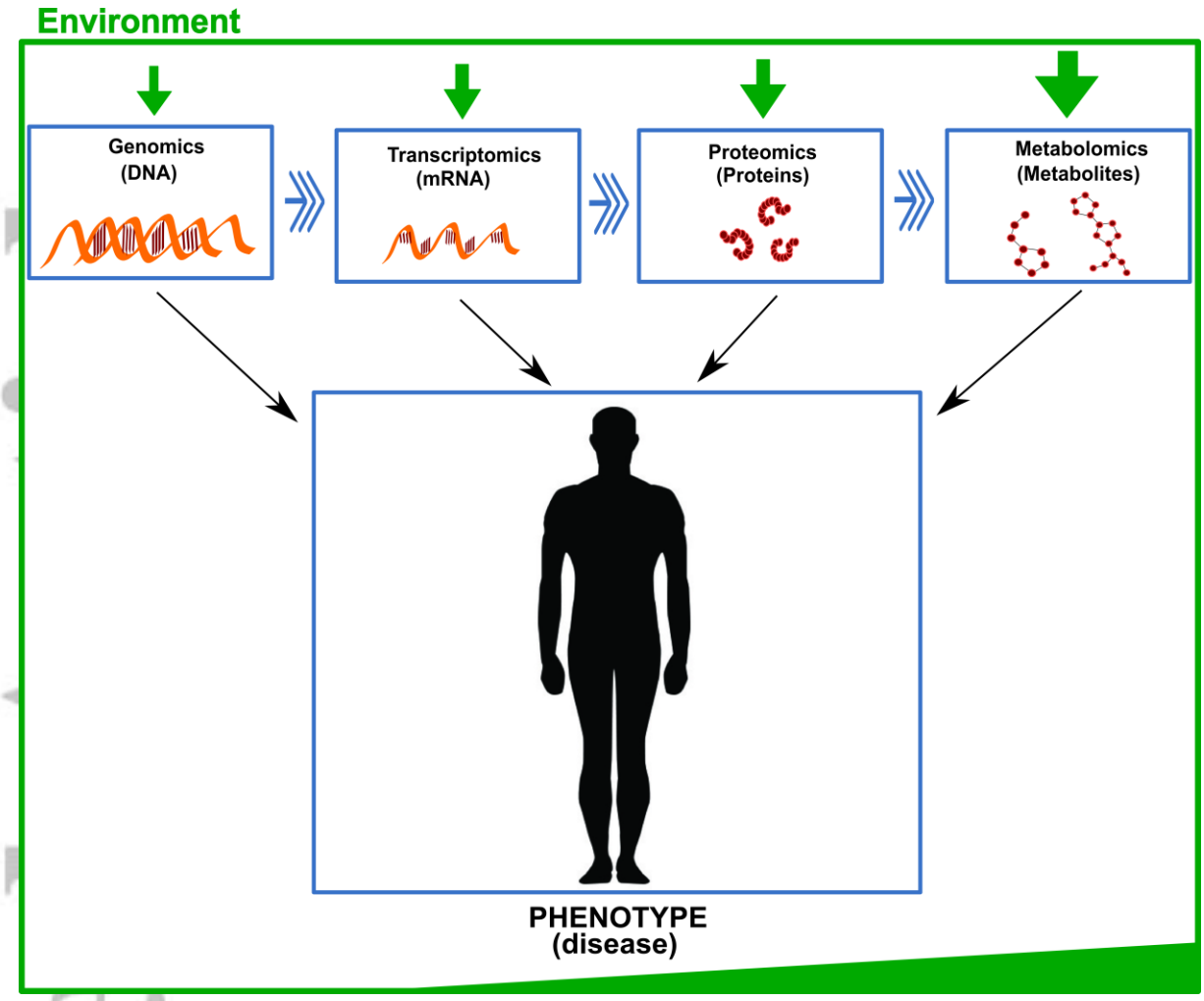

Figure 1. Each level of the "omics" cascade gives a different level of insight into the phenotype. The interaction of each component (genome, transcriptome, proteome, metabolome) with the environment (nutrition, stress, drugs) influences the resulting phenotype and can contribute to disease. The size of the green arrows indicates the influence of the environment increases on each level of the omics cascade, and is highly reflected by the metabolome. Although each omics technique can reveal important diagnostic and prognostic biomarkers of disease, the ability of metabolomics to capture both endogenous (*i.e.* genetic) and exogenous (*i.e.* environmental and drug-related) influences on the observed disease phenotype, its chemical diversity and dynamic nature, suggest that metabolic biomarkers may better represent the resulting phenotype of drug abuse.

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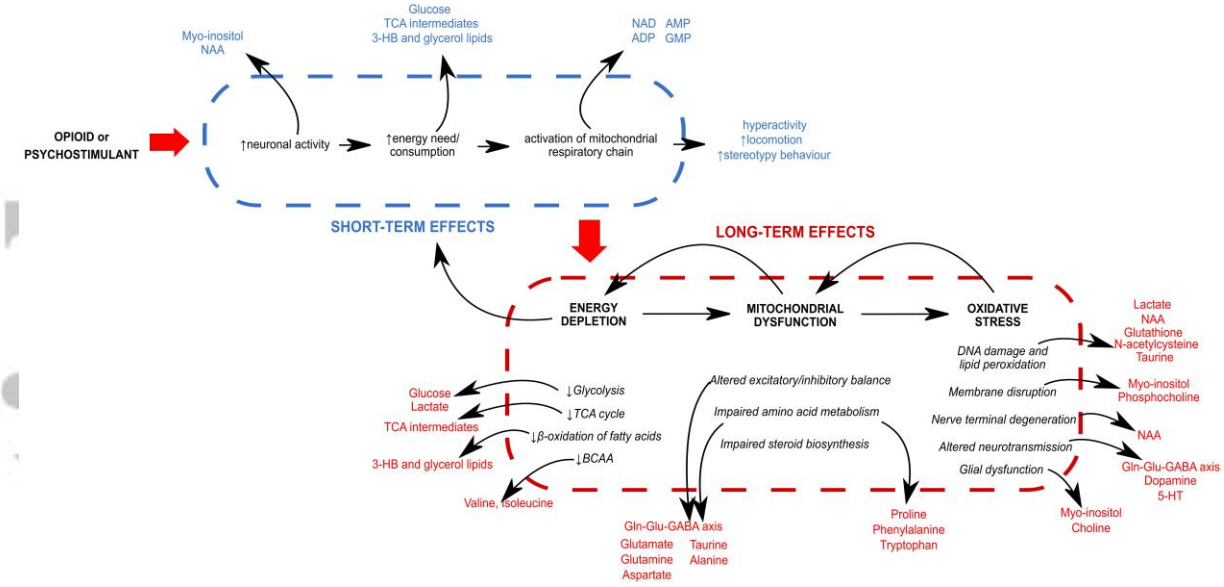

Figure 2. Diagram of metabolic alterations caused by drugs of abuse. Metabolites that are altered in response to drug exposure provide information on the underlying cascade of events leading to addiction. As most of the changes leading to an established addiction state are likely to involve adaptive mechanisms, it is important to investigate and discriminate between the short-term effects of acute drug exposure and the long-term, compensatory changes resulting from chronic drug administration (i.e. dependence) on the metabolome. A general mechanism of action of both opioids and psychostimulants seems to involve a short-term increase in energy demand, which leads to long-term energy depletion, mitochondrial dysfunction and oxidative stress. These long-term effects contribute to a cascade of events that feed the cycle of metabolic and pathophysiological derangements characterizing addiction, as shown with the backwards arrows.

3-HB – 3-hydroxybutyric acid, **5-HT** – 5-hydroxytryptamine, **Gln** – glutamine, **Glu** – glutamate, **NAA**, – N-acetylaspartate, **TCA** – tricarboxylic acid

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Table 1. Summary of animal and human studies. 5-HIAA – 5-hydroxyindoleacetic acid, **ADP** – Adenosine diphosphate, **AMP** – Adenosine monophosphate, **Cer –** Cerebellum , **DOPAC –** 3,4- dihydroxyphenylacetic acid, **GMP –** Guanosine monophosphate, **GPC –** Glycerol phosphocholine, **GSH/GSSG –** glutathione/oxidised glutathione, **Hi –** Hippocampus, **NAA -** *N*-acetylaspartate, **NAc –** Nucleus accumbens**, NAG –** *N*-acetyl-glycoprotein, **OAG –** *O*-acetyl-glycoprotein, **Pc –** phosphocholine, **PFC –** Prefrontal Cortex, **Str –** Striatum, **TCA –** Tricarboxylic acid**, Th –** Thalamus, **VTA –** Ventral tegmental area

Drug	Protocol	Species / Participants	Tissue(s)/ Fluid(s)	Main results	Altered metabolites (drug of abuse vs control)	Analytical method	Reference
Morphine	90 days escalating dose 3-15 mg/kg of drug 3 times/ day (s.c) followed by 7 days detoxification period (6 mg/kg methadone or 0.02mg/kg clonidine (i.g) 3 times/day)	Rhesus Monkeys	Hi, PFC	Shift in the equilibrium of glu-gln-GABA axis Significant membrane and energy metabolism disruption Methadone/Clonidine treatment reversed most metabolites except glutamate See Table 2 and 3 for details	Hi: ↑Glutamine, GABA, Lactic acid ↓Glutamate, GABA, Succinic acid semialdehyde, Glutathione, NAA, Phosphocholine, Myo-inositol, Taurine, Lactic acid, Creatine PFC: ↑Succinic acid semialdehyde, NAA, Phosphocholine, Lactic acid, Creatine ↓Glutamate, GABA, Myo-inositol, Taurine	^{1}H-NMR spectroscopy (600 MHz)	Deng et al., 2012
	14 days escalating dose 5-40 mg/kg drug 2 times/day (i.p) followed by detoxification with 10 mg/kg methadone or 0.2 mg/kg clonidine (i.g) (3 administrations at 12h intervals)	Male Sprague-Dawley Rats	Hi, PFC, NAc and Striatum	Shift in the equilibrium of glu-gln-GABA axis	Hi: ↑Glutamate, Succinic acid semialdehyde, NAA, Lactic acid, Creatine; ↓Glutamine, GABA, Phosphocholine, Myo-inositol	^{1}H-NMR spectroscopy (600 MHz)	Hu et al., 2012

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			Significant membrane and energy metabolism disruption	PFC: ↑GABA, Succinic acid semialdehyde, Lactic acid; ↓Glutamine, Glutamate, Glutathione, Myo-inositol, Creatine		
			Methadone and clonidine reversed most metabolites, but clonidine restored metabolites which methadone was ineffective for.	NAc: ↑ Glutamine, Glutamate, GABA, Succinic acid semialdehyde, Glutathione, NAA, Taurine, Lactic acid, Creatine; ↓ Myo-inositol		
			See Table 2 for details	Str: ↑ Glutamine, Glutamate, GABA, Succinic acid semialdehyde, Glutathione, NAA, Phosphocholine, Taurine, Lactic acid; ↓ Myo-inositol, Creatine		
10 mg/kg 2 times/day of drug for 1, 6 or 10 days (i.p)	Male Sprague-Dawley Rats	Hi, PFC	Significant disturbance of glu-glu-GABA axis and other metabolites after 10 days of administration (see Table 2)	Hi: ↑ Glutamine, Myo-inositol; ↓ Glutamate, GABA, NAA, Taurine	^{1}H-NMR spectroscopy (800 MHz)	Gao et al., 2007
10 day administration group underwent 1, 3 or 5 days of abstinence			Even 3 day long withdrawal period normalises metabolites	PFC: ↑ GABA, Myo-inositol; ↓ Glutamate		
	Male ICR mice	Whole brain	21 significantly altered metabolites	↑Carbodiimide, 2-Methyl-propanoic acid, Aminoethyl	GC-MS	Meng et al., 2012

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			between morphine and control groups	alcohol, l-proline, 3-Hydroxybutanoic acid, l-valine, Ethanedioic acid, Uracil, d-mannofuranoside, 4(3H)-Quinazolinone, 2-methyl-3-[2-methyl-3-[(trimethylsilyl)oxy]phenyl]-, Niacinamide, 2,3-dimethyl-3-hydroxyglutaric acid, Pyrogallol, Dodecanoic acid, 2-Hexenedioic acid, 2-Hydoxy-1,2,3-propanetricarboxylic acid, Myo-Inositol, d-Glucopyranose phosphate, Myo-Inositol phosphate		
alternating morphine [4 mg/kg (s.c.)] or saline [10 mL/kg (s.c.)] + 7 days of CPP			myo-inositol, myo-inositol phosphate, nicotinamide, proline and valine reflect abnormal intracellular signal transduction pathway in morphine-induced CPP			
			PCA and ROC analysis revealed clear separation with an area under the curve value of 0.947	↓Arabinonic acid, Cholesterol		
4 mg/kg + 10 days CPP	Male Sprague-Dawley Rats	Plasma, urine	Full separation of urine samples on TCA due to 12 metabolites Partial separation in plasma samples due to 4 metabolites PLS- regression model based on these could predict CPP scores	Plasma: ↑ N-propylamine; ↓ 3-hydroxybutyric acid, L-tryptophan, Cystine Urine: ↑ 2-Ketoglutaric Acid, Fumaric Acid, Malic Acid, L-Threonine; ↓ Glutamic Acid, Isoleucine, L-Valine, L-Aspartic Acid, Oxamic Acid, 2-Aminoethanol, Indoxyl Sulfate, Creatinine	GC-MS	Zaitsu et al., 2014

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			11 organic acids, 13 amino acids, 6 sugars and 6 fatty acids discriminated between groups	[Morphine vs control groups]: ↑ oxalic acid, aminomalonate, beta-d-glucose, linoleic acid, stearic acid, cholesterol; ↓ alanine, proline, glycine, serine, threonine, pyroglutamic acid, hydroxyproline, erythronic acid, glutamine, arabinose, rhamnose		
5 days of escalating dose of drug 10-50 mg/kg 3 times/day (i.p.) followed by 5 mg/kg naloxone (i.p.)	Male Sprague-Dawley Rats	Plasma	PLS-DA model could discriminate between euphoria, withdrawal and tolerance groups	[Naloxone vs control groups]: ↑ lactic acid, oxalic acid, aminomalonate, oxoglutaric acid, beta-dglucose, linoleic acid, cholesterol, palmitic acid; ↓alanine, valine, proline, glycine, serine, threonine, pyroglutamic acid, hydroxyproline, erythronic acid, glutamine, arabinose, rhamnose, isoleucine, succinate, 4-hydroxybutanoic acid, leucine	GC-MS	Liu et al., 2015
6-day inpatient detoxification with methadone (30 mg on day 1, tapered by 5mg daily)	14 opioid-dependent individuals enrolled in methadone detoxification programme; 10 non-	Plasma	Overactivity of oxidation-reduction pathways, purine and monoamice metabolism	↑GSH/GSSG, α- and γ-tocopherol ↑guanine, xanthosine; ↓guanosine, hypoxanthine, hypoxanthine/xanthine, xanthine/xanthosine ↑N-methylserotonin	liquid chromatography electrochemical array detection (LCECA)	Mannelli et al., 2009

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		dependent controls					
Heroin	10 days of escalating dose of drug 3-16.5 mg/kg 2 times/day (i.p) followed by 4 days of abstinence, followed by 4 days of relapse period (16.5 mg/kg heroin i.p. once a day for 4 days)	Male Sprague-Dawley Rats	Serum , urine	Urine and serum metabolites show similar trend of changes throughout the study Metabolome of withdrawn rats tend back to baseline, except: *myo*-inositol, citrate and threonate Metabolic state of relapse is closer to 10 days exposure group.	Serum: ↑Aspartate, Leucine, Valine, Phenylalanine, Threonine, Citrate, Myo-inositol-1-phosphate, Myo-inositol, Thiamine; ↓Hyrdoxyproline, Tryptophan, 5-hydroxytryptamine, Threonate, Creatine, Non-esterified cholesterol Urine: ↑Pyroglutamate, Spermidine, Tryptophan, Citrate, Aconitate, Myo-inositol, Glucuronate; ↓Aspartate, Glycine, β-Alanine, Hyrdoxyproline, Fumarate, Lactate, Octadecanoic acid	GC-MS	Zheng et al., 2013
	Chronic administration (15 mmol/kg, i.p., twice a day) for 12 days; withdrawal group given saline for the last 2 days	Male C57BL/6J mice	Brain	Altered amino acids, tricarboxylic-acid cycle intermediates, neurotransmitters, nucleotides and other compounds	Withdrawal: ↓histidine and melatonin; ↑phenylalanine and tryptophan, N-acetylserotonin; restored catecholamines	UPLC-TOF/MS	Li et al., 2017

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Self-administration: infusion (0.06 mg/kg); followed by 14 days of abstinence	Male Sprague–Dawley rats	Serum	Impaired energy production, altered fatty acid metabolism, altered neurotransmission	↑Choline, PC, GPC, glucose, 3-HB, acetoacetate, glutamine, phenylalanine, acetate, NAG, OAG ↓Fumarate, pyruvate, histamine, lactate, lipid	^{1}H-NMR spectroscopy (600 MHz)	Ning et al., 2018
Hair collected from vertex posterior region of the head consumption of heroin was confirmed by blood testing	58 heroin abusers and 72 non-heroin abusers	Hair	Possible upregulation of the HPA axis	↑Sorbitol, Cortisol ↓ Myristic acid, Linoleic acid, Arachidonic acid, Glutathione, 3 unknown compounds	UFLC-ITTOF	Xie et al., 2016
Acute 30 mg/kg administration (i.p.)	Male Sprague-Dawley Rats	Frontal cortex, left and right striatum and thalamus	Global metabolic analysis showed separation of control vs. cocaine treated brains Standard addition to characterise biogenic amine levels' response to acute cocaine	PFC: ↑ Serotonin, Norepinephrine, Glucose, Dopamine, DOPAC; ↓ 5-HIAA	IMMS for global metabolites	Kaplan et al., 2013
				Str: ↑ Norepinephrine; ↓ Glucose, Dopamine, DOPAC	Isotope dilution for [glucose]	
				Th: ↑ Serotonin, Dopamine, DOPAC, 5-HIAA; ↓ Norepinephrine, Glucose	Standard addition for NE, 5-HT,5-HIAA, DA, DOPAC	
	Male Wistar Rats	Nac, Striatum	Even single exposure causes oxidative stress (OS) and lasting changes in metabolites	[Repeated administration (CPP)]: NAc: ↑ Lactate, NAA, Acetylcystein, Glutamate, Succinate, GABA, Creatine, Taurine; ↓ Leucine, 3-Hydroxybutryc	^{1}H-NMR spectroscopy (600 MHz)	Li et al., 2012

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		Acid, L-Lysine, Cysteine, Myo-Inositol
Single administration: 20 mg/kg (i.p), sacrificed after 2, 24 or 48 hours		[Repeated administration (no CPP)]: Nac: ↑NAA, succinate, creatine, taurine
	Repeated treatment caused mitochondrial dysfunction and membrane disruption, neurotransmitter deviation and OS	[Repeated administration (CPP)]: St: ↑ NAA, GABA, taurine, choline; ↓ 3-HB, lactate, creatine, glycerol, myo-inositol
Repeated administration: 10 or 20 mg/kg (i.p) 7days CPP conditioning.		[Repeated administration (no CPP)]: St: ↑ GABA
	Rats treated with CPP and without CPP separated based on their metabolites with PLS-OSC	[Single exposure - 2 hrs]: ↑ NAA, Myo-Inositol, Cysteine; ↓ Lactate, Creatine, Choline, Taurine, Glycine, Phosphocholine
Repeated administration: 20mg/kg (i.p) 7days but with no CPP		[Single exposure - 24 hrs]: ↑ NAA, Myo-Inositol, Creatine, Taurine, Cysteine; ↓ Lactate, Choline, Glycine, Phosphocholine
	Separation also between 2h, 24h and 48h groups.	[Single exposure - 48 hrs]: ↑ NAA, Choline, Taurine, Glycine; ↓ Lactate, Myo-Inositol, Cysteine, Phosphocholine

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Repeated administration: 20 mg/kg (i.p.) 6 days CPP conditioning 20 mg/kg	C57BL/6J mice (6–8 weeks old)	VTA and Nac	Altered energy metabolism and epigenetic regulation	↑nicotinamide mononucleotide (NMN) and nicotinamide adenine dinucleotide (NAD), blocked by specific Nicotinamide phosphoribosyltransferase (NAMPT) inhibitor FK866	^{1}H-NMR spectroscopy (600 MHz)	Kong et al., 2018
20 mg/kg (i.p.)	Male Wistar rats	Brain (PFC, Cer, Hipp or St) and plasma	Altered neurotransmission	PFC: ↑acetylcholine Cer: ↑ glutamate, choline, acetylcholine, carnitine, creatinine Hipp: ↑ valine, leucine, GABA, glutamate, choline, acetylcholine, carnitine, acetylcarnitine, creatine, creatinine, adenosine St: ↑ choline, adenosine Plasma: ↑ choline, creatine; ↓creatinine	LC-MS/MS	Olesti et al. 2019
7-day pre-treatment with nicotine (50 μg/ml) followed by cocaine + 4 days CPP	Male C57BL/6J mice	Brain (NAc and St)	Nicotine enhances cocaine-induced CPP (2 mg/kg cocaine with nicotine pre-treatment was	20 mg/kg cocaine vs control: Nac: ↑ glutamate, tryptamine, glucose 1-methylhistidine, proline Nac: ↓ lactate, acetylcholine, L-glutamine, L-methionine, creatine, alpha-ketoglutaric acid, phosphocholine	^{1}H-NMR spectroscopy (600 MHz)	Li et al., 2014

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			similar to 20 mg/kg cocaine only)			
Five groups: (A) control (B) 2 mg/kg cocaine (C) 20 mg/kg cocaine (D) nicotine (E) nicotine +2 mg/kg cocaine.			Similar metabolic effects of nicotine pre-treatment and low cocaine dose and high cocaine only Altered neurotransmission, energy homeostasis and cell metabolism	St: ↑ glutamate, 1-methylhistidine, glucose St: ↓ lactate, alpha-ketoglutaric acid, L-glutamine Nicotine +2 mg/kg cocaine vs control Nac: ↑ glutamate, 1-methylhistidine, proline Nac: ↓ tryptamine, lactate, L-glutamine, L-methionine, creatine, alpha-ketoglutaric acid St: ↑ glutamate St: ↓ alpha-ketoglutaric acid, L-glutamine		
Self-administration for 6h/day or up to 50 infusions/day, followed by extintion (cocaine replaced with saline) for 8 days, and reinstatement (10 mg/kg i.p.)	Rats (species unspecified)	Serum	only a subset of features was investigated, due to the primary aim of the study to evaluate the	↑3-methylene-indolenine, (3-methoxy-4-hydroxyphenyl) ethylene glycol, kynurenic acid, Deoxyuridine, thymidine Estradiol, glutathione, 3-	UPLC-IM-MS and UPLC-IM-MSE	Goodwin et al., 2014

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			application of the MEDI method to discriminate phenotypic froups based on mass spectroscopy signals This method (based on self-organising maps) allows for the discrimination of cocaine addicted, cocaine non-addicted and cocaine naïve rats	indole carboxylic acid glucuronide, 5-hydroxy-6-methoxyindole, Glucuronide, dityrosine, LPE(20:5), LPC(18:2)		
10 mg/kg (i.p.) + 10 days CPP	Male Sprague-Dawley Rats	Plasma, Urine	No separation of urine and partial separation of plasma	Plasma: ↑ threonine, n-propylamine; ↓ cystine, spermidine; no differences detected in urine	GC-MS	Zaitsu et al., 2014
1-day or 3-week withdrawal after self-administration training	Male Sprague-Dawley rats	Brain (St, PFC, Nac)	Altered markers of oxidative stress, energy metabolism, membrane integrity	St (1-day): ↑ creatinine, pyroglutamic acid, adenosine; ↓ choline St (3-wk): ↑ creatinine, spermidine, carnosine, adenosine PFC (1-day): ↑ GABA, creatinine, adenosine; ↓ creatine, glutamate NAc (1-day): ↑ glutamate; ↓ niacinamide, glutamine, adenosine	HPLC-IMMS	Zhang et al., 2016

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	21 days of 105-min daily infusions of: (i) cocaine(15 mg/kg); (ii) alcohol (2 g/kg); (iii) cocaine + alcohol (15 mg/kg and2 g/kg, respectively); (iv) 0.9% (w/v) sodium chloride solution(control group)	Male and female Wistar rats	plasma	Altered amino acid metabolism	Cocaine vs control: ↑ Methionine, ↓ Argininosuccinic acid and N-ε-acetyl-l-lysine Cocaine+alcohol vs control: ↑ carnosine, spermidine, serotonin; ↓methionine, Argininosuccinic acid, N- ε-acetyl-l-lysine	LC-MS	Sánchez-López et al., 2017
	Blood collected in the morning after 8-hr fasting	44 crack-cocaine users and 44 healthy volunteers (males only)	Serum	Altered histamine and dopamine metabolism?	↑ lactate, histidine and tyrosine ↓ long chain fatty acid, carnitine and acylcarnitines	NMR	Costa et al., 2019
	>2 weeks of abstinence (monitored by urine drug screens three times a week for 2 weeks)	18 cocaine-dependent individuals and 10 healthy controls	Plasma	N-methylserotonin and xanthine can predict addiciton severity N-methylserotonin, xanthine, xanthosine, and guanine differentiated cocaine and control groups.	↑ n-methylserotonin and guanine ↓ hypoxanthine, anthranilate and xanthine	LCECA	Patkar et al., 2009
Methamphetamine	10 mg/kg administration every hour (4 injections) (*i.p*) Sample collection:	Male Sprague-Dawley Rats	Plasma, urine	Both urine and plasma of treated animals separated from controls on PCA scores after 24	Group A: Plasma: ↑ Glucose; ↓ 3-HB	GC-MS and CE-MS	Shima et al., 2011

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			hrs, but all metabolites restored after 96 hrs.			
Group A: 0-24 h (urine) or 24 h (plasma) after treatment Group B: 72-96h (urine) or 96 h (plasma) after treatment			Decreased TCA intermediates in both urine and plasma indicate energy production inhibition	Urine: ↑ 5-oxoproline, saccharic acid, and uracil; ↓ TCA cycle intermediates (including aconitate, alpha-ketoglutarate, malate, fumarate, succinate, oxaloacetate/pyruvate, and isocitrate/citrate), 3-HB, and adipic acid Group B: metabolic disturbances observed in Group A returned to baseline levels		
Single exposure:10 mg/kg followed by 10-30 mg/kg/day escalating dose for 5 days followed by 2 days of drug free detoxification	Male Sprague-Dawley Rats	Serum, urine	Disruption of amino acid, inositol and lipid metabolism and TCA cycle (energy interruption and increased physical activity).	Serum: ↑ Asparatate, Glutamate, Glutamine, Tryptophan, Lysine, Cis-9-hexadecenoic acid, 3-HB, Fumarate, Galactonolacetone; ↓ Alanine, Glycine, Proline, Serine, Threonine, Valine, Leucine, Isoleucine, Taurine, Monopalmitin, Oleic acid, Palmitic acid, Heptadecanic acid, Stearic acid, Glycerol, Glycerol-3-phosphate, α-aminoisobutyrate, Aminomalonic acid, α-ketoglutarate, Citrate, Pyruvate, Succinate,	GC-MS	Zheng et al., 2014

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					Glucose, Creatine, Indoleacetate, Myo-inositol, Lactate			
					Urine: ↑ Alanine, Glutamate, Glycine, Serine, Palmitic acid, 3-HB, Glycerol, Citrate, Pyruvate, Fumarate, Succinate, Myo-inositol, Lactate; ↓ Heptadecanic acid, Stearic acid, Hippurate			
	plasma collected immediately after 16 days of MA self-administration (SA) and after 12 and 24 h of abstinence	Male Sprague–Dawley Rats	Plasma	Greatest separation observed between 24 hr abstinence and control mice (untargeted) and between 24 hr abstinence and self-administering rats (targeted)	SA vs control: ↑hexadecanoylcarnitine, octadecanoylcarnitine, octadecadienylcarnitine, acetylcarnitine; serotonin; PC (aa 30:0, 32:0, ae 34:0, 34:1,); SM 18:0	UPLC-QTOF-ESI-MS (untargeted) and (LC)-QQQ-MS (targeted)	Kim et al., 2019

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				Variance explained largely by amino acid metabolism	↓carnitine; alanine, arginine, asparagine, citrulline, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithinine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, acetylornithine, asymmetric dimethylarginine, carnosine, creatinine, methionine sulfoxide, putrescine, spermidine, trans-4-hydroxyproline, dimethylarginine		
				Disrupted metabolic pathways involved in energy metabolism, the nervous system, and membrane lipid metabolism	↓lyso PC (16:0, 16:1, 17:0, 18:0, 18:1, 18:2, 20:3, 20:4, 26:0, 26:1); PC(aa 36:0, 36:2, 36:3, 36:4, 38:0, 38:5, 38:6, 40:4, 40:5, 40:6, 42:5, 42:6, ae 38:2, 38:4, 40:4, 40:5, 40:6, 42:1, 42:2, 42:3, 44:6); SM (OH) 22:2		
2 mg/kg i.p. + 10 days CPP	Male Sprague-Dawley Rats	Plasma, urine	No significant separation observed between treated and control animals, neither in plasma or urine. Few metabolite changes are not enough to have caused a PCA score separation and their		Plasma: ↑ N-propylamine; ↓ lauric acid Urine: ↑ lactose, spermidine, and stearic acid	GC-MS	Zaitsu et al., 2014

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			biological significance is unclear. Unchanged energy metabolites			
2.5 mg/kg drug twice a day for 7 days (*s.c*) Locomotor measurements on days 0, 1, 3, 5 following injections (sensitization)	Male Wistar rats	Hi, NAc, PFC	Metabolite shifts indicate, disruption of GABAergic function and membranes, OS and mitochondrial dysfunction. No significant correlation with locomotor sensitisation	Hi: ↑ Homocysteic acid, Myo-Inositol, Succinic acid semualdehyde, ADP; ↓ GABA, Glutamate, Glutamine, NAA, Glutathione, Acetylcysteine, Methionine, α-ketoglutarate, Succinate, Citrate NAc: ↑ Phosphocoline, Homocysteic acid; ↓ GABA, Glutamate, Glutamine, NAA, Dopamine, Serotonin, Taurine, Glutathione, Acetylcysteine, Methionine, α-ketoglutarate, Succinate, Citrate PFC: ↑ Phosphocoline, Myo-Inositol; ↓ GABA, Glutamate, Glutamine, NAA, Serotonin, Taurine, Glutathione, Methionine, α-ketoglutarate, Succinate, Citrate	^{1}H-NMR spectroscopy (600 MHz)	Bu et al., 2013

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Single 3 mg/kg dose or 5 daily injections (*i.p*)	8 different strains of male mice: 129S1/SvImJ, A/J, C3H/HeJ, C57BL/6J, CAST/EiJ, DBA/2J, NOD/ShiLtJ, PWD/PhJ	Whole brain	Metabolite deviations showed disruption of TCA cycle, fatty acid metabolism and damage to the mitochondrial electron transport chain in acute and chronic administration between strains. Different metabolites contributed to the separation of single exposure group from control than repeated exposure.	Single dose: ↓ fructose and serotonin; ↑ lactate, malate, 2-hydroxyglutarate, succinate fumarate, tryptophan and tyrosine Chronic administration: ↑2-hydroxyglutarate, fructose, sorbitol, putrescine, ergothioneine and phosphocholine	LC-MS and GC-MS	McClay et al., 2013
3 mg/kg drug once/day for 5 days (*i.p*)	8 different strains of male mice: 129S1/SvImJ, A/J, C3H/HeJ, C57BL/6J, CAST/EiJ, DBA/2J, NOD/ShiLtJ, PWD/PhJ	Whole brain	Homocarnosine, pantothenate, 4-guanidinobutanoate and *myo*-inositol explained 91% of behavioural sensitisation variation across the eight strains of mice. .	↑lactate, malate, 2-hydroxyglutarate, succinate, fumarate, tryptophan and tyrosine ; ↓fructose, serotonin	LC-MS and GC-MS	Adkins et al., 2013

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1 mg/kg (i.p.) + 6 days CPP	male Sprague-Dawley rats	Brain (NAc, dorsal and ventral Hipp)	Altered energy metabolism, amino acids metabolism, and phospholipids metabolism	Nac: ↓glucose, creatine, glutamate and glutamine, glutathione, PE(P-18:0/22:6) and PS(18:0/22:6) ; ↑ADP, aspartate, PA(18:0/22:6) Dorsal Hipp: ↓glucose, creatine, glutamate and glutamine, glutathione, PE(P-18:0/22:6), PE(16:0/18:1), PE(18:0/22:6) and PS(18:0/22:6); ↑GMP, ADP, and AMP, aspartate Ventral Hipp: ↑creatine, glutamate, glutamine and aspartate, PA(18:0/22:6) (Fig. 6I) and those three PEs ; ↓GMP, ADP and AMP	MALDI-MSI	Lin et al. 2019
Control diet or diet supplemented with 0.6% (w/v) METH	Virgin male Drosophila melanogaster	Whole organism	Increased dietary trehalose (sugar) partially alleviated the toxic effects of METH Altered carbohydrate metabolism, dysregulation of calcium and iron homeostasis, increased oxidative stress, and disruption of mitochondrial functions	↓ trehalose	GC-MS and MS/MS	Sun et al., 2011

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daily 2-h self-administration sessions (i.v., 0.05 mg/kg/injection)	male Sprague–Dawley rats	Urine and hair	More ion features were significantly altered in hair than in urine	Hair: ↑Acetylcarnitine, 5-methylcytidine, 1-methyladenosine and palmitoyl-(l)-carnitine; ↓(l)-norvaline/betaine/5-aminopentanoate/ (l)-valine, lumichrome, deoxycorticosterone, oleamide, stearamide and hippurate	LC-QTOF-MS	Choi et al., 2017
Sample collection at baseline (M1 = "drug-free"), after stable lever-press responding to methamphetamine (M2 = "reward"), after methamphetamine following extintion with saline (M3 = "relapse")			mineralocorticoid, fatty acid amides, mitochondrial fatty acid oxidation	Urine: potential early, late and recent markers of addiction were found but not assigned		
3 cm hair strands from the root	10 heavy MA abusers and 12 drug-free controls	Hair	Altered metabolism of acylcarnitines, amino acids and biogenic amines, glycerophospholipids and sphingolipids	↑carnitine, decadienylcarnitine, octadecadienylcarnitine; arginine; lysophosphatidylcholine acyl C160, C170, C181, C204; sphingomyelin C241, C221, C241 ↓octadecanoylcarnitine, valerylcarnitine; methionine; phosphatidylcholine diacyl C341, C362, C365, C366, C381, C383, C385, C403, C404, phosphatidylcholine acyl-alkyl C340, C360, C361, C365, C380, C381,	UPLC-QTOF-ESI-MS (untargeted); AbsoluteIDQ p180 kit (targeted)	Kim et al., 2020

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				C382, C383, C401, C402, C422, C423		
Blood collected in the morning at fasting	30 METH abusers and 30 healthy controls (males only)	Serum	Altered TCA cycle intermediates, amino acids and other biomolecules	↑ lactate, glutamine, ornithine,niacinamide, histidine, creatinine, acetylcholine, asparagine, glutamate, malic acid, 5-aminolevulinic acid, taurine, 1-methylnicotinamide, citrulline, guanidine acetic acid ↓ choline, succinate semialdehyde, threonine, glycine, serine, isoleucine, proline, xanthine, creatine, alanine, sulfate, methionine, uric acid, citrate, pyruvic acid, fumarate, N-acetyl-L-aspartate, ketoleucine, valine, succinate	UHPLC-MS/MS	Lin et al., 2019

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Table 2. Brain region specific changes in metabolite concentrations following chronic morphine treatment vs control animals. Studies conducted in rhesus monkeys and rats (see protocol in Table 1). ↑ - increase, ↓- decrease, ■ - rhesus monkeys (Deng et al., 2012) ■ - rats (Hu et al., 2012) ■ - rats (Gao et al., 2007) * -disagreement between Hu *et al.* (2012) and Gao *et al.* (2007).

Morphine treated *vs.* control					
		Hi	FCx	NAc	Striatum
Neurotransmitters	Glutamine	↑↓↑*	↓	↑	↑
	Glutamate	↓↑↓*	↓↓↓	↑	↑
	GABA	↑↓↓	↓↑↑	↑	↑
	Succinic acid semialdehyde	↑	↑↑	↑	-
Oxidative stress metabolites	Glutathione	↓	↓		
	NAA	↑↓*	↑	↑	↑
	Phosphocholine	↓↓	↑		↑
	Myo -inositol	↓↓↑*	↓↓↑*	↓	↓
	Taurine	↓↑↓*	↓	↑	↑
Energy metabolism	Lactic acid	↑↑	↑↑	↑	↑
	Creatine	↓↑	↑↓	↑	↓

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Table 3. Effectiveness of clonidine and methadone detoxification treatment on metabolite deviations caused by morphine treatment. Studies conducted in rhesus monkeys (Deng et al., 2012) and rats (Hu et al., 2012), (see protocol in Table 1).

✓- both clonidine and methadone treatment restored, ✗- neither clonidine nor methadone restored metabolite, clonidine-only clonidine restored, methadone – only methadone restored, a dash means there were no results reported

	Withdrawal intervention with clonidine or methadone					
	Rhesus monkey			Rats		
	Hi	FCx	Hi	FCx	NAc	Striatum
Glutamine	✓	-	Clonidine	✓	Clonidine	✗
Glutamate	✗	✗	Methadone	✓	✓	✓
GABA	✓	✗	-	Clonidine	Clonidine	✗
Glutathione	✗	-	-	✓	-	-
NAA	-	✓	✓	-	✓	✓
Phosphocholine	✓	✓	✓	-	-	✓
***Myo*-inositol**	✓	✓	Clonidine	✗	✓	-
Taurine	✓	Methadone	✓	-	Clonidine	✓
Lactic acid	✓	Clonidine	Clonidine	✗	Clonidine	Clonidine
Creatine	✓	✓	✓	Clonidine	✓	✓
Succinic acid semialdehyde	-	✗	✓	✓	✗	-

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