Cis-Expression Quantitative Trait Loci Mapping Reveals Replicable Associations with Heroin Addiction in *OPRM1*

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ABSTRACT

BACKGROUND: No opioid receptor, mu 1 (*OPRM1*) gene polymorphisms, including the functional single nucleotide polymorphism (SNP) rs1799971, have been conclusively associated with heroin/other opioid addiction, despite their biological plausibility. We used evidence of polymorphisms altering *OPRM1* expression in normal human brain tissue to nominate and then test associations with heroin addiction.

METHODS: We tested 103 *OPRM1* SNPs for association with *OPRM1* messenger RNA expression in prefrontal cortex from 224 European Americans and African Americans of the BrainCloud cohort. We then tested the 16 putative cis-expression quantitative trait loci (cis-eQTL) SNPs for association with heroin addiction in the Urban Health Study and two replication cohorts, totaling 16,729 European Americans, African Americans, and Australians of European ancestry.

RESULTS: Four putative cis-eQTL SNPs were significantly associated with heroin addiction in the Urban Health Study (smallest $p = 8.9 \times 10^{-5}$): rs9478495, rs3778150, rs9384169, and rs562859. Rs3778150, located in *OPRM1* intron 1, was significantly replicated ($p = 6.3 \times 10^{-5}$). Meta-analysis across all case-control cohorts resulted in $p = 4.3 \times 10^{-8}$: the rs3778150-C allele (frequency = 16%–19%) being associated with increased heroin addiction risk. Importantly, the functional SNP allele rs1799971-A was associated with heroin addiction only in the presence of rs3778150-C ($p = 1.48 \times 10^{-6}$ for rs1799971-A/rs3778150-C and $p = .79$ for rs1799971-A/rs3778150-T haplotypes). Lastly, replication was observed for six other intron 1 SNPs that had prior suggestive associations with heroin addiction (smallest $p = 2.7 \times 10^{-8}$ for rs3823010).

CONCLUSIONS: Our findings show that common *OPRM1* intron 1 SNPs have replicable associations with heroin addiction. The haplotype structure of rs3778150 and nearby SNPs may underlie the inconsistent associations between rs1799971 and heroin addiction.

Keywords: Genetic association study, Heroin, Multiancestry, Opioid, OPRM1, Prefrontal cortex

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According to the 2010 Global Burden of Disease Health Measurement Survey, around 15.5 million people worldwide were dependent on heroin and other opioid drugs (1). Three regions had prevalence rates significantly higher than the global rate: Australasia, western Europe, and North America (1). In the United States, the 2013 National Survey on Drug Use and Health estimated that 669,000 people aged 12 or older abused heroin in the past year, representing a 78% increase since 2007 (2). To address this public health burden, a better understanding of the pathogenesis leading to heroin addiction is needed. Genetic vulnerability is recognized as a major risk factor contributing to heroin and other opioid addiction, as evidenced by twin studies showing that genetic factors account for 40% to 60% of the population variability (3–6). A few genome-wide association (7–9) and numerous

candidate gene studies (10–19) in humans have implicated genes encoding opioid receptors (*OPRM1*, *OPRD1*, and *OPRK1*) and potassium channels (*KCNG1* and *KCNG2*) and others as contributing to heroin/opioid addiction phenotypes. In supporting the biological plausibility of the candidate genes, particularly genes in the opioid system (20), knockout mouse models have been used to study behavioral effects resulting from genetic perturbations. However, conclusively identified associations of specific genetic variants remain elusive.

The current study focuses on the opioid receptor, mu 1 (*OPRM1*) gene, the most widely studied candidate gene (17), which encodes a predominant target for heroin and other opioid molecules. The *OPRM1* missense polymorphism rs1799971 has been widely studied for its functional consequences, including reduced signaling efficiency and reduced

expression of the receptor (21–25). However, rs1799971 associations with heroin and other drug addictions (13–17) have been modest and often inconsistent. Additional *OPRM1* single nucleotide polymorphisms (SNPs) have been tested for association with heroin/opioid addiction (10,12,13,18,19), but none of the findings have been independently replicated.

To enhance the detection of replicable *OPRM1* SNP associations with heroin addiction, our study focused only on SNPs with evidence for altering *OPRM1* messenger RNA expression in human brain, thereby reducing the multiple testing burden with a limited number of plausible regulatory SNPs carried forward for disease association testing. This approach was motivated by prior findings that psychiatric and other disease-associated SNPs tag expression quantitative trait loci (eQTL) more often than unassociated SNPs (26–28). Other studies have successfully used cis-eQTL mapping to nominate SNPs and consequently find associations with complex diseases, such as Crohn's disease (29), chronic obstructive pulmonary disease (30), and amyotrophic lateral sclerosis (31). We used cis-eQTL mapping to identify SNPs associated with *OPRM1* messenger RNA expression in human prefrontal cortex from 224 BrainCloud cohort participants (32) who had no evidence of drug use/abuse at the time of death and tested the putative cis-eQTL SNPs for association with heroin addiction across three independent cohorts totaling 16,729 (4287 cases and 12,442 control subjects). Our findings revealed replicable and highly significant SNP associations with heroin addiction and provided strong support of *OPRM1* as an important susceptibility gene for heroin addiction.

METHODS AND MATERIALS

Figure 1 outlines our overall study design of conducting cis-eQTL mapping for *OPRM1* in nonaddicted BrainCloud participants and testing the nominated SNPs for association in heroin addiction case-control cohorts. All study protocols received Institutional Review Board approval at their respective sites, and all study participants or their legal next of kin provided informed consent.
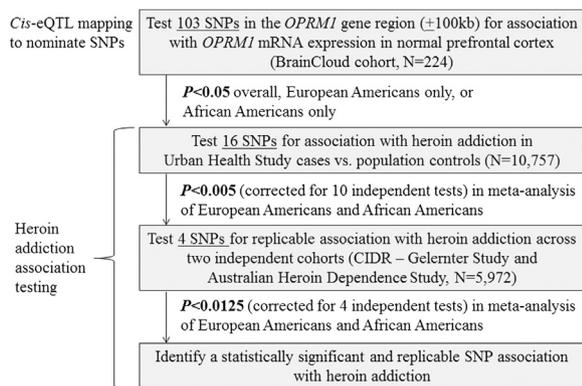

Figure 1. Overview of study design. CIDR, Center for Inherited Disease Research; eQTL, expression quantitative trait loci; mRNA, messenger RNA; SNP, single nucleotide polymorphism.

Cis-eQTL Mapping Using Human Prefrontal Cortex in the BrainCloud Cohort

To identify putative cis-eQTL SNPs for *OPRM1*, we utilized SNP genotype and gene expression data (Supplement 1) available on postmortem dorsolateral prefrontal cortex samples from 110 European Americans and 114 African Americans, ranging in age from 0 to 78 years old, who had no neuropathologic or neuropsychiatric diagnoses, no drug or alcohol abuse, and no positive toxicology result (32–34).

Normalized ratios of gene expression levels from the single *OPRM1* probe available in BrainCloud (Figure S1 in Supplement 1) were log-transformed and tested for association with additive three-level SNP genotypes using the BrainCloud software (32). Genotyped SNP association results were generated from the best fit general linear model with race, sex, age, life stage (infant, child, teen, and adult), and an age by life stage interaction included as the range of covariates. Inclusion of age, life stage, and the corresponding interaction was used to account for nonlinear trajectories of age-dependent gene expression over the life span (33). We evaluated associations overall and stratified by ancestry for the 103 SNPs genotyped across *OPRM1* and its 100 kilobase flanking regions. A p value threshold of .00125, which takes into account the correlations among the 103 *OPRM1* SNPs (α = .05/40 independent tests) (35,36), was used to declare statistical significance. Given the hypothesis-generating nature of our cis-eQTL mapping, SNPs associated with *OPRM1* expression at the nominal significance threshold of $p < .05$ were carried forward for association testing with heroin addiction.

Discovery Cohort for Heroin Addiction Association Testing: Urban Health Study Cases versus Population Control Subjects

European American and African American cases were drawn from the Urban Health Study (UHS), one of the largest studies of street-recruited injection drug users in North America (37). See Supplement 1 for further details on the UHS design. Stored serum samples from 3227 UHS participants were selected for genotyping on the Illumina Omni1-Quad BeadChip (Illumina, Inc, San Diego, California) (38). Over 60% of the genotyped UHS participants met the Office of National Drug Control Policy definition of heroin abuse (injecting 10+ times in the past 30 days) (39,40), which is highly correlated with clinical levels of dependence on the Severity of Dependence Scale (41,42) and with DSM-IV (43) heroin abuse/dependence in analyses of the National Household Survey on Drug Use and Health data (87% positive predictive value; see Supplemental Methods and Table S1 in Supplement 1). These UHS participants, who abused heroin an average of 80.9 times in the past month and were very likely dependent on it, are henceforth referred to as heroin addiction cases. The remaining genotyped UHS participants who were addicted to cocaine or other substances but not addicted to heroin were not included in the current study.

For comparison with the UHS heroin addiction cases, we used six study cohorts from the database of Genotypes and Phenotypes (dbGaP) as a source of control subjects. Several prior studies have been reliably conducted using a similar design with study cases and population control subjects (44–49). See Supplemental Methods, Table S2, and Figure S2 in

Supplement 1 for an outline of our cohort selection and quality control processes. There were no apparent biases underlying our control dataset, as indicated by genomic control inflation factor (λ_{gc}) $\leq$ 1.05 for all pairwise combinations of dbGaP cohorts (Tables S3 and S4 in Supplement 1). Our final analysis dataset included 7095 European Americans (711 UHS heroin addiction cases and 6384 population control subjects) and 3662 African Americans (1293 UHS heroin addiction cases and 2369 population control subjects).

To capture all of the nominated cis-eQTL SNPs, genotype imputation was conducted using IMPUTE2 (http://mathgen. stats.ox.ac.uk/impute/impute_v2.html) (50) with reference to all 1000 Genomes haplotype panels (http://www.1000genomes. org/) (51,52). To avoid potential bias due to UHS cases and population control subjects being genotyped on different arrays, imputations were based on the intersection of SNPs available across all participants (53). Further details on quality control and imputation are provided in Supplement 1.

Replication Cohorts for Heroin Addiction Association Testing

Two independent cohorts, comprised of heroin and other opioid abuse/dependent participants (henceforth referred to as heroin addiction cases) and their own control subjects, were used to replicate SNP associations with heroin addiction. First, we used African Americans from the "Alcohol Dependence GWAS in European- and African-Americans (CIDR–Gelernter Study)" (dbGaP accession number phs000425.v1.p1) (7). Our final analysis dataset included 852 African Americans (307 DSM-IV defined cases of heroin/other opioid abuse or dependence and 545 control subjects with no illicit drug abuse or dependence; Supplemental Methods and Table S2 in Supplement 1). European Americans from this cohort were not analyzed because there was an insufficient number of independent control subjects (<5) who met our inclusion criteria.

Second, we used DSM-IV defined cases of heroin dependence and general population control subjects gathered from Australian datasets, many of which are deposited in dbGaP (accession number phs000277.v1.p1). We henceforth refer to them as the Australian Heroin Dependence Study. A subset of this case-control cohort and descriptions of the study design have been previously described (10). Our final analysis dataset totaled 5120 Australians of European ancestry (1976 cases and 3144 control subjects; Supplemental Methods and Table S2 in Supplement 1).

Statistical Analyses: Association Testing with Heroin Addiction

Putative cis-eQTL SNPs were tested for association with heroin addiction across both ancestry groups in the discovery cohort (UHS vs. population control subjects). Significantly associated SNPs were then tested for replication in the CIDR–Gelernter Study and the Australian Heroin Dependence Study. Observed SNP genotypes or imputed SNP genotype dosages were tested for association using logistic regression models, separately by cohort and by ancestry group, with adjustment for sex and principal component eigenvectors (Supplement 1). SNP association results were compared using the Forest Plot Viewer (http://ntp.niehs.nih.gov/help/browsers/metadata/index-2.html) (54) and combined via fixed-effects sample size weighted

meta-analyses, as used in prior multiancestry meta-analyses (55,56). As shown in Figure 1, at each stage of the association testing, linkage disequilibrium among the tested SNPs was taken into account to compute the p value thresholds for declaring statistical significance (35,36) (α = .05/number of independent tests): meta-analysis $p <$ .0050 based on 10 independent tests in the discovery stage and meta-analysis $p <$ .0125 based on 4 independent tests in the replication stage.

Bioinformatics Analyses

Linkage disequilibrium patterns were discerned using Locus-Zoom (http://csg.sph.umich.edu/locuszoom/) (57) and Haplo-view (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview) (58) with reference to two 1000 Genomes panels: EUR (comprised of European Americans, Finns, Britons, Spaniards, and Italians) and AFR (comprised of African Americans, Kenyans, and Nigerians). SNPs were annotated according to their location in the longest principal isoform for *OPRM1*, according to the APPRIS database (http://appris.bioinfo.cnio.es) (59). Regulatory SNP annotations were taken from the HaploReg v2 (http://www.broadinstitute.org/mammals/haploreg/haploreg.php) database containing information on chromatin states, conservation, and regulatory motif alterations from the Encyclopedia of DNA Elements and elsewhere (60).

RESULTS

Cis-eQTL Mapping in Human Prefrontal Cortex

Sixteen of the 103 SNPs tested for association with *OPRM1* expression in the BrainCloud cohort were nominated as cis-eQTL SNPs (Figure 1; Table 1): 9 SNPs overall, 2 SNPs when analyzing only European Americans, and 5 SNPs when analyzing only African Americans. Three of the cis-eQTL SNPs were significantly associated with *OPRM1* expression at $p <$ .00125, based on an overall α = .05 corrected for 40 independent tests among the 103 *OPRM1* SNPs (35,36); the other 13 SNPs were nominally associated at $p <$ .05. Results of all tested SNPs are shown in Table S5 in Supplement 1.

HaploReg v2 (60) corroborated the regulatory potential of the putative cis-eQTL SNPs, showing that almost all of them are predicted to alter one or more of the regulatory motifs indicative of transcription factor binding sites (61,62) (Table S6 in Supplement 1). Moreover, rs3778150 resides in a highly conserved region, and two of the SNPs (rs562859 and rs13203628) are located in DNase hypersensitivity and enhancer sites (Table S6 in Supplement 1).

Testing cis-eQTL SNPs for Association with Heroin Addiction

The 16 putative cis-eQTL SNPs for *OPRM1* were tested for association with heroin addiction in UHS cases versus population control subjects (Figure 1; Table 1). There was no evidence to suggest heterogeneity for any of the 16 SNPs (smallest p = .15), and the imputation quality of all tested SNPs was high (info $\geq$ .9). Meta-analysis across the ancestry groups revealed 4 SNPs significantly associated with heroin addiction at $p <$ .005, based on an overall α = .05 corrected for 10 independent tests among the 16 cis-eQTL SNPs (35,36):

Table 1. Sixteen Putative cis-eQTL SNPs for *OPRM1* in the BrainCloud Cohort and Tested for Association with Heroin Addiction in European American and African American Cases from the Urban Health Study Versus Population Control Subjects

						Association with Heroin Addiction						
			Association with OPRM1 Expression in BrainCloud			African Americans (n = 1293 Cases and 2369 Control Subjects)			European Americans (n = 711 Cases and 6384 Control Subjects)			
SNP (Minor Allele)	Base Pair Position (NCBI Build 36)	SNP Type (Distance to *OPRM1* for Intergenic SNPs)	p, Overall	p, European Americans Only (n = 110)	p, African Americans Only (n = 114)	MAF	p	OR (95% CI)	MAF	p	OR (95% CI)	Meta-analysis p
rs9478495 (A)	154,365,602	intergenic (−36,533)	.22	.89	.015	.22	.019	1.16 (1.02–1.30)	.16	.0017	1.27 (1.09–1.47)	**8.9×10^{-5}**
rs3778150 (C)	154,425,351	intronic	.089	.042	.38	.19	.037	1.15 (1.01–1.30)	.16	.0012	1.28 (1.10–1.47)	**1.2×10^{-4}**
rs9384169 (C)	154,354,864	intergenic (−47,271)	.25	.90	.046	.31	.029	1.12 (1.01–1.25)	.32	.025	1.15 (1.02–1.30)	**2.0×10^{-3}**
rs562859 (C)	154,456,266	synonymous	.054	.016	.96	.38	.018	1.13 (1.02–1.25)	.33	.070	1.12 (.99–1.26)	**4.3×10^{-3}**
rs613355 (C)	154,491,542	intronic	.010	.012	.72	.39	.071	1.10 (.99–1.22)	.34	.067	1.12 (.99–1.26)	.011
rs13203628 (G)	154,502,815	intronic	.037	.010	.82	.34	.068	1.11 (.99–1.23)	.26	.11	1.11 (.97–1.28)	.019
rs671531 (A)	154,482,434	intronic	.015	.010	.92	.39	.11	1.08 (.98–1.20)	.33	.079	1.11 (.99–1.26)	.019
rs9479779 (G)	154,544,688	intronic	.30	.57	.042	.085	.57	1.05 (.88–1.25)	.059	.20	.84 (.65–1.10)	.48
rs558948 (T)	154,483,556	intronic	.0017	.00094	.71	.16	.76	1.02 (.89–1.16)	.25	.58	1.04 (.91–1.19)	.53
rs650825 (A)	154,470,229	intronic	.0034	.0017	.45	.17	.86	.99 (.87–1.12)	.25	.57	1.04 (.91–1.19)	.72
rs10485060 (A)	154,557,793	intronic	.039	.34	.24	.022	.23	.81 (.57–1.15)	.047	.66	1.06 (.81–1.39)	.73
rs647192 (G)	154,464,437	intronic	.0085	.0018	.63	.085	.80	.98 (.82–1.17)	.25	.60	1.04 (.91–1.18)	.78
rs647303 (C)	154,324,902	intergenic (−77,233)	.79	.28	.0010	.11	.29	1.09 (.93–1.27)	.26	.60	.97 (.85–1.10)	.85
rs6900805 (G)	154,329,725	intergenic (−72,410)	.79	.28	.0010	.11	.34	1.08 (.92–1.26)	.26	.63	.97 (.85–1.10)	.87
rs538174 (C)	154,494,229	intronic	.012	.0028	1.00	.076	.27	.89 (.74–1.09)	.24	.35	1.06 (.93–1.22)	.91
rs1852629 (T)	154,564,432	intronic	.044	.090	.38	.42	.96	1.00 (.90–1.10)	.43	.87	1.01 (.90–1.13)	.92

SNPs are sorted by the meta-analysis *p* value for association with heroin addiction, with statistically significant results shown in bold (meta-analysis *p* < .005).

CI, confidence interval; eQTL, expression quantitative trait loci; MAF, minor allele frequency; NCBI, National Center for Biotechnology Information; OR, odds ratio; SNP, single nucleotide polymorphism.

rs9478495, rs3778150, rs9384169, and rs562859. Minor alleles of these four SNPs were common (frequencies ranging from 16% to 38%), and they had consistent directions of association: odds ratios ranging from 1.12 to 1.28 across the ancestry groups. The two most significantly associated SNPs for heroin addiction (rs9478495 and rs3778150) were in moderate linkage disequilibrium in the 1000 Genomes EUR panel ($r^2 = .48$; Figure S3A in Supplement 1) but weak linkage disequilibrium in the AFR panel ($r^2 = .04$; Figure S3B in Supplement 1).

The four SNPs significantly associated with heroin addiction in the discovery cohort were then tested in African Americans from the CIDR–Gelernter Study and Australians of European ancestry from the Australian Heroin Dependence Study (Figure 1). Results are presented in Table S7 in Supplement 1. The rs3778150 association with heroin addiction was significantly replicated at $p = 6.8 \times 10^{-5}$, well below the corrected replication threshold $p < .0125$, based on an overall $\alpha = .05$ corrected for four independent tests (35,36). Meta-analysis of rs3778150 combining discovery and replication cohorts resulted in $p = 4.3 \times 10^{-8}$. As shown by the odds ratios estimates in Figure 2, the rs3778150 minor allele (C) was consistently associated with an increased heroin addiction risk in participants of European ancestry (1.28 in the UHS and 1.23 in the Australian Heroin Dependence Study) and African Americans (1.15 in the UHS and 1.20 in the CIDR–Gelernter Study), and it was associated at $p < .05$ in both UHS ancestry groups and the Australian Heroin Dependence Study. The direction of the rs3778150-C association was consistent in the CIDR–Gelernter Study but with $p = .17$; this cohort was smaller than the others and thus had less statistical power than the rest of the cohorts. The three other tested SNPs were not replicated (Table S7 in Supplement 1).

The replicated SNP rs3778150 was originally implicated as a cis-eQTL SNP at $p = .042$ in BrainCloud European Americans (Table 1), the C allele being associated with reduced *OPRM1* expression (Figure S4A in Supplement 1). Rs3778150 was not significantly implicated in the African Americans only ($p = .38$; Figure S4B in Supplement 1) or all BrainCloud participants ($p = .089$; Figure S4C in Supplement 1).

Testing rs3778150–rs1799971 Haplotypes for Association with Heroin Addiction

We next evaluated linkage disequilibrium with the widely studied SNP rs1799971 (r^2 values shown in Figure S5 in Supplement 1 and D′ values shown in Figure S6 in Supplement 1). Rs3778150 and rs1799971 had $r^2 = 0$ and D′ = .05 in the AFR panel. In the EUR panel, rs3778150 and rs1799971 had D′ = 1; the rs3778150 minor allele (C, frequency = 15%) occurred on the same haplotype background with the rs1799971 major allele (A, frequency = 84%), but a weak r^2 value ($r^2 = .03$) reflected the discrepant allele frequencies.

Rs1799971 was not significantly associated with heroin addiction in any of the cohorts or ancestry groups individually or in a meta-analysis ($p = .12$), although its direction of association generally suggested that the major allele (A) conferred an increased risk with heroin addiction (Figure S7 in Supplement 1). Given the indication of a shared haplotype background between rs3778150 and rs1799971 in the EUR panel, we constructed the two SNP haplotypes and tested their associations with heroin addiction across all case-control cohorts and ancestry groups. As shown in Table 2, the haplotype carrying both the rs3778150-C and rs1799971-A alleles was consistently and significantly associated with increased risk of heroin addiction for each cohort and for both European Americans and African Americans: meta-analysis $p = 1.5 \times 10^{-6}$. The haplotype carrying rs1799971-A without rs3778150-C was not associated with heroin addiction in any of the cohorts or ancestry groups: meta-analysis $p = .79$.

Testing Previously Implicated *OPRM1* Intron 1 SNPs for Association with Heroin Addiction

Rs3778150 has varying linkage disequilibrium patterns with six other SNPs located in *OPRM1* intron 1, which were previously reported for their associations with heroin/opioid addiction phenotypes (12,19) (Figures S8 and S9 in Supplement 1). The intron 1 location of these SNPs is designated according to the longest principal isoform for *OPRM1* (59). Levran *et al.* (12) reported rs510769 and rs3778151 as having nominally signifi-
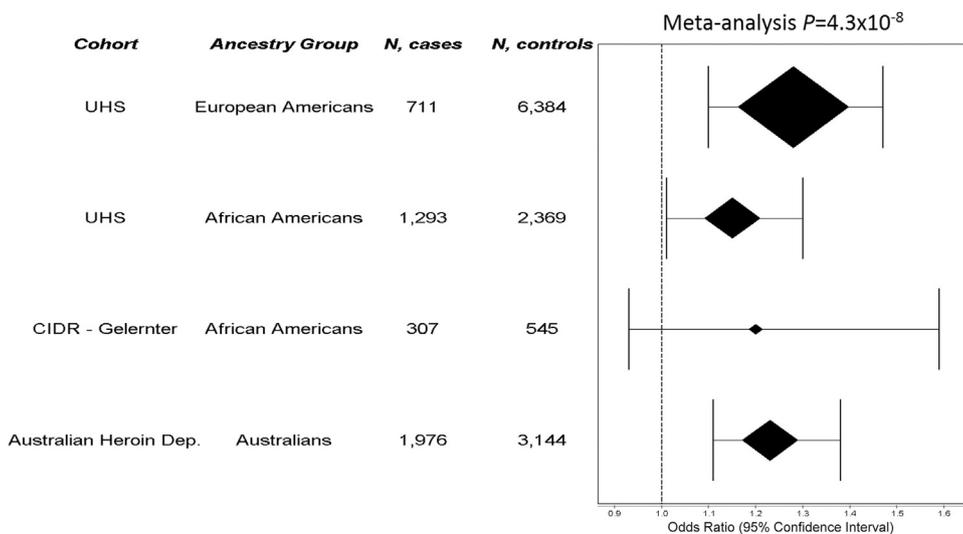

Figure 2. Forest plot of association results for the rs3778150-C allele. Results are shown across all heroin addiction case-control cohorts (UHS, CIDR–Gelernter Study, and Australian Heroin Dependence Study) and ancestry groups (European Americans, African Americans, Australians of European ancestry). CIDR, Center for Inherited Disease Research; Dep., Dependence; UHS, Urban Health Study.

Table 2. Haplotype Analyses of rs3778150 and the Widely Studied Missense SNP rs1799971 Across All Study Cohorts and Ancestry Groups

rs3778150 Allele	rs1799971 Allele	Urban Health Study, European Americans (n = 7095)			Urban Health Study, African Americans (n = 3662)			CIDR–Gelernter Study, African Americans (n = 852)			Australian Heroin Dependence Study, Australians of European Ancestry (n = 5120)			Meta-analysis p
		Freq	p	OR (95% CI)	Freq	p	OR (95% CI)	Freq	p	OR (95% CI)	Freq	p	OR (95% CI)	
C	A	.16	.0070	1.29 (1.07–1.55)	.19	.0056	1.24 (1.06–1.44)	.18	.40	1.17 (.82–1.66)	.17	.0045	1.26 (1.07–1.47)	1.5×10^{-6}
T	A	.70	.10	.79 (.60–1.05)	.79	.13	1.32 (.92–1.87)	.79	.81	.91 (.41–2.01)	.71	.80	1.02 (.89–1.16)	.79
T	G	.13	–	ref.	.03	–	ref.	.02	–	ref.	.12	–	ref.	–

The single SNP analyses suggested that the rs3778150 minor allele (C) and rs1799971 major allele (A) conferred increased risks for heroin addiction.

CI, confidence interval; CIDR, Center for Inherited Disease Research; Freq, frequency; OR, odds ratio; ref., reference group; SNP, single nucleotide polymorphism.

cant associations, whereas Zhang *et al.* (19) reported rs524731 as having a nominally significant association and rs511435, rs3823010, and rs495491 as having statistically significant associations that surpassed multiple testing correction. Nelson *et al.* (10), using a subset of the Australian replication cohort reported here, tested three of these SNPs (rs510769, rs3778151, and rs495491) and found a nominal association for rs3778151. To our knowledge, the six prior SNPs have not been tested elsewhere for independent replication. In a meta-analysis across all of our heroin addiction case-control cohorts, all six of the previously reported SNPs or their proxies were significantly replicated at $p < .01$ (overall $\alpha = .05$ corrected for five independent tests). Their meta-analysis p values ranged from 7.0×10^{-4} for rs510769 to 2.7×10^{-8} for rs3823010 (Table 3). Similar to rs3778150, the minor alleles were all associated with increased heroin addiction risk.

Rs3778150 and the top prior SNP rs3823010 were in perfect linkage disequilibrium ($r^2 = 1$ and D′ = 1) with equivalent minor allele frequencies at 15% in EUR, and although D′ = 1 in AFR, their moderate r^2 value of .54 reflected varied minor allele frequencies (20% for rs3778150 and 12% for rs3823010). In the African American cohorts from the current study where the SNPs were not collinear, both SNPs were associated with heroin addiction (African American specific meta-analysis $p = .013$ for rs3778150 and $p = .0087$ for rs3823010), but in follow-up regression analyses with both rs3778150 and rs3823010 included as predictors, neither SNP association remained (African American specific meta-analysis $p = .48$ for rs3778150 and $p = .38$ for rs3823010), suggesting that their associations with heroin addiction reflect the same underlying signal.

DISCUSSION

The focus of our study was to identify *OPRM1* polymorphisms consistently associated with heroin addiction. To increase the likelihood of detecting such associations, we followed the strategy of mapping cis-eQTLs altering *OPRM1* expression in human brain from nonaddicted participants, nominating this relatively small number of potentially regulatory polymorphisms and testing them for association with heroin addiction. Among 16 putative cis-eQTL SNPs, we found that rs3778150 had a highly significant association with heroin addiction across three independent cohorts, even surpassing the standard genome-wide significance p value threshold of 5×10^{-8}. Follow-up haplotype analyses showed that the widely studied missense SNP rs1799971 was associated with heroin addiction only in the presence of the rs3778150 risk allele, suggesting that rs3778150 and its nearby correlated SNPs may explain the inconsistent associations of rs1799971 observed across numerous studies of heroin/opioid addiction.

The rs3778150 minor allele (C) was associated with both reduced *OPRM1* expression and increased risk of heroin addiction, with its associations being strongest with European ancestry. It is common for cis-eQTL and disease risk associations to differ by ancestry, possibly due to unmeasured genetic or environmental modifiers (63). The direction of association between *OPRM1* expression and risk of heroin addiction has not been conclusively shown. Nonetheless, our observed patterns for the rs3778150 minor allele are consistent

Table 3. Associations of Previously Implicated *OPRM1* Intron 1 SNPs with Heroin Addiction Across the Cohorts Used in the Current Study (Urban Health Study, CIDR–Gelernter Study, and the Australian Heroin Dependence Study)

SNP (Minor Allele)	Ref.	Previously Reported p	Urban Health Study, African Americans (n = 1293 Cases and 2369 Control Subjects)			Urban Health Study, European Americans (n = 711 Cases and 6384 Control Subjects)			CIDR–Gelernter Study, African Americans (n = 307 Cases and 545 Control Subjects)			Australian Heroin Dependence Study, Australians of European Ancestry (n = 1293 Cases and 2369 Control Subjects)			Meta-analysis p
			MAF	p	OR (95% CI)	MAF	p	OR (95% CI)	MAF	p	OR (95% CI)	MAF	p	OR (95% CI)	
rs3823010 (A)	(19)	.001	.12	.025	1.19 (1.02–1.37)	.16	8.6×10^{-4}	1.28 (1.11–1.49)	.13	.17	1.25 (.91–1.72)	.17	2.4×10^{-4}	1.23 (1.10–1.37)	2.7×10^{-8}
rs3778151 (C)[a]	(10,12)	.003 (12)	.29	.029	1.12 (1.01–1.27)	.16	3.0×10^{-3}	1.25 (1.08–1.45)	.31	.33	1.12 (.89–1.41)	.17	3.2×10^{-4}	1.23 (1.10–1.37)	2.4×10^{-7}
		.031 (10)													
rs524731 (A)[a]	(19)	.011	.12	.065	1.15 (.99–1.33)	.21	1.4×10^{-3}	1.25 (1.09–1.43)	.12	.17	1.25 (.91–1.75)	.21	3.7×10^{-3}	1.16 (1.05–1.29)	1.2×10^{-6}
rs511435 (T)[a]	(19)	.002	.20	.15	1.10 (.97–1.23)	.21	1.4×10^{-3}	1.25 (1.09–1.43)	.19	.17	1.20 (.93–1.59)	.21	3.7×10^{-3}	1.16 (1.05–1.29)	3.0×10^{-6}
rs495491 (G)[a,b]	(19)	.002	.52	.015	1.14 (1.02–1.25)	.26	8.2×10^{-3}	1.19 (1.04–1.35)	.52	.25	1.14 (.91–1.43)	not available	not available	not available	1.8×10^{-4c}
rs510769 (T)	(12)	.0008	.24	.97	1.00 (.89–1.12)	.26	1.0×10^{-2}	1.19 (1.04–1.35)	.24	.29	1.15 (.88–1.52)	.26	8.4×10^{-3}	1.14 (1.03–1.25)	7.0×10^{-4}

These SNPs were reported for having nominally to statistically significant associations with heroin addiction (n = 412 cases and 184 control subjects, comprised of European Americans or Israelis with Jewish ancestry) (12), opioid dependence (n = 91 cases and 338 control subjects, comprised of all European Americans) (19), or heroin dependence (n = 1459 cases and 531 control subjects, comprised of Australians of European ancestry) (10). None of these SNPs were previously tested for independent replication. SNPs are sorted by meta-analysis p value.

CI, confidence interval; CIDR, Center for Inherited Disease Research; EUR, 1000 Genomes panel comprised of European Americans, Finns, Britons, Spaniards, and Italians; GWAS, genome-wide association study; MAF, minor allele frequency; OR, odds ratio; Ref., reference; SNP, single nucleotide polymorphism.

[a]These SNPs were not available as genotyped SNPs in the Heroin Dependence GWAS, so the following SNPs in high linkage disequilibrium were used: rs514980 as a proxy SNP for both rs511435 and rs524731 ($r^2 = 1$ and D′ = 1 in 1000 Genomes EUR) and rs3778153 as a proxy SNP for rs3778151 ($r^2 = .98$ and D′ = 1 in 1000 Genomes EUR). The best proxy for rs495491 was the other previously reported SNP rs510769 ($r^2 = .98$ and D′ = 1 in 1000 Genomes EUR).

[b]For rs495491, G is the minor allele in populations of European ancestry, but A is the minor allele in African Americans. The presented allele frequencies correspond to the G allele.

[c]Meta-analysis was computed using the results that were available.

with one prior report noting reduced *OPRM1* expression levels in thalamus and secondary somatosensory cortex samples of heroin addicts compared with control subjects and postulating that downregulation of *OPRM1* may play a role in increasing risk of heroin addiction by disrupting the opioid system of a key compensatory response to heroin (25). More specifically, heroin exposure results in reduced μ receptor function, and in response, the opioid system upregulates *OPRM1* expression to make more μ receptors available. It is plausible that genetic polymorphisms attenuating *OPRM1* expression could hinder this compensatory mechanism, leading to a greater quantity of heroin being needed to trigger a physiological effect and thus increasing risk of becoming addicted to heroin.

One prior study reported a nominal association between rs3778150 and heroin dependence. Using a subset of the Australian Heroin Dependence Study reported here ($n = 1459$ cases and 531 general population control subjects), Nelson *et al.* (10) found that rs3778150 was associated with DSM-IV defined heroin dependence at $p = .024$, but this finding did not pass multiple testing correction in that study. The rs3778150 association signal was greatly strengthened with the larger sample size in this study: $p = 1.7 \times 10^{-4}$ in the Australian Heroin Dependence Study ($n = 5120$) and meta-analysis $p = 4.3 \times 10^{-8}$ across all cohorts (total $n = 16,729$). Beyond rs3778150, six other *OPRM1* intron 1 SNPs have been reported for their associations with heroin or other opioid addiction phenotypes. Levran *et al.* (12) reported that rs510769 and rs3778151 were nominally associated in 596 European American severe heroin addicts and healthy control subjects, but these findings did not surpass multiple testing correction. Zhang *et al.* (19) found that rs524731, rs511435, rs3823010, and rs495491 were nominally to significantly associated in 429 European American DSM-defined opioid dependent cases and control subjects, who were ascertained in parallel with the African Americans from the CIDR–Gelernter Study that we included in our study. We tested the prior SNPs or their proxies across the cohorts used in the current study and found statistically significant evidence to support their associations (smallest meta-analysis $p = 2.7 \times 10^{-8}$ for rs3823010; Table 3). Rs3823010 was not associated with *OPRM1* expression (smallest $p = .12$; Table S5 in Supplement 1). Nonetheless, the directions of association for the previously reported SNPs were consistent with our rs3778150 finding: the minor alleles being associated with increased risk. The regulatory potentials of the prior SNPs were hypothesized given their positions in a noncoding region (12,19,64).

Our findings show, for the first time, that *OPRM1* intron 1 contains SNPs that have replicable associations with heroin addiction. Our findings further suggest that rs3778150 and possibly other intron 1 SNPs may tag a cis-eQTL that influences *OPRM1* expression and underlies the disease associations. Our tested SNPs also included rs563649, which is located in intron 1 and was previously reported for being associated with *OPRM1* expression in vitro (65); we did not corroborate this association with *OPRM1* expression (smallest $p = .26$; Table S5 in Supplement 1) and did not find evidence for association with heroin addiction in our discovery cohort (meta-analysis $p = .57$). Fine-mapping in the intron 1 region, particularly in diverse ancestry groups with varied linkage disequilibrium patterns, is needed to delineate the variants that influence both *OPRM1* expression and heroin addiction.

Our findings were made possible by mapping putative cis-eQTL SNPs in prefrontal cortex, a highly relevant brain region when studying addiction and other psychiatric diseases (66). The prefrontal cortex is the center for mammalian purposive action including self-control/response inhibition, emotional regulation, flexibility/control of attention, and planning and goal formation. Dysregulation of this region can increase impulsivity and risk taking and stress reactivity and bias attention and reward anticipation toward immediate rather than delayed gratification, all characteristics associated with greater risk of addiction (66). Differences in prefrontal cortex activity have been observed in numerous human brain imaging studies comparing healthy control subjects with those with heroin/opioid or other drug addictions (66).

Limitations of our study include reliance on SNP genotyping and gene expression microarrays for the cis-eQTL mapping means that structural or rare genetic variants were not captured, coverage of common genetic variants was more complete for the participants of European ancestry compared with the African Americans, and only a single *OPRM1* probe was represented. Future studies capturing more complete genetic variation and more localized expression patterns and measuring other brain regions relevant to addiction (67) may lead to the identification of other important regulatory polymorphisms associated with heroin addiction.

When testing the putative cis-eQTL SNPs for association with heroin addiction, many of the cases used in our study were derived from the UHS, where no study control subjects were available, and they were compared with a set of population control subjects obtained via dbGaP. This study design enabled us to create a large sample size for heroin addiction association testing. Stringent quality control was implemented to reduce the chances of introducing artifactual biases, and our top findings were corroborated in independent cohorts including DSM-IV assessed cases and study control subjects. Greater power for identifying variants associated specifically with heroin addiction could be achieved with a large sample of control subjects who had used heroin but never became addicted. No such large sample of exposed but never addicted control subjects exists, but their absence does not diminish the observed associations of rs3778150 and other intron 1 SNPs with heroin addiction.

A more complete understanding of the biological factors that underlie the risk of developing heroin addiction is needed to combat its high prevalence and societal impact. To date, no specific genetic variants have been conclusively identified despite much research focused on *OPRM1* and its missense SNP rs1799971. Our hypothesis-generating approach of converging cis-eQTL mapping and SNP-disease association testing led to the identification of minor alleles at two *OPRM1* intron 1 SNPs (rs3778150 and rs3823010) that are consistently associated with increased risk of heroin addiction across independent cohorts with p values that even exceed the stringent genome-wide significance threshold ($p < 5 \times 10^{-8}$). Our findings highlight the importance of *OPRM1* intron 1 and its underlying haplotype structure that provides an explanation for the widely studied but largely inconsistent association observed for rs1799971, giving credence to the prior suggestion that haplotypes carrying noncoding regulatory SNPs may explain the rs1799971 inconsistencies (19,64). Consideration of

these intronic SNPs and their regulatory effects on *OPRM1* expression in disease-relevant human brain tissues is needed to better understand the role of *OPRM1* in influencing risk of heroin and other addictions.

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