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Special Issue: Biomarkers of Substance Abuse

Opinion

Focusing on the Opioid System for Addiction Biomarker Discovery

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Substance use disorders (SUD) and behavioral addictions are devastating conditions that impose a severe burden on all societies, and represent difficult challenges for clinicians. Therefore, biomarkers are urgently needed to help predict vulnerability, clinical course, and response to treatment. Here, we elaborate on the potential for addiction biomarker discovery of the opioid system, particularly within the emerging framework aiming to probe opioid function in peripheral tissues. Mu, delta, and kappa opioid receptors all critically regulate neurobiological and behavioral processes that define addiction, and are also targeted by major pharmacotherapies used in the management of patients with SUD. We propose that opioid biomarkers may have the potential to improve and guide diagnosis and therapeutic decisions in the addiction field.

A Clinical Need for Biomarkers in Drug and Behavioral Addictions: The Opioid System

SUDs affect up to one in ten subjects over their lifetime [1], a prevalence that is even higher when including **behavioral addictions** (see Glossary) [2]. SUDs associate with substantial burden through somatic and psychiatric comorbidities, neuropsychological impairments, and a high risk of death by suicide and overdose [1]. Addictive disorders are the consequence of a complex interplay between life experiences (including trauma during childhood) and social environments, as well as individual genetic and neurobiological vulnerability factors. Although SUDs and behavioral addictions both involve dysregulation of the brain reward system (Box 1), their clinical expression is heterogeneous [2]. In addition, emergence of an addictive disorder and determinants of the disease phenotype remain highly unpredictable, even in vulnerable populations. Pharmacotherapies for addictive disorders are currently limited and only available for SUDs, not for behavioral addictions (see examples in Box 2). In light of these challenges, and because of the difficulty of assessing brain function in affected individuals, there has been growing interest in the identification of putative addiction **biomarkers** over the past decade.

Substances associated with a variable risk of abuse are widely used in the general population (e.g., alcohol and nicotine) and in daily medical practice (e.g., **opiates** for pain management or **L-Dopa** for the treatment of Parkinson's disease [3]). As a consequence, biomarkers are needed to help predict vulnerability to addiction in the general population, as well as in specific subgroups. Biomarkers are also being developed to evaluate the prognosis of affected individuals, such as the risk of overdose, the intensity of craving, or the risk of relapse during abstinence. Furthermore, biomarkers could help predict medication dosage, response, and tolerance during pharmacological interventions. Overall, while a major hurdle in modern psychiatry stems from the absence of objective biological measures that can be used for

Highlights

Recent findings suggest that opioid receptors, critically regulating reward, mood, and cognitive processes, represent promising targets for the discovery of clinically useful addiction biomarker across all drugs of abuse.

Biomarkers may include genetic, epigenetic, transcriptomic, and imaging measures of opioid signaling in the brain and peripheral tissues.

Medications that activate or antagonize mu opioid receptors are used across many drug and behavioral addictions, and call for biomarkers to determine which medication should be used, at what dose, and at which stage of the disease, as well as to predict risks of overdose and relapse.

Molecular profiling of single cell types is revealing heterogeneous properties and reactivity in health and disease. This gears animal models of addiction and translational applications towards exploiting cell type-specific strategies that focus on well-identified populations of opioid receptor-expressing cells.

Peripheral samples, as well as cells and organoids derived from addicted individuals, constitute necessary models to probe interindividual variability in drug-induced and addiction-related opioid dysfunction.

Longitudinal studies over the full course of addictive disorders, as well as the use of combinations of multiple biomarkers to evaluate addiction, may significantly improve the management of affected individuals.

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Box 1. Mammalian Opioid Neurobiology

Addiction can be described as a three-stage vicious cycle, and studies in animal models using pharmacological approaches, genetically modified animals, and, more recently, **optogenetics** concur to establish that opioid receptors have major roles at each of these stages, with the potential for risk, diagnostic, or prognostic biomarkers (see Figure 1 in the main text) [76].

MOR represents a key molecular player for reward processing of drugs of abuse [76]. Opiates, as well as alcohol, tetrahydrocannabinol (THC), and nicotine, all produce reinforcement at least in part by recruiting MOR signaling [76]. These effects are notably due to the activation of MOR along the **mesolimbic pathway**, leading, through a disinhibition mechanism documented across rodent and human species, to increased dopamine signaling [92]. By contrast, the dynorphin/KOR pathway is an antireward system that inhibits dopamine neurons [76], thereby triggering dysphoric states and antagonizing reinforcing properties of drugs of abuse (including opiates, alcohol, cocaine, nicotine, and THC). Therefore, drug reward is bidirectionally regulated by MOR- and KOR-dependent modulations of dopaminergic signaling [93].

KOR also critically contributes to relapse, a major aspect of addiction. A large body of evidence indicates that: (i) reinstatement of drug seeking is blocked or attenuated by genetic or pharmacological blockade of KOR in rodents, across several drugs of abuse (cocaine, alcohol, heroin, and nicotine); and (ii) these processes rely on multiple interactions between KOR and **monoaminergic systems** [94].

Emotional comorbidities and neurocognitive processes implicated in addiction are also modulated by opioid signaling [95,96] (see Figure 1 in the main text). During the development of an addiction, drug use initially engages goal-directed and impulsive actions, while executive control over drug intake progressively weakens, leading to habitual behaviors and compulsive drug use [76]. Rodent models suggest that MOR and DOR contribute to all of these aspects [76]. Moreover, a role for MOR expressed by striatal GABAergic neurons was recently shown in mice to underlie motivational, but not reinforcing, properties of drug (heroin) or natural (chocolate) stimuli [97], unraveling an anatomofunctional dissociation between MOR populations.

The molecular processes recruited following opioid receptor activation have been extensively investigated. These receptors selectively recruit inhibitory G_i-proteins and globally repress neuronal activity. Upon opioid peptide binding, and to a certain extent constitutively [98], their signaling involves both G-protein-dependent and independent pathways [99]. The former recruits α_i subunits, which inhibit adenylate cyclase activity and stimulate G-protein-gated inwardly rectifying potassium channels, and also β/γ subunits that inhibit voltage-gated calcium channels and neurotransmitter release [99]. The latter engages arrestins, which in turn activate phosphorylation cascades, including mitogen-activated protein kinase signaling pathways (MAPK, including JNK, ERK1/2, and p38α) [99]. These processes are controlled through internalization and recycling of receptors [99], during which their redistribution and continued signaling within endosomal and Golgi compartments further extend their signaling diversity *in vivo* [100]. Beyond these mechanisms for acute opioid signaling, chronic adaptations have been described during long-term drug abuse, which may represent additional candidate biomarker targets (see the discussion in the main text).

diagnostic or therapeutic decisions, developing addiction biomarkers represents an endeavor that could significantly advance our ability to manage these conditions.

In this context, here we explore the potential of the **opioid** system for biomarker development for all known drugs of abuse and, in particular, carefully consider the emerging hypothesis that opioid function might be probed outside the brain to infer addiction-related processes. The opioid system comprises three receptors, the mu, delta, and kappa opioid receptors (MOR, DOR and KOR), activated by a family of endogenous peptides, namely endorphins, enkephalins, and dynorphins. Opioid receptors are major actors in addiction pathophysiology across almost all drugs of abuse, and several treatments currently used for SUDs directly modulate opioid signaling (Figure 1, Key Figure; Box 2). Moreover, opioid receptors are expressed in many nonbrain tissues (such as blood or skin; Box 3), where addiction-associated biological processes might be probed at the molecular level. Here, we briefly summarize opioid neurobiology in relation to addictive disorders (Box 1), and review the most-recent achievements in the development of genomic, transcriptomic, epigenetic, and imaging biomarkers related to the opioid system. Lastly, we speculate on immediate research avenues.

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Box 2. Clinician's Corner

Several drugs have received approval by the US Food and Drug Administration and the European Medicines Agency for different SUDs. These include medications for the reduction of drug use (and possibly the maintenance of abstinence) as well as replacement therapies [1]. While the efficacy of these medications has represented a historical breakthrough in psychiatry, it appears that treatment response, as well as treatment management (i.e., dosage and adverse effects) varies among individuals, and that available clinical predictors remain insufficient, underlining the need for the development of biomarkers to guide their use [1].

Labeled medications that target the opioid system fall under two categories: nonspecific opioid receptor antagonists and replacement therapies. Opioid receptor antagonists, such as naltrexone and nalmefene, are mainly used in alcohol SUD, and are thought to reduce drug use by decreasing the rewarding effects of alcohol mediated by MOR, and the dysphoric effects associated with the risk of relapse mediated by the KOR (see Box 1 in the main text) [1]. Replacement therapies include methadone (a MOR agonist), buprenorphine (a partial MOR agonist and a KOR antagonist, which lowers risk of overdose), and a buprenorphine–naloxone combination (naloxone being added to guard against intravenous buprenorphine abuse). These are thought to act by alleviating withdrawal symptoms through long-term occupancy of MOR, thereby helping opiate addicts to reduce compulsive drug use and resume normal interests and activities [101]. The daily dose of methadone has been shown to predict retention in treatment [101], and is increasingly used as a target phenotype in biomarker studies.

A longstanding clinical question relates to the potential utility of MOR-targeting pharmacotherapies in the context of nonopiate addictions. Considering that all drugs of abuse produce reinforcement at least in part by activating MOR, it is easy to speculate that MOR antagonists might help reduce drug use (whatever the drug considered, or in polydrug abusers), or, alternatively, that MOR agonists might serve as substitutive medications for nonopiate drugs. In support of these hypotheses, a recent meta-analysis concluded that naltrexone shows some efficacy as a therapeutic for alcohol, opiate, nicotine, and stimulant SUDs, as well as behavioral addictions [102], while methadone maintenance in opiate addicts has been shown to reduce concurrent cocaine usage [103]. While stimulating, these clinical data on the potential *trans*-diagnostic generalization of opiate-based therapies highlight our limited understanding of these complex conditions, as illustrated by the aforementioned putative therapeutic utility of either MOR agonist or antagonist agents.

Finally, research efforts are currently devoted to the development of new opiates, and to the understanding of their potential properties for **biased agonism** [104–106]. Such compounds hold great promise for the selective recruitment of beneficial opiate effects at the expense of historical adverse effects and, therefore, may help reduce the addiction risk in individuals receiving opiate pain therapies.

Opioid-Induced Adaptations in Peripheral Tissues

A common framework regarding biomarkers of complex psychiatric phenotypes posits that measures of biological variables in peripheral samples may reflect molecular processes occurring in the brain [4,5], a concept that has recently received convincing support in the context of alcohol SUDs [6]. Although SUDs are mental disorders whose primary mechanisms lie within the brain, it is possible to hypothesize that such chronic and severe conditions also manifest in other organs and tissues. While such inferences are difficult in the face of our limited knowledge of addiction pathophysiology, we argue that a reductionist approach focusing on well-described substrates, such as opiate-induced adaptations, has the potential to provide testable hypotheses. Recent research further suggests the notion that the different organs of the human body do not function as isolated entities and are, in fact, communicating with each other. Accordingly, molecular adaptations detectable in peripheral tissues, whether distinct from those occurring in the brain, may nevertheless be their cause or consequence. Along these lines, **extracellular vesicles**, may hold promise as putative biomarkers. Indeed, accumulating data suggest that they fulfil physiological functions in the brain, where they contribute to neural development, synaptic communication, and nerve regeneration (reviewed in [7]), while also enabling the potential monitoring of disease states. As an example, the release of extracellular vesicles in peripheral blood from glioblastoma (GBM) tumors has been observed in humans, suggesting that these exosomes could be exploited as putative predictive biomarkers of GBM [8].

Glossary

Behavioral addiction: syndrome analogous to substance addiction, with a behavioral focus other than the use of a psychoactive substance. It corresponds to a reinforcement derived from the performance of a specific behavior, and notably includes gambling disorder (now in DSM-5), sexual addiction, compulsive buying, and eating disorders.
Biased agonism: phenomenon whereby G-protein-coupled receptors exist under various agonist-dependent conformations that form specific ternary complexes (agonist + receptor + effector), which in turn recruit specific downstream signaling.
Binge-eating disorders: characterized by frequent and recurrent binge-eating episodes, with associated negative psychological and social problems.
Bioavailability: in PET scan studies, refers to the amount of radiotracer that binds to its target receptor, reflecting the protein levels of the receptor, its occupancy by competing endogenous ligands, and the pharmacokinetics of the tracer.
Biomarker: a characteristic measured as an indicator of normal biological or pathogenic processes, or responses to an exposure or intervention. They are classically classified according to their clinical purpose (e.g., risk or diagnostic) and type of measurement (e.g., genetic or metabolic).
CpG site: a cytosine is followed by a guanine in the linear DNA sequence; corresponds to the most-frequent site where DNA methylation occurs in mammals.
DNA methylation: chemical modification of DNA corresponding to the addition of a methyl group to a cytosine (typically at CpG sites). It affects DNA activity notably by repressing the expression of nearby genes.
Epigenetic: chemical and physical processes that regulate the architecture and activity of the genome (with no change in the DNA sequence). These include DNA methylation, histone modifications, and modulation by noncoding RNAs (e.g., miRNAs).

While the potential role of extracellular vesicles in addictive disorders remains unknown, an example of such brain–periphery communication was recently uncovered in relation to the opioid system [9]. In mice, ultraviolet light was shown to trigger the release of endorphins by keratinocytes in the skin, which then induced reinforcement, tolerance, and physical dependence in these animals [9]. Although it remains to be determined whether such endorphins can act in the brain via blood circulation, or locally, through opioid receptors, this work nevertheless indicates that opioid peptides synthesized in the periphery may have central brain effects [9]. Future studies will be necessary to determine the extent of such potential opioid communication across tissues.

Genetics of the Opioid System in SUDs

Human genetic studies, initially focused on comparisons between patients and healthy controls, have consistently shown that **opioid genes** are essential components of the genetic architecture of addiction (reviewed in [10,11]). With rapid technical progress in our capacity to investigate the entire genome (**genome-wide association studies**, GWAS), it has become clear that individual variants can account for a minor proportion of the total variance associated with addiction, with huge numbers of samples required to detect such effects [12,13]. This limitation is best exemplified by the A118G **single nucleotide polymorphism** (SNP): this variant has received considerable interest based on candidate studies suggesting its association with nicotine, opioid, or alcohol SUD [14]; however, GWAS studies have failed to replicate these findings [15]. Overall, the existing literature suggests that individual variants in opioid genes, when considered on their own, have limited clinical utility as potential vulnerability or diagnosis biomarkers. Nevertheless, it is expected that the future identification of combinations of alleles (polygenic effects), using machine-learning approaches for example [16], may allow clinicians to better identify high-risk individuals.

Recently, opioid genetic polymorphisms were explored in combination with gene expression studies or addiction-related phenotypes to improve biomarker utility. Accordingly, a study focusing on heroin addiction took the following two-step approach: first, it prioritized SNPs shown to affect MOR expression in brain tissue; second, it assessed those specific SNPs in a large cohort of patients and healthy controls (N = 16 729) [17]. Results revealed replicable associations between heroin addiction and SNPs located within MOR intron 1 (Figure 2). In another approach, two recent studies correlated genotype with drug-induced euphoria. Specifically, subjects that reported experiencing positive feelings during first heroin use consumed more drugs than did subjects reporting negative feelings, and allele frequencies significantly differed across the two groups for three SNPs in MOR intron 1 [18]. In the second study, amphetamine-induced euphoria was significantly associated with three groups of SNPs within the MOR locus in healthy volunteers (N = 162) [19]. Another important achievement came recently from studies on **neonatal abstinence syndrome** (NAS), which frequently affects infants born to mothers taking opiates during pregnancy. The severity of this condition in affected children (length of hospitalization and/or need for medication) was significantly associated with the A118G polymorphism in MOR [20] and with polymorphisms in other opioid receptors, namely the KOR rs702764C allele, and the DOR rs204076 A allele [21].

Finally, **pharmacogenomic** approaches can provide important data related to therapeutic outcomes. A recent GWAS (N = 1410) study revealed an association between genotype and opiate therapeutic dosing [22]. The only SNP that showed a genome-wide significant association with the dose of methadone administrated (a potent MOR agonist used as **maintenance therapy** for heroin use disorder) was identified 300 kilobases upstream of the MOR gene [22].

Extracellular vesicles: cell-derived vesicles present in almost all eukaryotic fluids (including saliva, blood, and urine). They contain proteins, nucleic acids, or specialized lipids. They are increasingly recognized as a new dimension of intercellular crosstalk (with the ability to send directional instructions to specific recipient cells), and are currently intensively explored as potential biomarkers relevant to numerous neuropsychiatric phenotypes

Functional magnetic resonance imaging (fMRI): methodology that measures brain activity by visualizing changes associated with blood flow.

Gα: one of the subunits of the membrane-associated heterotrimeric G proteins, which are typically activated by, and couple with, 7-transmembrane G-protein-coupled receptors to inhibit, for example, ($G\alpha_i$) or stimulate ($G\alpha_s$) adenylate cyclase.

Genome-wide association studies (GWAS): observational studies of a genome-wide set of genetic variants in different individuals to determine their degree of association with a trait of interest.

Induced pluripotent stem cells: type of pluripotent stem cell that can be generated directly by reprogramming mature cells, and that can be converted to every other cell type in the body, including neurons.

L-Dopa: L-3,4-dihydroxyphenylalanine: precursor to dopamine, noradrenaline, and adrenaline neurotransmitters; mainly used as a treatment for Parkinson's disease.

Maintenance therapy: opioid addiction treatment based on prescribing opiates to substitute for illicit opiates.

Mesolimbic pathway: dopaminergic neurons located in the mammalian midbrain ventral tegmental area; send axonal projections to forebrain structures, including the nucleus accumbens and prefrontal cortex; can predict and encode the salience of environmental stimuli and natural rewards, and is disrupted in addictive disorders.

Monoaminergic systems: networks of neurons that utilize monoamine

This association, limited to African Americans, was replicated by the authors in the context of opiate treatment for surgical pain ($N = 241$) [22]. Naltrexone efficacy in the treatment of alcohol use disorders (Box 2) similarly appears to be modulated by MOR SNPs. Accordingly, several studies and a meta-analysis globally confirmed the moderate utility of A118G as a predictive biomarker for naltrexone response, with the minor G allele associated with better outcomes [23] (although negative results have also been published [24]). In parallel, preclinical studies have directly addressed, using humanized mice expressing the A or G allele, the potential neurobiological and behavioral consequences of the A118G polymorphism. While the minor G allele was shown to associate with greater efficacy of naltrexone in reducing alcohol intake (consistent with human data [25]), effects related to the sensitivity towards the acute properties of drugs of abuse have been conflicting: increased reward and striatal dopamine release were reported for alcohol [26] or nicotine [27], while enhanced [28], unchanged [29], or diminished [30,31] rewarding effects of opiates were observed across a variety of experimental paradigms. Reconciling these findings and being able to translate them back to the clinic is proving challenging [32,33]; nevertheless, such research endeavors should continue.

Epigenetics of the Opioid System in SUDs

It is widely accepted that **epigenetic** processes remain plastic throughout life. By providing molecular substrates explaining how life experiences may interact with each individual's genetic make-up, they are ideally suited to reconcile 'nature and nurture' [34]. Given that these changes can be long lasting, they might represent a form of 'memory' that may contribute to chronic psychiatric phenotypes, including addiction.

Recent data suggest that interindividual differences in **DNA methylation** observed in peripheral samples may be used to infer methylation changes in the brain [4] and, as such, a limited number of epigenome-wide studies have recently investigated DNA methylation in peripheral blood samples of alcoholics [35–37]. The earliest of these reports focused on a moderate-sized cohort ($N = 534$) and detected increased DNA methylation in alcoholics compared with controls in the *POMC* gene, which encodes endorphins [37]. However, in a recent study of >13 000 subjects, no significant epigenetic adaptation was found within any opioid genes among the top-30 **CpG sites** where DNA methylation most robustly associated with continuous alcohol intake; this suggested that other nonopioid genes could be more informative of alcohol SUD [36]. Of note, a recent study compared heroin addicts who were grouped according to methadone daily dosage, and found that this was associated with numerous differentially methylated CpG sites, with an overlap in 13 genes across two French and Swiss cohorts [38]. Surprisingly, there are no postmortem genome-wide analyses of DNA methylation changes potentially occurring in the human brain as a function of SUDs, despite evidence from rodent studies suggesting that epigenetic modulation of neural-specific loci exhibits some degree of plasticity to drugs of abuse, as shown for cocaine [39,40].

In parallel, a handful of candidate studies focusing on MOR have been published in the context of opiate addiction. Overall, results indicate that opiate exposure associated with increased DNA methylation levels in the MOR gene promoter (Figure 2), as assessed using peripheral blood from methadone-maintained Caucasian heroin abusers [41,42], or Iranian opium abusers [43], compared with control groups. Of note, opiate-induced hypermethylation in the MOR promoter was also detectable in the saliva or cord blood of infants with NAS, and associated with worse outcomes [44]. Considering the aforementioned genetic findings from the same group [20], combining the characterization of the A118G variant with DNA methylation measures in MOR promoter and exon 1, proximal to the A118G position (Figure 2) may provide a prognostic biomarker for NAS.

neurotransmitters; involved in the regulation of numerous physiological functions.

Neonatal abstinence syndrome (NAS): withdrawal syndrome of infants after birth caused by *in utero* exposure to licit or illicit drugs, especially opiates.

Opiate: exogenous alkaloid compound derived from opium, either natural or synthetic; typically binds to opioid receptors and hijacks endogenous opioid signaling.

Opioid: refers to three opioid receptors, mu, delta, and kappa, which are activated under physiological conditions by endogenous opioid peptides.

Opioid genes: eight human opioid genes have been identified based on DNA sequence similarity. The *POMC, PENK,* and *PDYN* genes encode three groups of opioid peptides: endorphins, enkephalins, and dynorphins, respectively, with preferential affinity for MOR, DOR, and KOR, respectively (encoded by the *OPRM1, OPRD1,* and *OPRK1* genes, respectively). The *PNOC* and *OPRL1* genes encode nociceptin and the opioid-related nociceptin receptor 1, which do not cross-react with other opioid peptides and receptors.

Optogenetics: biological technique that uses light to control cells in living tissue, typically neurons, that have been genetically modified to express light-sensitive ion channels.

Pharmacogenomics: study of the influence of interindividual genetic variation on responses to medications and/or drugs.

Positron emission tomography (PET): fMRI technique used to observe brain functional and metabolic processes.

Ribonucleoproteic complexes: an association between RNA and a RNA-binding protein.

Single nucleotide polymorphism (SNP): variation in a single nucleotide that occurs among individuals at a specific position in the DNA sequence.

Key Figure

Potential for Opioid Biomarker Development at Every Stage of the Addiction Cycle

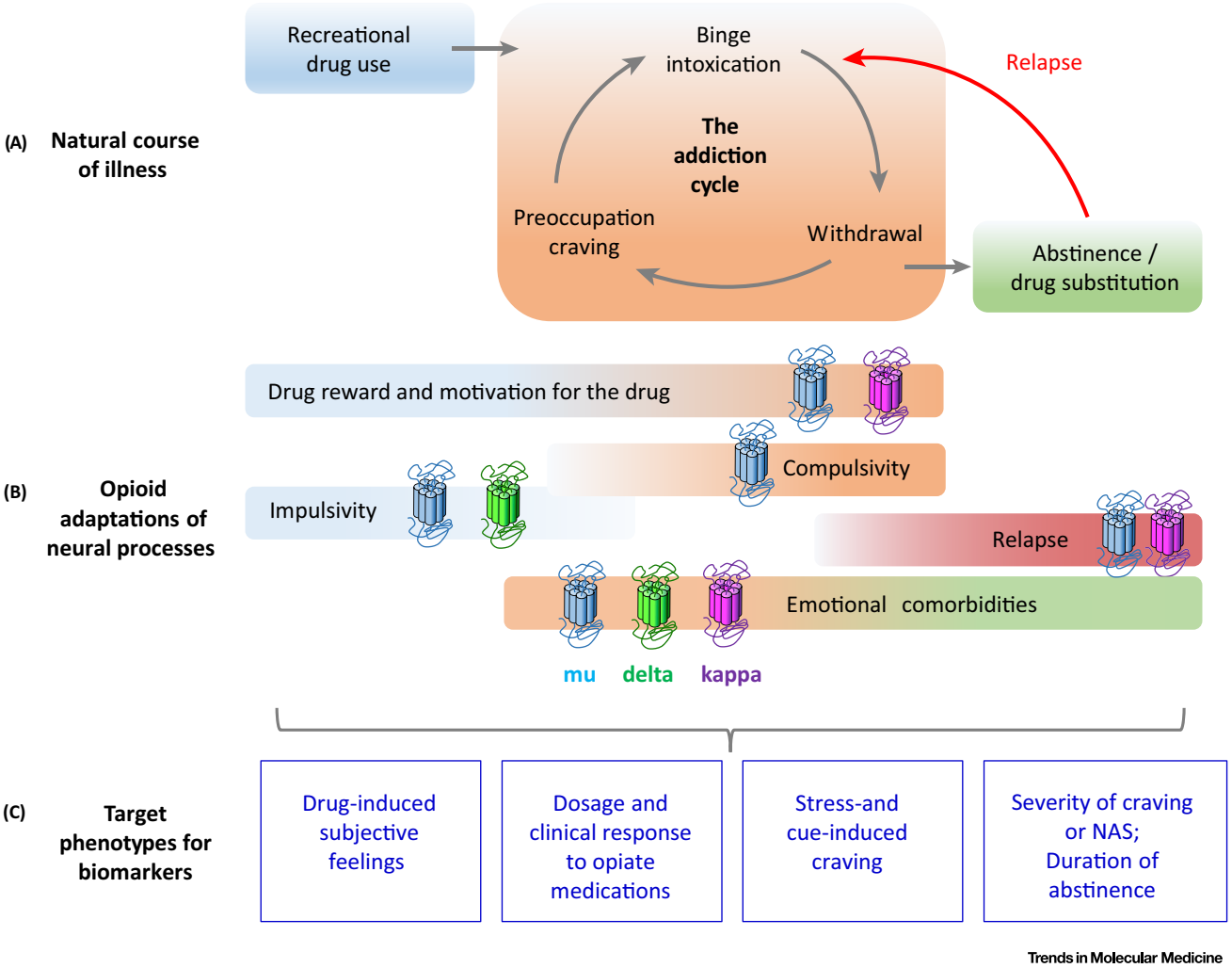

Figure 1. (A) Addiction is a chronic disorder that develops from an initiation phase during which the drug is consumed recreationally. In susceptible individuals, control over drug intake gradually declines, leading to compulsive drug use. The addicted individual has entered a vicious cycle, involving recurrent binge and/or intoxication, withdrawal, and preoccupation/craving stages. (B) Opioid receptors are implicated in several addiction-related processes over the course of the disease. The mu and kappa opioid receptors (MOR and KOR, respectively) exert opposing control over reward processing, with MOR increasing and KOR decreasing drug reinforcement [93], while the role of the delta opioid receptor (DOR) is more complex [114]. Recently, an unexpected inhibitory activity on motivational processes was uncovered for MOR expressed by forebrain inhibitory neurons [97]. MOR and DOR oppositely regulate inhibitory controls, with MOR and DOR activities increasing and decreasing motor impulsivity in rodents, respectively [115]. In addition, the three opioid receptors have distinct roles in emotional comorbidities of addiction, with pro- and antidepressant-like activities for KOR and DOR, respectively, and a complex role for MOR [96,116]. Finally, KOR likely has a critical role in relapse, as shown using stress- or drug-induced reinstatement of drug seeking in animal models [94]. (C) Genetic, epigenetic, transcriptomic, and imaging biomarkers of opioid receptor function have been investigated at several stages of the addiction cycle. While a large number of studies have been conducted using case/control designs, others have also investigated specific phenotypes (see the discussion in the main text). Abbreviation: NAS, neonatal abstinence syndrome.

Box 3. Practical Considerations for Peripheral Opioid Biomarkers

While addiction-related measures of opioid function and regulation have been almost exclusively investigated within the brain, opioid receptor and peptides appear to be readily expressed, quantifiable, and functional in peripheral tissues, including in skin, blood, and cells of the immune system [107,108]. In the context of biomarker development, blood, urine, and saliva can easily be collected in daily practice, and constitute accessible tissues, sometimes referred to as 'liquid biopsies' [109].

Although more painful, skin biopsies are also feasible, notably for iPSC-based approaches, although these are only starting to be explored in the addiction field [110,111]. Opioid peptides and receptors are expressed in the skin by many cell types (including keratinocytes, as well as fibroblasts and melanocytes), where they contribute to homeostasis and regeneration [107]. However, to date, there has been no study exploring skin opioid signaling in the context of addictive disorders in humans.

In the blood, expression of the mu opioid receptor by B and T lymphocytes, as well as by monocytes, has been well documented and shown to contribute to the immunosuppressive effects of opiates, and possibly to the increased infectious risk in drug addicts [112]. While blood is extensively used in peripheral biomarker studies, it is nevertheless a complex tissue that comprises rapidly recycled red blood cells, platelets, and heterogeneous populations of leukocytes, all bearing distinct transcriptional and epigenetic profiles. As a consequence, studies focusing on whole blood are especially susceptible to false positive and false negative findings, which may result from untracked changes in cellular composition and, therefore, ideally require monitoring blood counts in all patients under study [66]. Alternatively, urine and saliva may offer interesting opportunities to study epigenetic marks, based on the epithelial cells they contain, and to a lesser extent, on cell-free DNA. Saliva or urine collection is less invasive, requires no professional intervention, and appears less challenging from a logistical point of view than blood collection, which can prove difficult in long-term intravenous drug users.

At the genome-wide level, both methylomes and transcriptomes have shown some degree of correlation across blood and brain tissues in cohorts of healthy individuals (from $r = 0.90$ to $r = 0.66$ for methylomes, and from $r = 0.64$ to $r = 0.24$ for transcriptomes), as evidenced from pair-wise correlations between blood and tissues from various brain regions [5]. Similar comparisons have been drawn for saliva (with limitations due to potential bacterial contamination) and DNA methylation profiles might be more similar across brain and saliva than across brain and blood, although this requires further investigation [113]. Moreover, biological fluids can be used to characterize circulating RNAs (such as miRNAs or other noncoding RNAs), either within extracellular vesicles or **ribonucleoproteic complexes** [7], although the functional role of these molecular actors remains elusive.

The previous findings should be interpreted with caution because the functional significance of opiate-induced DNA methylation changes remains elusive; indeed, MOR expression was not assessed in most of these studies. These effects also appear to be modulated by ethnicity: while increased DNA methylation was observed in the MOR promoter of Hispanic heroin abusers (Figure 2), an opposite effect was found in African Americans [45]. These results emphasize the well-established notion that ethnicity and the DNA sequence strongly determine epigenetic states and plasticity. This has led some groups to compare pairs of monozygotic twins discordant for a given psychiatric disorder, aiming to control for genetic heterogeneity [46]; however, there have been no similar reports for drug addiction [47]. These results also suggest that combining genetic and epigenetic measures could yield more-robust biomarkers. Accordingly, a postmortem study found that the A118G minor G allele, which introduces a new CpG site for DNA methylation, was associated with increased methylation in opiate abusers, suggesting a possible genetic–epigenetic modulation, but this remains to be further investigated [48].

Finally, while no addiction study has focused on the epigenetic regulation of KOR or DOR, a recent postmortem investigation provided important findings regarding life stress and KOR. In this study [49], a history of child abuse (a form of early-life stress with lifelong detrimental consequences) associated specifically with decreased KOR expression in the anterior insula, a brain region where KOR availability was recently shown via **positron emission tomography** (PET) to mediate the expression of trauma-related symptomatology [50]. Child abuse-related

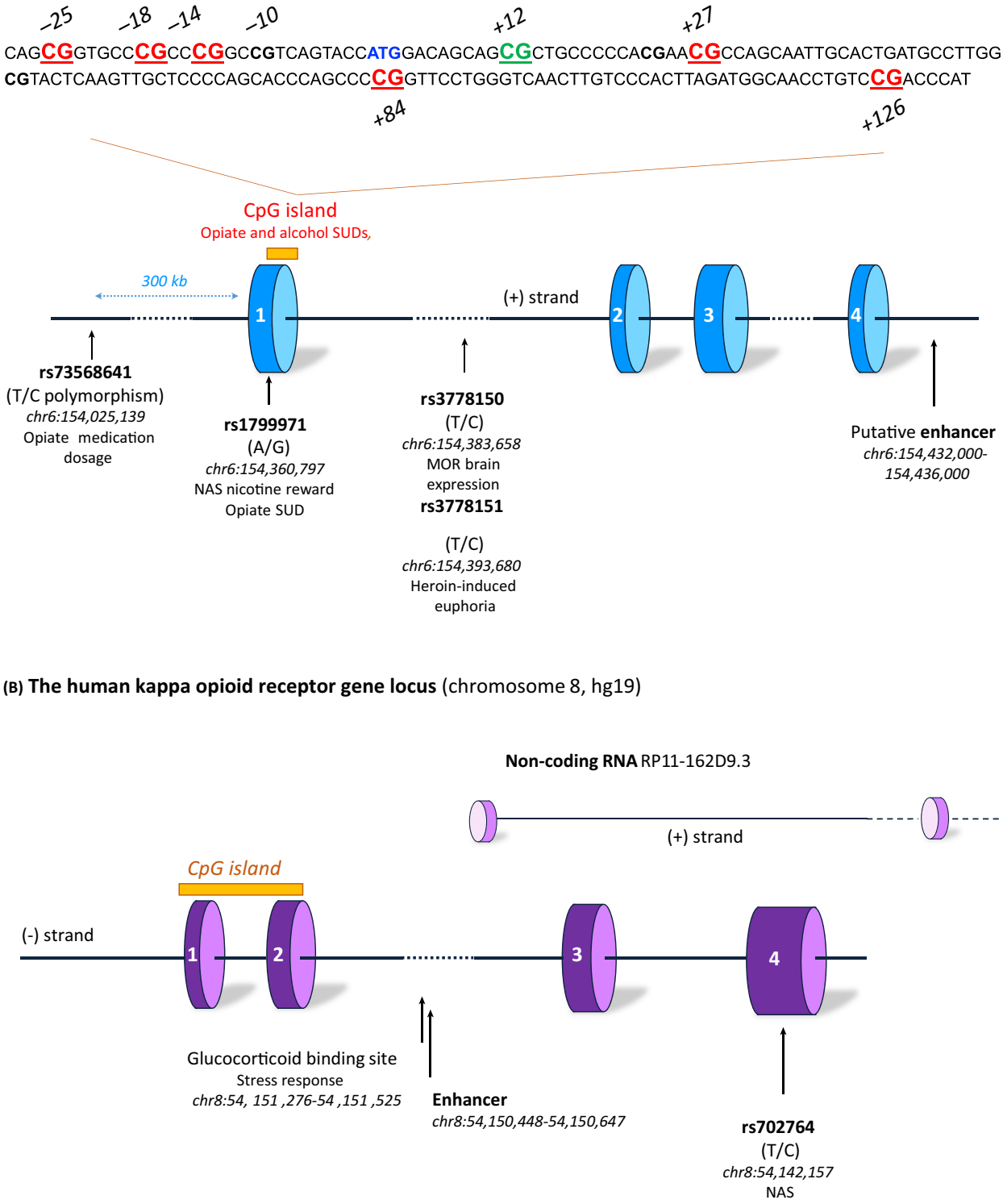

Figure 2. Putative Biomarkers at the Human mu and kappa Opioid Receptors (MOR and KOR). Depicted are schematic representations of the MOR and KOR opioid receptor genes, and potential addiction biomarkers. (A) For MOR, the rs1799971 (A118G) single nucleotide polymorphism (SNP) is located in the coding part

(Figure legend continued at the bottom of the next page.)

downregulation of KOR in brain tissue was associated with decreased DNA methylation of intron 2. Furthermore, *in vitro* reporter assays revealed that this intronic region acts as an enhancer site whose activity is regulated by levels of DNA methylation, which notably modulate local glucocorticoid receptor binding [49]. Given that the glucocorticoid receptor represents a critical mediator of the stress axis, these results uncover a new epigenetic mechanism that might contribute to the potentiation of dynorphin/KOR signaling during stressful experiences, including for SUDs [49]. Of note, brain and blood epigenetic patterns at the *PDYN* locus (which encodes dynorphins) were recently compared in psychiatrically healthy individuals [51], providing a foundation for combining central and peripheral biomarker investigations of KOR and its endogenous ligand.

While the information content of DNA methylation has only begun to be investigated [52], future studies will require large efforts to be able to assess the entire set of 27 million CpGs in the human genome [53]. Moreover, there is an ongoing debate on whether epigenome-wide association studies (EWAS) should be conducted in cohorts as large as in GWAS, because the relative strengths of the association between complex traits and genetic versus epigenetic variation remain uncharacterized [54]. Nevertheless, longitudinal studies using peripheral tissues and serial sampling at multiple stages of the addiction cycle, as well as rigorous analyses of postmortem brains, might allow us to assess dynamic epigenetic changes as well as potential similarities among central and peripheral adaptations to drugs of abuse, with promising leads for biomarkers. Lastly, while new mechanisms of opioid receptor regulation are being progressively uncovered at the level of other epigenetic layers (noncoding RNAs, including miRNAs [55,56], and histone modifications [57]), these molecular pathways and functions, as well as their potential to be exploited as suitable biomarkers, remain poorly explored.

Transcriptomics of the Opioid System in SUDs

Numerous studies have described transcriptional adaptations occurring in the brain during exposure to substance abuse, and across various brain structures. These studies notably indicate that chronic opiate exposure may trigger a complex reorganization of alternative splicing at the MOR locus, a gene with multiple isoforms in humans and rodents; this may lead to the expression of isoforms encoding MOR with six or even one transmembrane domain (s) [58], or with variable C-termini [59]. It is possible that the receptors expressed as a result of such alternative splicing may differentially contribute to opiate tolerance, physical dependence, and reward, but this remains hypothetical. Nevertheless, decades of animal and human research indicate that, following MOR activation, opiates can trigger signaling changes in downstream second messenger pathways, including the activation of adenylate cyclase/cAMP, as well as the ERK and AP-1 family of transcription factors [60]. Accordingly, there is strong *in vitro* [61] and mouse *in vivo* evidence that, while pharmacological acute MOR activation can inhibit **$G\alpha_i$ signaling** (Box 1), chronic MOR activation can lead to a preferential

of its first exon, corresponding to an exchange of asparagine for aspartic acid at a putative *N*-glycosylation site. Other variants associating with addiction are located in intron 1 (e.g., rs3778150 [17] and rs3778151 [18]); it is unclear how they might regulate the receptor function. The MOR gene also harbors a CpG island in its promoter (orange square), representing the sole focus of candidate epigenetic studies of MOR in addiction: chronic opiate exposure associates with decreased (green) or increased (red) DNA methylation at several CpGs (positions are indicated relative to the ATG). (B) Fewer studies have focused on KOR. Early-life stress has been associated with DNA methylation changes in KOR intron 2, a genomic site where the stress axis regulates its expression [49]. Freely available data sets are uncovering new opportunities for opioid biomarkers discovery at MOR and KOR loci. An online tool enables the visualization of brain–blood DNA methylation correlations (https://redgar598.shinyapps.io/BECon/ [117]), identifying positive correlations for three CpGs in the MOR promoter (cg22370006, cg14262937, and cg12838303). Epigenetic regulation of MOR is also documented by ENCODE (www.encodeproject.org/), and a region located next to exon 4 may correspond to an enhancer. Noncoding RNA RP11-162D9.3 (or G080834; www.mitranscriptome.org/) overlaps with KOR, and may regulate its expression. Abbreviations: Kb, kilobase; NAS, neonatal abstinence syndrome; SUD, substance use disorder.

expression of constitutively hyperactive adenylate cyclases 1 and 8, which can contribute to opiate physical dependence (as assessed classically in rodents using precipitated withdrawal) [62]. These signaling changes have been observed across several brain structures in rodents, such as the locus coeruleus and dorsal raphe nucleus [63], suggesting that these represent ubiquitous mechanisms potentially conserved in nonbrain tissues. In humans, a recent postmortem study reported decreased MOR protein levels and dysregulation of ERK and ELK1 signaling in the striatum of heroin abusers relative to healthy controls [64]. While these series of brain adaptations should also be investigated in the periphery, potential opiate-induced changes in blood expression of MOR splice variants, and of MOR-dependent signaling players, are currently unknown. Limited available evidence comes from studies of methadone-maintained heroin abusers, in whom decreased MOR and DOR gene and protein expression in peripheral blood lymphocytes were observed relative to controls [65]. The latter result appears to be consistent with the aforementioned MOR downregulation in the brain striatum [64] and MOR promoter hypermethylation in blood [41–43]. Generally, while transcriptomic analyses of peripheral tissues have been conducted in relation to several psychiatric phenotypes (>100 reports [66]), such studies are strikingly scarce for addictive disorders [67]. Altogether, rigorous studies in animal models and in clinical cohorts are required to unravel potential transcriptomic biomarkers that may be relevant for addiction disorders.

Brain Imaging of the Opioid System in SUDs

Brain imaging has been used extensively to understand how opioid receptor function is modified in patients with addictive disorders. These studies have almost exclusively focused on the investigation of MOR bioavailability for [^{11}C]-carfentanil binding, using PET, exploring cocaine, alcohol, opiate, and nicotine SUDs.

MOR binding potential was found to be increased in cocaine addicts relative to nonaddicted controls [68], to be transiently potentiated during cocaine abstinence, and to be correlated in specific brain regions with craving, a tendency to relapse, as well as cognitive behavioral therapy outcomes [69]. Similar adaptations were detected during abstinence from alcohol addiction, with increased MOR binding potential in patients abstinent from 5 days to up to 3 weeks [70,71], although another group described opposite findings during a shorter 3-day abstinence [72], suggesting complex kinetics. The classical question raised by PET studies relates to the interpretation of changes in binding potential, because an increase may reflect either enhanced receptor expression or lower occupancy by endogenous ligands [73]. In the context of alcohol addiction, a recent study combined PET scans in living individuals with receptor autoradiography in brain postmortem tissue [73]: postmortem results indicated that MOR expression was decreased in the striatum, while high MOR binding potential was associated with intense craving during abstinence. This led the authors to formulate a hypothetical allostatic model postulating that the release of endogenous opioid peptides might be potentiated during chronic alcohol consumption (leading to a compensatory decrease in MOR expression); by contrast, the concentrations of opioid peptides would decrease during abstinence, accounting for the increased binding potential that was observed [70,71,73].

In the context of opiate addiction, PET studies have focused on maintenance therapies, in particular buprenorphine. As expected, a relationship between buprenorphine doses and MOR bioavailability has been documented [74]. It was shown that, as buprenorphine doses increase, withdrawal symptoms are first attenuated (requiring approximately 50% MOR occupancy), while the blockade of reinforcing and subjective effects of illicit opiates requires higher buprenorphine doses (achieving approximately 80% MOR occupancy) [74].

Finally, MOR availability has also been investigated in smokers. Subjective effects of smoking (such as nervousness, alertness, or craving for a cigarette) were significantly associated with activation of MOR signaling (i.e., decreased binding potential) [75], consistent with the notion that this receptor represents a gateway to drug reward [76]. More recently, the relationship between the A118G polymorphism, smoking-induced subjective feelings, and MOR availability was investigated. Results indicated that subjective feelings and MOR availability were positively correlated in carriers of the minor G allele but not in carriers of the A allele; this finding was surprising, given that it contrasted with a neuropsychological study reporting decreased subjective smoking reinforcement in G carriers compared with A carriers [77].

Altogether, results from these studies concur to establish that MOR imaging can be used to detect acute, chronic, as well as withdrawal effects for several drugs of abuse in living brains (with potentially complex kinetics along the natural course of the disease). This may in turn lead, when adequately combined with clinical assessment (craving severity in particular), to the development of prognostic or predictive biomarkers to estimate the risk of relapse or to assess the efficacy of opioid maintenance therapies, respectively. By contrast, the potential utility of DOR or KOR [78] remains largely unexplored, mostly due to a longstanding lack of radiotracers. However, as mentioned above, KOR ligands have been recently synthesized and validated (e.g., [^{11}C]-LY2795050) [50]. These KOR radiotracers will hopefully prove useful in addiction research across a variety of SUD phenotypes.

Investigations of behavioral addictions have recently emerged in MOR-imaging PET studies. While no change in MOR-binding potential has been detected between pathological gamblers and healthy volunteers at baseline, individuals in the former group exhibited a blunted activation of MOR signaling following oral amphetamine administration, as well as decreased subjective amphetamine effects (alertness and euphoria) relative to controls [79]. By contrast, another report found decreased MOR binding potential at baseline in the cingulate cortex of pathological gamblers, an effect that has also been noted in multiple brain structures from patients with **binge-eating disorders** [80]. While these studies suggest a possible dysregulation of opioid neurotransmission in behavioral addictions, extensive work is needed to begin to define similarities and differences among behavioral addictions, and between those and drug addiction disorders.

Finally, the use of **functional magnetic resonance imaging** (fMRI) is expanding rapidly in psychiatric research to interrogate brain connectivity patterns under a resting state, as well as their putative correlation with genetics factors in healthy subjects [81], and their possible use as risk [82] or predictive [83] biomarkers for SUDs. Whether opioid system genes shape functional connectivity in normal or addicted individuals remains to be tested. However, a first mouse resting-state fMRI study with translational potential showed remarkable reorganization of reward-aversion connectional patterns in mice lacking the MOR gene, relative to wild-type littermates [84]. This new field may open intriguing avenues in the search for biomarkers, which may utilize opioid receptor-based or opiate therapy-based [85] whole-brain connectivity signatures. Furthermore, these imaging technologies should now be harnessed to explore the dynamics of brain activity in the context of rodent behavioral models of addiction, in combination with genetic tools.

Concluding Remarks

Here, we have synthesized existing knowledge on brain and peripheral opioid processes in the context of developing addiction biomarkers. Current studies indicate that related transcriptomic adaptations have been primarily conducted in the brain, while most epigenetic studies

Outstanding Questions

Is it possible to combine a selection of genetic variants, such as SNPs, that would collectively better predict the risk of addiction, drug abuse-related phenotypes, and clinical outcomes, or responses to opiate medications?

What are the molecular pathways that are modulated by the mu opioid receptor A118G polymorphism and that may help establish a converging model for its complex behavioral and clinical effects?

What are the epigenetic or regulatory processes explaining how noncoding genetic variants, such as those located in intronic or intergenic sites, impact opioid receptor function and expression?

Beyond the mu opioid receptor promoter and the kappa opioid receptor intron 2, what are the genomic sites at opioid gene loci that can be epigenetically reprogrammed as a function of life experiences, and that potentially contribute to addiction?

Is there any crosstalk between the brain and peripheral organs that significantly contributes to the pathophysiological processes of addiction?

Can we identify gene expression or DNA methylation changes in the peripheral tissues of addicted individuals that significantly correlate with either similar adaptations occurring in the brain, or known molecular or behavioral phenotypes contributing to the disease?

Are opiate-induced genetic and epigenetic adaptations similar across the variety of opioid receptor-expressing neurons and cell types, in various brain regions and peripheral tissues?

Can we combine genetic and epigenetic biosignatures with quantitative clinical variables to yield diagnostic or monitoring biomarkers?

Which changes in delta and kappa opioid receptors can we identify using PET in the brain of living addicted individuals?

have used peripheral samples. Therefore, a major goal in the coming years will be to more systematically apply similar technologies at both the peripheral and central levels. To this end, it is essential to encourage and promote the development of repositories for human brain and peripheral tissues from well-characterized cohorts of addicted individuals, and to maximize the availability of such precious samples to all researchers.

Opioid receptors represent a common molecular substrate for addiction-related biological processes across all drugs of abuse. Therefore, it is possible that future studies, including meta-analyses, may have the capacity to unravel universal opioid processes mediating the dysregulation of reward, motivation, or cognitive function, by investigating large cohorts of patients that may be subgrouped as a function of clinically meaningful endophenotypes (e.g., opioid medication dosage or drug-induced subjective feelings) rather than by the type of drug of abuse.

Despite the rapid increase in publications reporting the characterization of putative addiction biomarkers, to the best of our knowledge there has been no evaluation of their clinical accuracy and cost-effectiveness [86], and none are currently available in routine clinical practice (see Outstanding Questions and Box 2). Given the chronic and recurrent course of addictive disorders, it appears essential to conduct longitudinal studies, ideally using a prospective design and over long-term periods, to capture stage-specific opioid adaptations in the course of the disease. This may also enable us to distinguish between pharmacological actions of drugs of abuse and neurobiological substrates that may underlie personality traits predisposing to disease.

Another major objective will be to combine distinct measures of opioid function (genetic, epigenetic, and imaging) to generate more-meaningful biomarkers. These efforts are also expected to benefit from an on-going potent trend in the molecular psychiatry fields to recognize that mammalian brain tissue comprises multiple intermingled cell types that exhibit huge transcriptomic [87,88] and epigenetic [89] diversity. Such complexity severely hampers the detection of subtle cell type-specific adaptations driving complex behaviors [90,91], which may go undetected in studies performed in whole-tissue homogenates. Therefore, the implementation of rigorous cell type-specific strategies and single-cell analyses will be needed in the field of biomarker discovery. In the context of addiction, focusing on MOR is an appealing possibility, because opiate-induced adaptations may be tracked precisely in specific cell populations expressing this receptor, in both the brain and periphery. In the same vein, with the rapid dissemination of **induced pluripotent stem cell (iPSC) technologies,** aiming to recapitulate individual genomic characteristics, it is foreseeable that opioid-expressing neuronal and non-neuronal cells from addicted individuals might be available for experimental manipulations. Assessment of these populations may open exciting new research avenues towards personalized biomarker development.

During recreational drug use or in early stages of disease, can MOR bioavailability be used as a risk biomarker before the emergence of withdrawal states and compulsive drug seeking?

Can biased agonism be harnessed to develop opiate analgesics that target the mu opioid receptor but which lack addictive potential?

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