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Common and specific liability to addiction: Approaches to association studies of opioid addiction

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Abstract

Background—Opioid addiction, whether to opiates such as heroin and morphine, and/or to non-medical use of opioids, is a major problem worldwide. Although drug-induced and environmental factors are essential for the vulnerability to develop opioid addiction, the genetic background of an individual is now known also to play a substantial role.

Methods—The overall goal of this article is to address the common and specific liabilities to addiction in the context of approaches to studies of one addiction, opioid addiction. Literature on identifying genetic variants that may play a role in the development of opioid addiction was reviewed.

Results—A substantial number of genetic variants have been reported to be associated with opioid addiction. No single variant has been found in any of the reported GWAS studies with a substantial effect size on the vulnerability to develop heroin addiction. It appears that there is a complex interaction of a large number of variants, some rare, some common, which interact with the environment and in response to specific drugs of abuse to increase the vulnerability of developing opioid addiction.

Conclusions—In spite of the inherent difficulties in obtaining large well-phenotyped cohorts for genetic studies, new findings have been reported that are being used to develop testable hypotheses into the biological basis of opioid addiction.

Keywords

Genome-wide association studies; opioid; genetics; heroin; addiction

1. Introduction

Addiction to opiates and the illicit abuse of prescription opioids is a major problem in modern society. Since opioid addiction is a chronic brain disease, with high relapse rates, it causes major social, economic, and medical problems. Opioid addiction results from the use

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of illicit opioids such as heroin, morphine, or illicit prescription opioids, and from the treatment of chronic or acute pain. There are three major aspects that factor into the vulnerability to develop opiate addiction after self-exposure. These are: 1) drug-induced factors that produce neurobiological changes that result in changes in behaviors, 2) genetic factors contribute 40–60% to the variability of developing opioid addiction (Kendler et al., 2003; Tsuang et al., 1996; Tsuang et al., 1998), and 3) the influence of the environment on the individual, which includes external stressors (and the resultant stress), cues, and conditioning (for review see Kreek et al., 2005a).

Drug addiction vulnerability is impacted by a combination of genetic, epigenetic and environmental factors coupled with drug-induced effects. Early in the development of addiction, genetic variants that influence impulsivity and risk-taking have major role in the general liability of addiction (Kreek et al., 2005b). Recent studies from our laboratory have shown that former heroin addicts in methadone maintenance treatment have higher impulsivity than controls, regardless of comorbid cocaine dependence (Nielsen et al., 2012). Variants in pathways and systems specific to the drug of abuse are more important in the progression from intermittent to regular drug use, and the transition from abuse to addiction. Furthermore, variants in the stress hypothalamic–pituitary–adrenal (HPA) axis may, in general, affect these later stages of addiction. Studies have demonstrated that HPA activity predicted relapse to drug use and the amounts of drug used. Addictions to most drugs of abuse have been found to involve the endogenous opioid pathway. Variants in opioid receptors and ligand are also common liability factors in the development of addiction.

2. Association Studies

Linkage analysis has been a traditional method for identifying chromosomal regions with large genetic effects on specific disease (Cui, et al., 2010). The foundation of linkage study is to assess the recombination rate between a genetic marker and a disease predisposing region with family-based pedigree data. When the mode of inheritance of a disease follows a known pattern, a parametric linkage analysis (logarithm of the odds (LOD) score analysis) can be applied. However, linkage mapping has several limitations and has low power to detect multi-genetic variants with small effect. This limitation can be overcome by association studies. The basic idea of association analysis is to assess correlations between genetic variants and a disease phenotype in a population. The disease–gene association is assessed through the disease–marker association, where we expect markers associated with a disease phenotype to be in high linkage disequilibrium (LD) with disease variants. Case–control association studies compare the distribution of affected subjects with that of controls and test for significant difference that indicates that the locus may increase the disease risk or may be in LD with a causal variant.

Studies aimed at identifying genes involved in addiction can be designed using families or unrelated subjects. The use of families allows linkage of chromosomal regions with behavioral phenotypes, but suffers from low resolution and from difficulties in finding family members of addicted individuals. A more powerful and practical approach is to use unrelated subjects. These subjects usually are classified as cases or controls. Association studies with case/control design can be performed in two ways. The first approach is the hypothesis-driven candidate gene study and the second is the genome-wide association study (GWAS). In the first approach, genes are selected based on previous findings or on knowledge of the biological functions of the product of that gene. In the second, up to a million genetic variants, both in genes and in intergenic regions, are interrogated simultaneously using high-throughput technologies.

An example of the hypothesis-based approach is the study of the mu opioid receptor gene, *OPRM1*. The *OPRM1* gene encodes the receptor of the endogenous opioid beta-endorphin and the exogenous opiate morphine, and has been demonstrated to play a central role in opioid dependence and tolerance. Ligand binding to this G-coupled receptor has been shown to inhibit cyclic AMP formation and to activate potassium currents (e.g. Gong et al., 1998). Resequencing of the coding regions of this gene identified a common A to G transition at nucleotide 118 that encodes for a substitution of an aspartic acid for an asparagine (118A>G, Asn40Asp, rs1799971) (Bond et al., 1998; Bergen et al., 1997) and removes an *N*-glycosylation site from the N-terminal extracellular domain. *In vitro* functional studies have demonstrated the Asp40 (118G) receptor's role in enhanced binding of beta-endorphin, and in increased activation of the G protein-activated inwardly-rectifying K^+ channels (GIRKs) (Bond et al., 1998). In stable transfected cell lines, the expression of the variant receptor showed lower forskolin-induced cAMP accumulation and lower receptor binding site availability (Kroslak et al., 2007). The variant receptor also has reduced agonist-induced receptor signaling efficacy, but not binding, in human postmortem brain (Oertel et al., 2009). In autopsy brain samples, an allelic imbalance of expression of the two variant alleles was reported (Zhang et al., 2005). The 118A allele was expressed at a higher level, which probably would increase receptor density and function.

Association between the 118G variant and opioid dependence as well as other substance dependencies was reported by several studies (Kreek et al., 2005a; Bart et al., 2004; Kapur et al., 2007; Deb et al., 2010). In a study in central Sweden, the 118G variant was associated with alcoholism (Bart et al., 2005) and, in two studies, with the pharmacotherapeutic response to naltrexone treatment for alcoholism (Anton et al., 2008; Oslin et al., 2003). *OPRM1* single nucleotide polymorphisms (SNPs) in intron 1 were found to be associated with opioid and cocaine dependence in European Americans (EA) (Zhang et al., 2006) and with positive response to heroin after first use in Chinese (Zhang et al., 2007). However, two meta-analyses of case-control studies of opioid or substance dependence found a lack of evidence for an association with the 118G allele (Glatt et al., 2007; Arias et al., 2006).

In addition to *OPRM1*, several other genes have been associated with heroin addiction in linkage and/or candidate gene association studies (for review see (Kreek et al., 2005a, 2005b, 2009; Kreek and LaForge, 2007; Li and Burmeister, 2009)). This list includes delta and kappa opioid receptors (*OPRD1* and *OPRK1*) (Yuferov et al., 2004; Gerra et al., 2007; Zhang et al., 2008), dopamine receptors D2 and D4 (Szilagyi et al., 2005; Xu et al., 2004; Hou and Li, 2009), serotonin receptor 1B (*HTR1B*) (Proudnikov et al., 2006), serotonin transporter (*SLC6A4*) (Gerra et al., 2004), gamma-aminobutyric acid (GABA) receptor gamma 2 (*GABRG2*) (Loh et al., 2007), catechol-*O*-methyltransferase (*COMT*) (Oosterhuis et al., 2008), period circadian protein (*PER3*) (Zou et al., 2008), proenkephalin (*PENK*) (Xuei et al., 2007), proopiomelanocortin (*POMC*) (Xuei et al., 2007), tryptophan hydroxylase 1 and 2 (*TPH1* and *TPH2*) (Nielsen et al., 2008a), brain-derived neurotrophic factor (*BDNF*) (Cheng et al., 2005), and melanocortin receptor type 2 (*MC2R,* ACTH receptor) (Proudnikov et al., 2008).

We have performed a hypothesis-driven case-control association study of 130 genes (Levran et al., 2008; Levran et al., 2009) encoding drug receptors, neurotransmitters, transporters, and drug metabolism enzymes as well as genes encoding proteins that are involved in reward modulation, behavioral control, cognitive function, signal transduction, and stress response. The first study, in Caucasians, included 412 cases and 184 controls. The second study, in African Americans (AA), included 202 cases and 167 controls. The platform of choice was the 1,536-plex GoldenGate Custom Array designed by Dr. D. Goldman's group at NIAAA (Hodgkinson et al., 2008). The array contained 1,350 SNPs and 186 ancestry informative markers (AIMs) that were selected based on allele frequencies in the Caucasian,

African, and Chinese populations of the HapMap project (Enoch et al., 2006). Two independent methods, STRUCTURE (Pritchard et al., 2000) and genomic control (Devlin et al., 2001), were applied to exclude population stratification between cases and controls. AIMs analysis showed a clear distinction between the AA and EA. To minimize the effect of stratification, tests for association were performed separately for each population.

Ascertainment of patients and controls was made by personal interview using several instruments including the Addiction Severity Index (ASI) (McLellan et al., 1992; McLellan et al., 1980), Kreek-McHugh-Schluger-Kellogg (KMSK) scale (Kellogg et al., 2003), and the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV) (First et al., 1998). The subjects completed a three-generation family history questionnaire. In order to maximize the power of the study, cases were selected from the extreme phenotype range (i.e., former severe heroin addicts with a history of at least one year of daily multiple uses of heroin) and the controls were healthy volunteers that were selected by detailed personal interview and stringent criteria. Each of the following was used as an exclusion criterion from the control category: a) At least one instance of drinking to intoxication, or any illicit drug use, in the previous 30 days; b) A past history of alcohol drinking to intoxication, or illicit drug use, more than twice a week, for more than 6 consecutive months; c) Cannabis use for more than 12 days in the prior 30 days, or past use for more than twice a week for more than 4 years.

The nine variants that showed the most significant associations ($P = 0.009 – 0.0003$) in the EA population (Table 1a) were in non-coding regions of the following genes: the opioid receptors (mu, kappa and delta), galanin (involved in the regulation of food intake, behavioral and neurochemical effects of opiates, and stress response), the 3B subtype of the serotonin receptor, and the casein kinase epsilon (the human homolog of the drosophila protein DOUBLETIME that participates in important signaling pathways, including the phosphorylation of the circadian rhythm protein, period and DARPP-32).

Interestingly, the *OPRM1* SNPs that were found to be associated with vulnerability to develop heroin addiction in this study were from intron 1, in concordance with two previous studies (Zhang et al., 2006; Zhang et al., 2007). To follow up on these findings, we have analyzed the linkage disequilibrium (LD) data in the HapMap EA population (CEU). We found a haplotype block that spans intron 1 and also includes several variants in the 5′ flanking region, including rs1074287 that was identified in the 10K genome scan (see below) (Nielsen et al., 2008b).

Notably, the association test results in the EA and the AA populations yielded distinct, non-overlapping results. The most significant results in the AA population were obtained for the gene encoding glutamate receptor, ionotropic, N-methyl D-aspartate (NMDA) subtype 2A (*GRIN2A*). Four *GRIN2A* variants located within a 32 kb region of intron 3 (5′ to the translation start site) accounted for the strongest signals in single SNP analyses as well as in a haplotype analysis (Levran et al., 2009). The NMDA receptor family is involved in multiple cognitive processes. NMDA receptors are composed of subunits GRIN1 and GRIN2A-D. Expression of the GRIN2A subunit begins around puberty. *Grin2a* (*Nr2a*) knockout mice exhibit retarded discrimination learning (Brigman et al., 2008). Several *GRIN2A* polymorphisms have been found to be associated with attention-deficit/ hyperactivity disorder (ADHD), autism, and schizophrenia (Adams et al., 2004; Barnby et al., 2005; Itokawa et al., 2003; Tang et al., 2006; Turic et al., 2004). In our 100K GeneChip study (presented below) a different variant in *GRIN2A* was found to be associated with addiction vulnerability in AA.

In addition, SNPs in genes encoding several receptors, adrenergic alpha 1A (*ADRA1A*), arginine vasopressin 1A (*AVPR1A*), cholinergic muscarinic 2 (*CHRM2*), dopamine D1 *(DRD1*), GABA-A beta 3 (*GABRB3*), and serotonin 3A (*HTR3A*), as well as alcohol dehydrogenase 7 (*ADH7*), glutamic acid decarboxylase 1 and 2 (*GAD1* and *GAD2*), the nucleoside transporter (*SLC29A1*), and diazepam binding inhibitor (*DBI*), showed point-wise significant association with heroin addiction (Table 1b).

Several results corroborated previously reported associations with alcohol and drug (cocaine and/or opioid) addiction. An association of a galanin gene (*GAL*) haplotype with alcoholism was reported in Finnish and Native American males (Belfer et al., 2006). The *DRD1* SNP rs5326, from this study, is in strong LD with the functional 3′ UTR SNP rs686 that was found to be associated with nicotine dependence in an AA sample (Huang et al., 2008). The *ADH7* SNP rs971074 previously was found to be associated with substance dependence and personality traits (Luo et al., 2006, 2007, 2008). The 5′ UTR *AVPR1A* SNP rs3759292 is in close proximity to the two microsatellite polymorphisms (rs9325177 and rs11283312) that were found to be associated with autism and personality (Knafo et al., 2008; Wassink et al., 2004; Meyer-Lindenberg et al., 2009). The *DBI* SNP rs2289948 is in complete LD with SNP rs8192506 that was found to be associated with alcoholism in Asians (Waga et al., 2007) and with anxiety in EA (Thoeringer et al., 2007). The *DBI* SNP rs12613135 is located in the same haplotype block with SNPs rs2289948 and rs8192506. *GAD2* SNP rs2058725 is in complete LD with SNP rs701492, which was found to be associated with alcoholism in Han Taiwanese (Loh et al., 2006), and other *GAD2* SNPs showed association with alcoholism in EA (Lappalainen et al., 2007). The *CHRM2* 5′ UTR SNP rs2350780 is in strong LD with SNP rs1455858 that was found to be associated with drug dependence and affective disorders in EA and AA (Luo et al., 2005), and three *CHRM2* intronic SNPs (rs1824024, rs2061174, and rs324650) were found to be associated with alcohol dependence and major depressive syndrome Wang et al., 2004).

The possibility that the specific gene variants contributing to the vulnerability to develop heroin addiction differ among ethnic groups is intriguing. A difference in allele frequencies between the two populations was observed for the majority of the SNPs indicated. Other factors, such as LD pattern or modifier genes, also may explain these results.

This study suggests that testing a large number of genes, based on a biological hypothesis, in a well-characterized population with a well-defined phenotype may facilitate the identification of population-specific genetic variants that are associated with vulnerability to develop heroin addiction. This approach is more economical than whole-genome scans and may increase the power of the study due to the smaller number of tests performed.

3. Genome-wide Association Studies

GWAS use high-density microarrays to examine up to a million variants in each subject without a specific biological hypothesis. The development of a high-throughput GWAS was facilitated by the Human Genome Project and the International Human Haplotype Map Project (HapMap), along with the development of high-throughput genotyping technologies. However, the large number of variants has introduced a multiple-testing problem. Since the statistical analyses of these dense microarrays must take this problem into account, the power for finding a significant association decreases, necessitating the use of very large cohorts. For example, it is estimated that for a GWAS of type-2 diabetes, 800 variants would have to be identified to account for its heritability of 40% (Pawitan et al., 2009). To accomplish this goal, it is estimated that 50,000 cases and 50,000 controls would be required.

In past years, GWAS have identified many associations of specific genetic variants with complex disorders (for reviews see (Hardy and Singleton, 2009; Manolio et al., 2009)). A number of GWAS using relatively small sample sizes have found large effects for common variants. Studies on age-related macular degeneration (96 cases and 50 controls) (Klein et al., 2005), exfoliation glaucoma (195 cases and 14,474 controls) (Thorleifsson et al., 2007), and serum-transferrin levels (215 parents and 461 children from 169 nuclear families) (Benyamin et al., 2009) are notable examples. There have also been a number of large studies that have demonstrated that the genetic components of complex diseases are numerous and are of low penetrance. For example, the Wellcome Trust Case Control Consortium studied the genetic basis of seven common diseases (Wellcome Trust Case Control Consortium, 2007). Using 14,000 cases and 3,000 shared controls they identified associations with 12 variants that were previously identified in candidate gene studies, as well as 13 novel variants (one for bipolar disorder, one for coronary artery disease, nine for Crohn's disease, nine for hypertension, three for rheumatoid arthritis, seven for type 1 diabetes, three for type 2 diabetes, and one for both rheumatoid arthritis and type 1 diabetes). A series of studies of nicotine dependence including a large-scale GWAS of lung cancer (Thorgeirsson et al., 2008) implicated variants in the alpha 5-alpha 3-beta 4 nicotinic cholinergic receptor gene cluster (for review see (Bierut, 2010)). Notably, these variants explain only a small proportion of the expected genetic contribution.

Many of the variants identified by GWAS have mapped to intergenic regions, the chromosomal regions between the coding sequences. These variants may be functional and act by altering gene expression or by modifying unknown cellular functions. Current investigations are revealing various functions for the intergenic regions (Carninci, 2009; Mercer et al., 2009). Over twice as much non-coding sequence than coding sequence is transcribed in the genome (Kapranov et al., 2007). The resultant non-coding RNAs (ncRNAs) have been shown be involved in transcriptional and post-transcriptional regulation, and in chromatin remodeling (reviewed in (Mercer et al., 2009)). It appears that most polymerase II promoters are transcribed in both directions, producing mRNAs in one direction and stable promoter-associated long RNAs (PALRs) in the other direction (Kapranov et al., 2007). In addition, relatively unstable promoter upstream transcripts (PROMPTs) are transcribed from ~500 to 2,500 nucleotides upstream of active transcription start sites (Preker et al., 2008). Shorter RNAs known as PASRs (Affymetrix ENCODE Transcriptome Project; Cold Spring Harbor Laboratory ENCODE Transcriptome Project 2009), TSSa-RNAs, and NRO-RNAs (less than 200 nucleotides) are also transcribed from promoters in the antisense direction (Seila et al., 2008; Core et al., 2008). Transcription of these RNAs occurs in a tightly regulated and developmentally controlled fashion, but their properties are ill-defined. In addition, other small RNAs, such as microRNAs (miRNAs, 21–24 nucleotides) and Piwi interacting RNAs (piRNAs, 26 – 31 nucleotides) (Ghildiyal and Zamore, 2009; Ambros, 2004) are transcribed from intergenic regions and are required for gene regulation and replication.

Another factor in GWAS is that the human genome contains numerous regions that differ between individuals. Many regions have been shown to be deleted or amplified to produce copy number variants (CNVs) (Zhang et al., 2009). CNVs may be inherited or created *de novo*. These have been associated with risk for a large number of psychiatric disorders including autism and schizophrenia (Stefansson et al., 2008). When these regions are analyzed in finer detail, their structure appears quite complex. At one region, 15q13.3, a recurrent 680 kb deletion has been found in a number of individuals (Shinawi et al., 2009). This deletion creates a fusion of the ion channel neuronal acetylcholine receptor subunit alpha-7 gene (*CHRNA7*) with the RNA gene family with sequence similarity 7, member A1 (*FAM7A1*) to create a new gene, *CHRFAM7A*. Deletion of this region is always accompanied by a ~90 kb duplication at the breakpoint. Inversion of this region in

individuals without this deletion was reported to occur at a frequency of 44%. It will be important that GWAS take into account these complex regions in the interpretation of their results.

4. Genome-wide Association Studies of Opioid Addiction

A number of other groups have performed linkage analysis on opioid addiction. In a family study of 393 families, the Gelernter group identified five clusters linked to opioid dependence (Yu et al., 2008). Two regions had LOD scores greater than 3, an accepted cutoff for a significant linkage finding. These were both located on chromosome 17. In a different study of 296 families, the chromosomal region 14q was “suggestive” of linkage to opioid dependence in a Hispanic group (Lachman et al., 2007). The group of Tsuang found point-wise significant linkage with heroin dependence of two chromosomal regions (chromosome 4 and 17) in Han Chinese (Glatt et al., 2006).

Our laboratory has reported a GWAS designed to identify genes and genetic variants that may be involved in determining the vulnerability to develop heroin addiction using the Affymetrix 10K 2.0 GeneChip, which interrogated 10,000 SNPs (Nielsen et al., 2008b). This study was performed using data from 200 Caucasian subjects, 100 former severe heroin addicts and 100 control subjects. This was essentially a subgroup of the cohort analyzed in the hypothesis-driven array study (Levran et al., 2008). Analyses were performed separately for autosomal and X chromosomal variants. The variants found to be associated with the highest significance with heroin addiction by allele frequency were the autosomal variants rs965972, rs1986513, and rs1408830 ($P = 6 \times 10^{-6}$, 4×10^{-5}, 1×10^{-4}, respectively, Table 2). SNP rs965972 is located in the Unigene cluster Hs.147755, a cluster of expressed sequence tags cloned from testis and kidney, and alters a consensus CREB-binding site and is located in a region of predicted high regulatory potential. The closest annotated gene to rs965972 is the hyperparathyroidism 2 tumor suppressor gene (*HRPT2*) that is located 243 kb away. rs1408830 (also located at chromosome 1q31.2) is located in the twelfth intron of *HRPT2*. This variant is also 43,232 nucleotides upstream of the *B3GALT2* gene, a gene located in an intron of the *HRPT2* gene in the opposite orientation to the *HRPT2* gene. SNP rs1986513 is located in an intergenic region of high mammalian conservation and creates several new consensus transcription factor-binding sites, including TFIID. When genotype frequencies of the alleles were examined for association with heroin addiction, the three variants with the strongest association were in the second intron of the transcription factor myocardin (rs1714984, $P = 2.2 \times 10^{-5}$) and in intergenic regions (rs965972 and rs1867898, $P = 8 \times 10^{-5}$, 2.8×10^{-4}, respectively) (Table 2). Myocardin is expressed in smooth and cardiac muscle and functions as a muscle-specific transcriptional co-activator in conjunction with serum response factor (SRF) (Pipes et al., 2006).

We employed a strategy to identify common genotype patterns of unlinked alleles that may be associated with heroin addiction. Variants from the three most significant genotypic associations were analyzed together as genotype patterns. The common AG-TT-GG genotype pattern explained 27% of the population-attributable risk and was associated with heroin addiction vulnerability (odds ratio=6.25). A second common pattern, GG-CT-GG, of these three variants was associated with protection from developing heroin addiction (odds ratio=0.13). Lacking this genotype pattern explained 83% of the population-attributable risk ($P = 2.7 \times 10^{-9}$).

In addition, we compiled a list of 240 hypothesis-driven genes that may be involved in addiction vulnerability based on previous animal or human studies (see supplement to (Nielsen et al., 2008b)). We also utilized a second list compiled by the Gershon/Liu laboratory (The University of Chicago), hypothesized to be involved in affective disorders

(Hattori et al, 2005) for a total of 393 genes (Nielsen et al., 2008b). From these lists, SNPs from 183 genes were represented on the 10K array. Evidence for the involvement of five genes was found (Table 3). The genes were those coding for the nuclear receptor NR4A2, cryptochrome 1 (photolyase-like), the metabotropic receptors mGluR6 and mGluR8, and the mu opioid receptor ($P = 0.004 – 0.03$). Interestingly, the *OPRM1* variant (rs1074287) indicated in this study is located 11 kb upstream of the translation start site and is in the same LD block as the intron 1 variants that were associated with heroin addiction in our hypothesis-driven association study (Levran et al., 2008). This GWAS demonstrated that, given accurate phenotyping and careful analysis, valuable genetic information that can be used for the development and testing of hypotheses can be obtained using moderately sized cohorts.

6. Epigenetics

A factor in the interpretation of GWAS is epigenetic effects, and these effects may explain some of the addiction vulnerability that is not encoded in the DNA sequence. While some of the non-genetic influences may be drug-induced and environmental factors, epigenetic modifications, such as DNA methylation and chromatin remodeling, may be of importance.

Epigenetic inheritance is the transmission of information not encoded in the DNA sequence. The two primary molecular mechanisms are DNA methylation and covalent modification of histone proteins. DNA is wrapped around a histone core, which is composed of an octomer of histone monomers, two each of histone H3, H4, H2A, and H2B monomers, to form a nucleosome. DNA is packaged in nucleosomes to compact the DNA and to regulate gene expression. The amino acid tails of these histones protrude from the nucleosome and are accessible to covalent modifications, including acetylation and methylation. When the chromatin is tightly packed, the DNA is less accessible to the transcription machinery and, therefore, has reduced gene expression. Some histone modifications cause compaction of nucleosomes while other modifications relax the chromatin. For example, acetylation of histone H3 or H4 opens DNA and enhances transcription (Eberharter and Becker, 2002). Studies in rats have reported that chronic cocaine administration produces chromatin remodeling at the *bdnf* and *cdk5* promoters in rat striatum, while a single cocaine injection results in chromatin remodeling at *cFos* (Kumar et al., 2005). Studies in mice suggest that this may be due to reduced histone deacetylase HDAC5 function (Renthal et al., 2007). It has been reported recently that repeated administration of cocaine reduced global levels of histone 3 lysine 9 (H3K9) dimethylation in the nucleus accumbens through repression of the lysine dimethyltransferase G9a (Maze et al., 2010). When this dimethyltransferase was over-expressed in the nucleus accumbens, mice exhibited decreased cocaine preference.

The second major epigenetic mechanism regulating gene expression is DNA methylation. Methyl groups are added by DNA methyltransferases to cytosines nucleotides that are in cytosine : guanine (CpG) dinucleotides. The DNA methyltransferase DNMT1 maintains the methylation state of these CpG dinucleotides after cell division by methylation of unmethylated cytosines in hemi-methylated DNA, while DNMT3a and 3b can methylate DNA *de novo* (Bestor, 2000). When CpG dinucleotides are clustered together, they form “CpG” islands; 45,000 CpG islands are found in the human genome, many upstream of the promoter regions of genes (Antequera and Bird, 1993; Gardiner-Garden and Frommer, 1987). About 70% of the CpG dinucleotides in the genome are methylated while, in general, those found in “housekeeping” genes are unmethylated. Genes without CpG islands are often repressed by DNA methylation (Robertson and Wolffe, 2000).

The major cause of this repression is disruption of the transcription factor binding sites that have a CpG dinucleotide within their binding site [e.g. (Alikhani-Koopaei et al., 2004;

Douet et al., 2007)]. Another mechanism of repression by DNA methylation is due to binding of methyl-CpG binding proteins. Binding of these proteins may lead to the recruitment of histone modifying enzymes, such as histone methylases and deacetylases. For instance, a well-studied methyl binding protein, MeCP2, can bind mSin3A, which in turn binds histone deacetylase causing deacetylation of nearby histones (Michelotti et al., 2007). However, MeCP2 can also bind CREB, leading to the activation of nearby genes (Nan et al., 1998).

Recently, we examined the methylation of CpG sites in the promoter region of the mu opioid receptor gene in former heroin addicts in methadone maintenance treatment and controls (Nielsen et al., 2009). We found in peripheral lymphocytes that two of 16 CpG dinucleotide sites examined were hypermethylated in the cases compared to the controls. These sites could potentially alter gene expression, as the two hypermethylated CpG sites were located in Sp1 transcription factor binding sites. The hypermethylation of these sites could be a result of major life events prior to heroin use, to heroin use itself, or to methadone maintenance pharmacotherapy. In future studies, it will be important to take into account these epigenetic modifications.

7. Conclusions

Addictions are complex disorders for which a single gene analysis at the molecular level may contribute only a small amount of information. Addictions involve a complex interactive system with a wide range of complicated cellular mechanisms and environmental factors. Also, behavioral traits that contribute to addictions have mechanisms involving the products of many genes. Any individual technology platform or study may be limited, and there is a need for an integrative analytic approach to combine data across technology platforms and studies. Genetic testing for predisposition to any addiction may be problematic because of small effect sizes, epistatic (gene-gene) effects, and gene-environment interactions. On the other hand, identifying predisposing gene variants may enhance the understanding of the relevant biological processes and allow treatment development.

Case-control association studies of opioid addiction have a limitation in that the control group, for the most part, has never been self-exposed to an illicit opiate. The case group may represent people with specific behaviors related to the initial illicit drug taking (Kreek et al., 2005b). Similarly, if a variant discourages the use of a drug because of adverse effects (for example, acetaldehyde accumulation in alcohol drinking), it is not directly involved in the addiction process. Definitions of addiction phenotypes are also a major problem; the collection and accurate phenotyping of subjects requires significantly more effort than other studies of more easily definable phenotypes, such as hypertension.

We cannot be certain if the variants that have been shown to be involved in the development of heroin addiction are heroin-specific or contribute to common liability. The heritability of heroin addiction has been shown to be due to genetic factors which are shared by addiction to all drugs of abuse and factors specific to heroin addiction. In a large study of male twin pairs from the Virginia Twin Registry, it was found that 23% of the variance of opiate abuse/dependence was genetic, and that this variance was not specific to opiate addiction, but rather shared among addictions to all the drugs studied (opiates, cannabis, cocaine, hallucinogens, sedatives, and stimulants) (Kendler et al., 2003). In another large study of male twin pairs from the Vietnam Era Twin Registry, it was shown that opiate abuse/dependence had a genetic variance of 54%, with 16% shared across addictions to the drugs studied (opiates, marijuana, stimulants, sedatives, and psychedelics) and 38% specific to heroin addiction (Tsuang et al., 1996; Tsuang et al., 1998).

Several important GWAS findings for three psychiatric disorders (schizophrenia, autism and bipolar disorder) have been published recently (for review see (Psychiatric GWAS Consortium Coordinating Committee, 2009)) and have revealed new hypotheses about the causal mechanisms (primarily with the finding of various copy number variations (CNVs) and possible novel treatment targets. Importantly, a finding of an association of a variant with a disease does not necessarily pinpoint the causal factor, but, rather, identifies a chromosomal region where the functional variant(s) may be located. Confirmation of the findings from GWAS requires replication in similar, independent cohorts. The failure of many GWAS to replicate previous studies may be due to the architecture of the various microarrays used (such as poor coverage of alleles in a particular region or absence of the functional allele), low penetrance of the variant of interest, or low statistical power. For example, a recent analysis revealed that a substantial number of genes thought to be relevant to specific addictive diseases are missing from all the major commercial arrays (Saccone et al., 2009). To address this problem, Dr. D. Goldman's group at NIAAA has designed hypothesis-driven custom arrays with 130 genes encoding proteins that are involved in addictions, drug metabolism reward, and stress response (Hodgkinson et al., 2008). Intriguingly, a recent study showed that the effect of specific variants may depend on the paternal origin (Kong et al., 2009).

The success of GWAS is dependent on many factors including the frequency of risk alleles, sample size, and individual effect sizes, as well as the representation of markers on the array. GWAS may not be applicable to disorders caused by multiple rare SNPs or small, undetectable CNVs. Low frequency variants that are not well tagged by common SNPs, and are not well represented in the current commercial arrays, can be detected by the new high-density sequencing technologies (e.g., Illumina Solexa, ABI SOLiD, and Roche 454). Projects like the 1000 Genomes Project, which is a world-wide collaboration to produce an extensive catalog of human genetic variation by sequencing the genomes of over 1000 individuals from around the world using next-generation sequencing technologies, will facilitate these studies. GWAS results allow the development of hypotheses that can be tested in more directed studies with specific goals.

Although several investigators propose that an extremely large number of subjects are needed to identify all the variants involved in a disease, smaller studies have proven valuable in addiction research. The lack of accurate and complete phenotyping cannot be replaced by using larger numbers of individuals. Large studies can suffer from an increase in the diversity of ethnic backgrounds and co-morbidities. Several GWAS have used modest sample sizes of subjects with carefully defined phenotypes and well-defined ethnicities, and have provided the basis for novel hypotheses.

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Glossary

Allele	One form of a gene that varies between chromosomes or individuals that may determine a particular trait
cAMP	3′-5′-cyclic adenosine monophosphate is an intracellular second messenger involved in the transduction of signals within the cell
DNA	The material located inside the cell nucleus that carries genetic information and is found in all cells of the body
Epigenetics	The study of traits that are passed on from generation to generation or from cell to cell and are not encoded in the sequence of the DNA
Genes	The fundamental units of heredity responsible for how certain traits are passed down from parents to their biological children
Genetics	The study of heredity or how traits are passed down from parents to their offspring
Glycosylation	The post-translational addition of a glycan to a protein
GWAS	Genome-wide association study (GWAS) is an investigation of genetic variation across the entire genome
Heroin	Heroin or diacetylmorphine is a semi-synthetic opioid drug synthesized from the opium poppy-derived drug morphine
Ligand	A molecule that binds to a receptor
Opiates	Alkaloids naturally found in the resin of the opium poppy that bind to opioid receptors to produce analgesia
Opioids	Chemicals that bind to the opioid receptors to produce analgesia
Receptor	A protein molecule that is embedded in the cell surface membrane and which, after being bound by a specific ligand, transmits a signal to the inside of the cell
SNP	Single nucleotide polymorphisms (SNPs) are differences that occur at a single nucleotide between chromosomes
Traits	The characteristics inherited from parents that are passed down generation to generation. Examples of traits are eye color, height, and disease susceptibility
Transcription	The process whereby DNA is copied into messenger RNA (mRNA)
Translation	The process whereby ribosomes decode mRNA to produce an amino acid chain or peptide
Vulnerability	The susceptibility of a person or group to develop a disease

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Table 1

Hypothesis-driven study: the most significant results for association with vulnerability to develop opioid addiction

SNP	Gene Symbol	Protein	Location	Chr.	Position (Build 36.2)	*p*-value (Allele test)
a. European Americans						
rs510769	*OPRM1*	mu opioid receptor	IVS 1	6	154403712	0.0003
rs3778151	*OPRM1*	mu opioid receptor	IVS 1	6	154435373	0.0007
rs6473797	*OPRK1*	kappa opioid receptor	IVS 2	8	54315535	0.0009
rs1534891	*CSNK1E*	casein kinase 1, epsilon	IVS 7	22	37025045	0.0016
rs694066	*GAL*	galanin	IVS 2	11	68209561	0.0019
rs2236861	*OPRD1*	delta opioid receptor	IVS 1	1	29012343	0.0029
rs2236857	*OPRD1*	delta opioid receptor	IVS 1	1	29034196	0.0125
rs3766951	*OPRD1*	delta opioid receptor	IVS 1	1	29042146	0.0165
rs3758987	*HTR3B*	serotonin receptor 3B	−381	11	113280485	0.0174
b. African Americans						
rs486179	*ADRA1A*	adrenergic receptor, alpha 1A	5′ near gene	8	26779851	0.0251
rs3759292	*AVPR1A*	arginine vasopressin 1A receptor	5′ near gene	12	61833580	0.0079
rs971074	*ADH7*	alcohol dehydrogenase 7	Exon 6	4	100560884	0.0011
rs2350780	*CHRM2*	cholinergic receptor, muscarinic 2	IVS 3	7	136243509	0.0205
rs2289948	*DBI*	diazepam binding inhibitor	3′ near gene	2	119846668	0.0156
rs12613135	*DBI*	diazepam binding inhibitor	IVS 1	2	119838810	0.0181
rs5326	*DRD1*	dopamine D(1) receptor	5′ UTR	5	174802802	0.0108
rs7165224	*GABRB3*	GABA-A receptor subunit beta 3	5′ near gene	5	24575429	0.009
rs2058725	*GAD1*	glutamic acid decarboxylase1	IVS 5	2	171398367	0.005
rs8190646	*GAD2*	glutamic acid decarboxylase 2	IVS 7	10	26560513	0.0363
rs1650420	*GRIN2A*	glutamate receptor 2A	IVS 3	16	10175831	0.0009
rs4587976	*GRIN2A*	glutamate receptor 2A	IVS 3	16	10163636	0.009
rs6497730	*GRIN2A*	glutamate receptor 2A	IVS 3	16	10153171	0.0025
rs1070487	*GRIN2A*	glutamate receptor 2A	IVS 3	16	10143493	0.0046
rs1176724	*HTR3A*	serotonin receptor 3A	IVS 1	11	113353010	0.017
rs897687	*HTR3A*	serotonin receptor 3A	IVS 5	1	113361021	0.0265
rs731780	*SLC29A1*	solute carrier family 29 member 1	IVS 1	6	44301684	0.0004

Abbreviations: SNP, single nucleotide polymorphism; Chr, chromosome; TSS, transcription start site; UTR, untranslated region; IVS, intervening sequences.

Table 2

Variants identified in the GWAS to be associated with the vulnerability to develop opioid addiction

SNP	Gene Symbol	Protein	Location	Chr.	Position (Build 36.2)	*p*-value
rs965972	*Unigene cluster Hs.147755*		Intergenic	1	191730723	5.55×10^{-6}[a] 7.95×10^{-5}[b]
rs1986513			Intergenic	4	126286678	3.89×10^{-5}[a]
rs1408830	*HRPT2* *B3GALT*	hyperparathyroidism 2[c] UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	IVS 13 5′ near gene	1	191464280	1.28×10^{-4}[a]
rs1714984	*MYOCD*	myocardin	IVS 2	17	12558676	2.24×10^{-5}[b]
rs1867898			Intergenic	2	134236291	2.84×10^{-4}[b]

[a] Point-wise significance based on allele frequency

[b] Point-wise significance based on genotype frequency

[c] CDC73; Parafibromin (Cell division cycle protein 73 homolog)

Table 3

Hypothesis-derived genes identified in the GWAS to be associated with vulnerability to develop opioid addiction

SNP	Gene Symbol	Protein	Location	Chr.	Position (Build 36.2)	*p*-value by allele frequency	*p*-value by genotype frequency
rs1034576	*GRM8*	glutamate receptor, metabotropic 8	3′ near gene	7	125850917	0.0052	0.0058
rs1861591	*CRY1*	cryptochrome 1 (photolyase-like)	IVS 1	12	105941307	0.0040	0.0125
rs953741	*GRM6*	mGluR6	3′ near gene	5	178262702	0.0071	0.0068
rs1074287	*OPRM1*	mu opioid receptor	5′ near gene	6	154390752	36.0055	0.0309
rs1405735	*NR4A2*	nuclear receptor subfamily 4, group A, member 2	5′ near gene	2	156942328	0.0312	0.024