Addiction Biology

HUMAN GENETIC STUDY

doi:10.1111/j.1369-1600.2012.00445.x

Association of *OPRD1* polymorphisms with heroin dependence in a large case-control series

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ABSTRACT

Genes encoding the opioid receptors (*OPRM1, OPRD1* and *OPRK1*) are obvious candidates for involvement in risk for heroin dependence. Prior association studies commonly had samples of modest size, included limited single nucleotide polymorphism (SNP) coverage of these genes and yielded inconsistent results. Participants for the current investigation included 1459 heroin-dependent cases ascertained from maintenance clinics in New South Wales, Australia, 1495 unrelated individuals selected from an Australian sample of twins and siblings as not meeting DSM-IV criteria for lifetime alcohol or illicit drug dependence (non-dependent controls) and 531 controls ascertained from economically disadvantaged neighborhoods in proximity to the maintenance clinics. A total of 136 *OPRM1, OPRD1* and *OPRK1* SNPs were genotyped in this sample. After controlling for admixture with principal components analysis, our comparison of cases to non-dependent controls found four *OPRD1* SNPs in fairly high linkage disequilibrium for which adjusted *P* values remained significant (e.g. rs2236857; OR 1.25; $P = 2.95 \times 10^{-4}$) replicating a previously reported association. A *post hoc* analysis revealed that the two SNP (rs2236857 and rs581111) GA haplotype in *OPRD1* is associated with greater risk (OR 1.68; $P = 1.41 \times 10^{-5}$). No *OPRM1* or *OPRK1* SNPs reached more than nominal significance. Comparisons of cases to neighborhood controls reached only nominal significance. Our results replicate a prior report providing strong evidence implicating *OPRD1* SNPs and, in particular, the two SNP (rs2236857 and rs581111) GA haplotype in liability for heroin dependence. Support was not found for similar association involving either *OPRM1* or *OPRK1* SNPs.

Keywords Association study, case-control, heroin dependence, *OPRD1*, *OPRK1*, *OPRM1*.

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INTRODUCTION

Opioid dependence remains a major societal problem worldwide (Degenhardt *et al.* 2004). Family and twin studies have established that a substantial component of risk for this disorder is attributable to genetic factors (e.g. Merikangas *et al.* 1998; Tsuang *et al.* 1998). As with other complex traits, it has proved challenging to determine the specific genes responsible for this contribution.

Single nucleotide polymorphisms (SNPs) in the opioid receptor genes (*OPRM1*, *OPRD1* and *OPRK1*) are obvious candidates for involvement in liability for heroin dependence. *OPRM1*, which encodes the mu-opioid receptor (MOR) at which heroin and other commonly used opioids exert their primary effects (e.g. analgesia, reward and dependence; Le Merrer *et al.* 2009) has understandably been the most highly investigated of these genes. 118 A > G (rs1799971) is an exonic *OPRM1* SNP that results in an amino acid substitution (Bond *et al.* 1998), which reportedly reduces mRNA expression (Zhang *et al.* 2005) and alters stress responsivity (Wand *et al.* 2002). Initial excitement over reported association of this seemingly ideal candidate with heroin dependence (e.g. Bart *et al.* 2004) has been tempered by subsequent

findings. Although meta-analyses (Glatt *et al.* 2007; Coller *et al.* 2009) have concluded that the preponderance of evidence does not support a significant role in opioid dependence liability for this SNP, its potential involvement in the pathophysiology of other addictive disorders continues to be actively investigated (Ray *et al.* 2011). Overall, no associations involving *OPRM1* SNPs have been consistently replicated; preliminary findings include reports of association with a greater positive response to heroin (Zhang *et al.* 2007) and risk for heroin dependence (Zhang *et al.* 2006; Levran *et al.* 2008).

More recently, investigators have turned their attention to *OPRD1* and *OPRK1* (Levran *et al.* 2008; Zhang *et al.* 2008). Opioid drugs bind delta opioid receptors (DORs), but with a much lower affinity than MORs. DORs play an important role in the development of opioid tolerance (Daniels *et al.* 2005) and are involved in the rewarding and analgesic effects of opioids (Le Merrer *et al.* 2009). Overall, kappa opioid receptor (KOR) actions are commonly in opposition to those of MORs. Administration of KOR agonists results in conditioned place aversion rather than conditioned place preference (CPP; Le Merrer *et al.* 2009). KORs may play a major role in the dysphoria experienced with opioid withdrawal (Le Merrer *et al.* 2009).

Several early association studies (e.g. Franke *et al.* 1999) that focused on coding *OPRD1* SNPs in small samples produced largely negative findings. A more recent examination (Zhang *et al.* 2008) of opioid and other substance dependence observed significant opioid dependence risk associated with rs1042114, a coding SNP in *OPRD1*, but not with other *OPRD1* or *OPRK1* SNPs. Another investigation (Levran *et al.* 2008) that had a larger sample ($n = 412$) of heroin-dependent cases and used Goldman's 'addiction chip' (Hodgkinson *et al.* 2008) reported putative association with three SNPs in *OPRD1* and one in *OPRK1*.

The current investigation examines whether *OPRM1*, *OPRD1* and *OPRK1* SNPs are associated with risk for heroin dependence in a very large Australian sample of cases ($n = 1459$) receiving maintenance treatment for heroin dependence in New South Wales (NSW) and non-dependent controls ($n = 1495$ unrelated individuals) ascertained from samples of twins and family members as not meeting DSM-IV criteria for lifetime alcohol or illicit drug dependence. The substantially larger sample size provides an opportunity to investigate more definitively the involvement of these genes. Cases are also compared to a second control group ($n = 531$), termed neighborhood controls, ascertained from economically disadvantaged neighborhoods in close proximity to maintenance clinics. Because these individuals have higher rates of lifetime drug use and nicotine, alcohol and non-opioid illicit drug dependence, this comparison can provide insight into the degree to which association findings are specific for heroin dependence or shared with other addiction phenotypes.

MATERIALS AND METHODS

The Comorbidity and Trauma Study (CATS), a collaboration of investigators at Washington University School of Medicine, the Queensland Institute of Medical Research (QIMR) and the National Drug and Alcohol Research Centre of the University of New South Wales, examined genetic and environmental factors contributing to liability for heroin dependence using a case-control design. A description of the methods used for data collection has been given in previous reports of phenotypic data (Shand *et al.* 2010). In addition to case and control subjects recruited after funding was obtained, we include here 25 cases and 25 neighborhood controls from the CATS pilot study for whom other protocols were identical and assessment was comparable.

Participants

Cases were recruited from clinics providing opioid replacement therapy (ORT) in the greater Sydney region. Prior to enrollment in ORT, NSW guidelines require an extensive clinical assessment documenting that the individual meets DSM-IV diagnostic criteria for opioid dependence and is suitable for the form of maintenance treatment recommended. For cases, inclusion criteria were age 18 years or older, an adequate understanding of English and current or past participation in ORT consisting of either methadone or buprenorphine for heroin dependence. Participants reporting recent suicidal intent or known to be currently experiencing psychosis were excluded. Neighborhood controls were recruited from geographic areas in proximity to ORT clinics. The use of opioids recreationally more than 10 times lifetime was an exclusion criterion for controls; the inclusion and exclusion criteria except for ORT participation were otherwise identical to cases. Written informed consent was obtained from all participants. Institutional review board (IRB) approval was obtained from the University of New South Wales, Washington University, the Queensland Institute of Medical Research and the area health service ethics committees governing the participating clinics. Participants were reimbursed AU$50.00 for out-of-pocket expenses.

Interim analyses of phenotypic data revealed high rates of licit and non-opioid illicit drug dependence in neighborhood controls (41.6%, 28.1% and 36.0% for nicotine, alcohol and illicit drug dependence, respectively in the final sample), which raised concerns that comparisons with these individuals would be ill suited to identify

genetic variants associated with liability that is shared across classes of drug dependence. These concerns prompted a decision to revise the study design to include a non-dependent control group of unrelated individuals selected for the study's primary genotypic analyses from a large Australian Twin Registry (ATR) pool of twins and family members (see Hansell *et al.* 2009 for a description of this sample). Inclusion criteria for this group included an adequate DNA supply available and existing IRB approval allowing additional genotyping for substance dependence and related phenotypes. Exclusion criteria were lifetime diagnoses of any DSM-IV illicit drug or alcohol dependence at prior interview. Individuals without a lifetime DSM-IV diagnosis of nicotine dependence were also preferentially selected. The resulting non-dependent control sample ($n = 1500$) had a lifetime prevalence of nicotine dependence (12.5%) below that of the Australian general population.

Assessment

All participants completed semi-structured diagnostic interviews. Interviews for case and neighborhood control participants were completed in person; interviews for twin sample controls were completed via telephone. Substance dependence diagnostic sections of the interview were based on the Semi-Structured Assessment for the Genetics of Alcoholism—Australia (Bucholz *et al.* 1994) enabling DSM-IV and DSM-III-R lifetime diagnoses of opioid, cannabis, sedative, stimulant, cocaine and alcohol abuse and dependence. The nicotine dependence section of the interview was modified from the Nicotine Addiction Genetics Study (Hansell *et al.* 2009) assessment which was derived from the World Health Organization Composite International Diagnostic Interview (CIDI; Andrews & Peters 1998). Similar diagnoses were obtained for CATS pilot project participants (25 cases and 25 neighborhood controls) via the CIDI (Andrews & Peters 1998).

Marker selection

The pair-wise option of Tagger (de Bakker *et al.* 2005), implemented in Haploview (Barrett *et al.* 2005), was applied to HapMap European ancestry panel data using a threshold of $r^2 \geq 0.8$ for most genes and a higher threshold (≥ 0.9) for high-priority candidates (e.g. opioid receptors) to select a custom set of 1536 SNPs that provided coverage of 72 candidate genes and 47 additional SNPs for which prior studies reported association. A set of 30 ancestry-informative markers (AIMs) distributed physically across the genome was selected from SNPs for which the greatest allele frequency differences were found between populations with European and East Asian ancestry in Hapmap2 data for use in principal components analyses (PCAs). As summarized above, because SNPs from opioid receptor genes are obvious first-pass candidates, we prioritized examining these SNPs in the first stage of a two-stage analytic process (the remaining SNPs will be analyzed in stage 2). Data are thus reported here for the 142 SNPs genotyped in the opioid receptor genes: *OPRM1*, *OPRD1* and *OPRK1* (for details, see Table 1, Supporting Information Tables S1 and S2). We provide data cleaning information below for the entire set of markers since we utilized all retained SNPs to generate the principal components (PCs) used to control for admixture.

Genotyping

Genotyping was performed on an Illumina BeadStation using the GoldenGate technology (Peters *et al.* 2008). DNA samples from CEPH trio 1334 (obtained from the Coriell Cell Repository) served as internal controls for quality of clustering and reproducibility. The primary analysis of the genotyping data with the Illumina BeadStudio software was followed by visual inspection and assessment of data quality and clustering.

Statistical analyses

Data cleaning

Data were excluded from one individual (an ATR control) whose DNA did not genotype successfully. Initial quality control found 23 SNPs for which genotyping failed (i.e. Gencall score = 0). An additional nine SNPs that had a call rate below 95% were removed from further analyses. Genotypic data revealed the presence of 32 duplicate samples; further scrutiny found that these individuals had participated in the project more than once. For individuals who had participated both in the pilot and funded phases of the project, interview data from the funded phase were retained. For those who participated multiple times in the funded project, data from the first interview were used for analyses (case-control status was consistent throughout). Data from an additional three participants were excluded on the basis of mismatch of genotypic and phenotypic gender. Data from an additional 47 SNPs with minor allele frequency (MAF) less than 2% were not included in analyses. Twenty-seven SNPs were removed from further analyses because deviations from Hardy–Weinberg equilibrium in ATR controls exceeded Bonferroni correction for the total number of remaining SNPs ($0.05/1457 = 3.43 \times 10^{-5}$). An examination for cryptic relatedness found 25 instances in which the calculated proportion of shared alleles identical by descent exceeded 0.5, indicative of greater sharing than expected for unrelated individuals; in each case, the participant with the higher

Table 1 *OPRD1* SNP features and association with heroin dependence—comparison of cases ($n = 1459$) to non-dependent controls ($n = 1495$).

SNP	*Location*[a]	*Classification*	*Minor allele*	*Minor allele freq*		*P value*	*Odds ratio (95% confidence interval)*
				Cases	*Controls*		
rs569356	29136686	Flank 5′ UTR	G	0.133	0.146	0.48	0.95 (0.81–1.10)
rs1042114	29138975	Cod non-syn	C	0.133	0.146	0.52	0.95 (0.82–1.11)
rs2236861	29139756	Intron 1	A	0.247	0.231	0.030	1.15 (1.01–1.30)
rs204047	29145124	Intron 1	A	0.190	0.181	0.15	1.11 (0.97–1.26)
rs678849	29145188	Intron 1	G	0.473	0.458	0.053	1.11 (1.00–1.24)
rs419335	29151844	Intron 1	G	0.341	0.311	0.00112	1.20 (1.08–1.35)
rs204055	29159373	Intron 1	A	0.473	0.458	0.00998	1.15 (1.03–1.28)
rs2236857	29161609	Intron 1	G	0.273	0.243	**0.000295**	**1.25** (1.11–1.41)
rs2236855	29161999	Intron 1	A	0.273	0.243	**0.000295**	**1.25** (1.11–1.41)
rs760589	29162465	Intron 1	A	0.337	0.309	0.00109	1.20 (1.08–1.35)
rs2298897	29165837	Intron 1	C	0.283	0.254	**0.000469**	**1.24** (1.10–1.39)
rs3766951	29169559	Intron 1	G	0.340	0.307	**0.000411**	**1.22** (1.09–1.37)
rs529520	29174946	Intron 1	A	0.470	0.445	0.00193	1.18 (1.06–1.32)
rs581111	29175373	Intron 1	A	0.276	0.263	0.045	1.13 (1.00–1.27)
rs680090	29175461	Intron 1	A	0.479	0.490	0.016	0.88 (0.79–0.98)
rs12749204	29176213	Intron 1	G	0.194	0.186	0.09	1.12 (0.98–1.28)
rs2298895	29178924	Intron 1	T	0.052	0.045	0.18	1.18 (0.92–1.51)
rs508448	29181525	Intron 1	G	0.457	0.460	0.18	0.93 (0.84–1.03)
rs4654327	29190138	3′ UTR	G	0.477	0.471	0.24	1.07 (0.96–1.18)
rs204076	29190390	Flank 3′ UTR	T	0.351	0.362	0.86	1.01 (0.91–1.13)
rs204069	29194818	Flank 3′ UTR	A	0.352	0.356	0.43	1.05 (0.94–1.17)

[a]NCBI build 37.2.
cod = coding; flank = flanking; non-syn = non-synonymous; UTR = untranslated region.

project identifier number was excluded. For the final sample, the mean call rate for 136 opioid receptor SNPs that remained after data cleaning exceeded 99.9%.

The current report's primary analyses compared 1459 heroin-dependent cases [888 male; 571 female; mean age 36.5 (SD 8.6)] to 1495 non-dependent controls [972 male; 523 female; mean age 45.0 (SD 9.5)]; cases were compared to 531 neighborhood controls [235 male; 296 female; mean age 34.7 (SD 10.5)] in additional analyses aimed at identifying SNPs with effects specific to heroin dependence liability.

Admixture

PCAs were conducted with data from cases and ATR controls using the smartpca program in the Eigensoft 3.0 package (Patterson, Price & Reich 2006). Due to the dense coverage of high-priority candidate genes, the kill r^2 option was set at 0.8 for these analyses. Our inclusion of data from AIMs in these analyses prevented using Tracy-Widom statistics to determine the number of PCs to be used as covariates. As such, we opted to include all PCs for which case-control differences reached at least a trend level of significance. *Post hoc* analyses, which included a larger number of PCs (10) as covariates, were performed to demonstrate that our primary findings remained significant despite this additional control for admixture (see limitations).

Association

Single Nucleotide Polymorphism Spectral Decomposition (Nyholt 2004; Li & Ji 2005) was first used to calculate the appropriate correction for multiple testing for analyses of data for the 136 opioid receptor SNPs that remained after data cleaning. As the respective linkage disequilibrium (LD) plots demonstrate (see Fig. 1, Supporting Information Figs S1 and S2), we genotyped a number of SNPs in high LD for these genes. Based on the calculated effective number of independent loci markers (57.69), the significance threshold necessary to keep the Type 1 error rate at 5% was determined to be 8.89×10^{-4}. Association analyses were then performed using PLINK (Purcell *et al.* 2007). Logistic regression, including the smartpca-derived PCs in the model as independent variables to control for admixture, was used to examine the association between the log-additive effects of minor allele dosage and status (case versus non-dependent control). Although PC covariates were not needed for the secondary comparisons of cases versus neighborhood controls (see PCA results below), logistic regression was again used to obtain results comparable to the above.

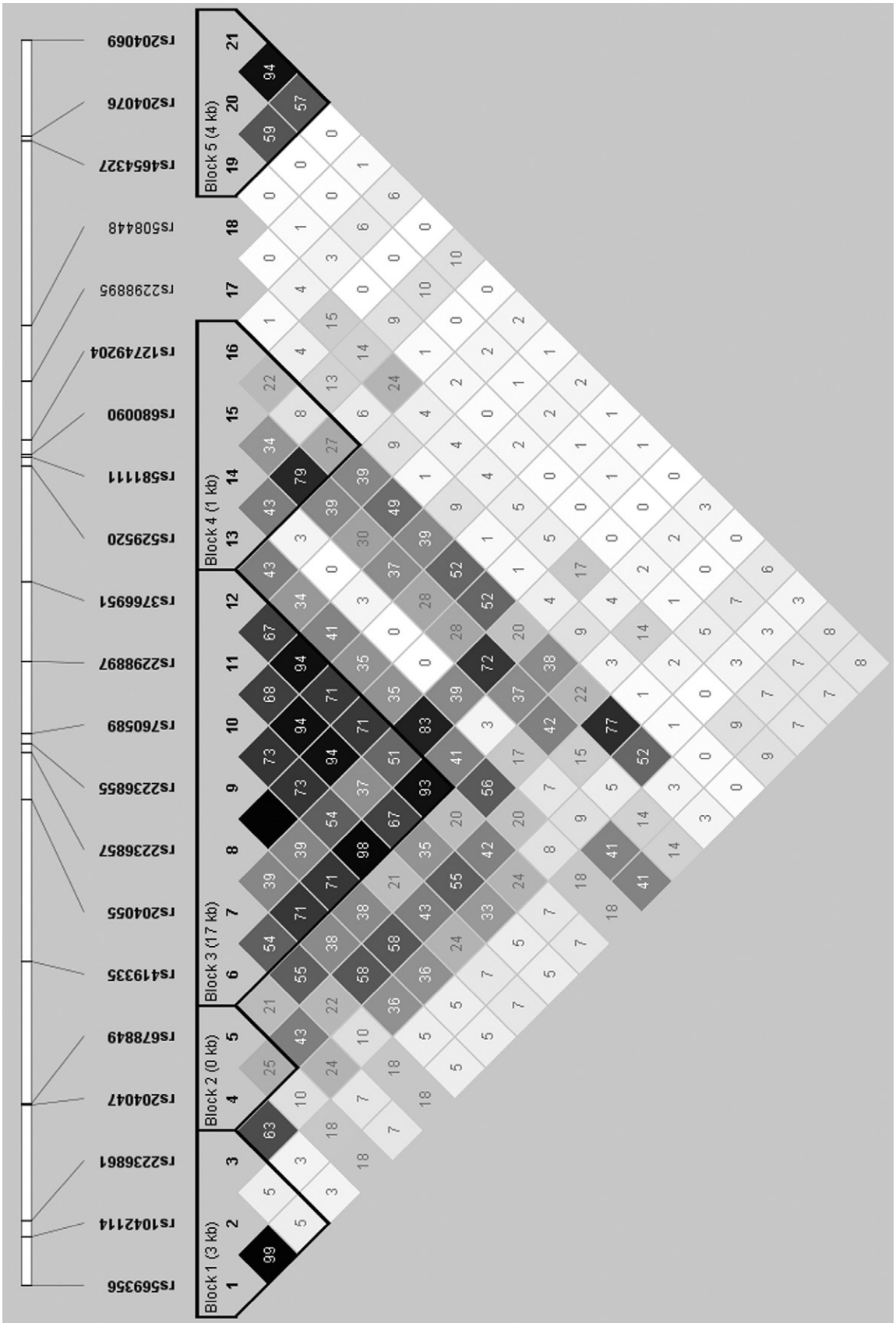

Figure 1 Linkage disequilibrium analysis of *OPRD1* SNPs (r^2 values are shown)

Several *post hoc* analyses were undertaken to provide a better understanding of our primary findings. An additional logistic regression comparing cases and non-dependent controls was performed to control for the allelic dosage of the most significant *OPRD1* SNP using the condition option in PLINK to examine whether evidence of more than a single association signal was found. Based on the results of this analysis, the haplotype command in PLINK was used to assess risk associated with a single 2 SNP *OPRD1* haplotype. Finally, consistent with the exclusion criteria for the non-dependent controls, we divided neighborhood controls into those who did not meet criteria for dependence on alcohol or a non-opioid illicit (i.e. cannabis, stimulants, sedatives or cocaine) drug ($n = 275$) and those who did ($n = 256$). We then separately compared cases to each of these groups to estimate the risk for heroin dependence risk associated with the most significant *OPRD1* SNP.

RESULTS

The PCA retained data from 1113 of the 1457 SNPs (including AIMs) remaining after data cleaning. As case-non-dependent control differences reached at least a trend level of significance for four PCs, a conservative decision was made to include all four PCs as covariates in comparisons of these groups. The respective *P* values for population differences along these eigenvectors were 1.1×10^{-12}, 2.4×10^{-11}, 1.1×10^{-4} and 0.064. Although both populations are primarily of European ancestry, the various combinations of PCs 1, 2 and 3 (see scatter plots in Supporting Information Fig. S3a–c) identify individuals of Asian ancestry (i.e. mapping to Han Chinese and Japanese HapMap populations) more prevalent among cases. PC4 appears to be identifying a Northern European ancestry subgroup. A similar examination in cases and neighborhood controls found no significant or trend level population differences and thus no covariates were used for those comparisons.

Unadjusted MAFs and the results of association analyses for *OPRD1* SNPs comparing cases to non-dependent controls are displayed in Table 1. Associations of at least nominal significance are seen for 10 of the 21 *OPRD1* SNPs. For four SNPs (for which pairwise r^2 ranged from 0.68 to 1.0, see Fig. 1), the *P* values are less than the significance threshold calculated to correct for multiple comparisons. Based on the LD relationships of the *OPRD1* SNPs, it appears likely that these results could largely be explained by a single strong association signal (minimal *P* value $= 2.95 \times 10^{-4}$ for rs2236857 and rs2236855). To test this assertion, association for those *OPRD1* SNPs of at least nominal significance was reexamined, conditioned on rs2236857 allelic dosage. The only residual signal observed was for rs581111, for which only mild attenuation was found ($P = 0.059$), consistent with the lack of LD ($r^2 = 0$) for this SNP with either rs2236857 or rs2236855. A *post hoc* two locus association analysis of rs2236857 and rs581111 (Table 2) found that the GA haplotype consisting of the coupled minor alleles (prevalence 0.077) is more strongly associated with an odds ratio (OR) of 1.68 ($P = 1.41 \times 10^{-5}$).

Table 2 Association of *OPRD1* two SNP (rs2236857 and rs581111) haplotype with heroin dependence—comparison of cases ($n = 1459$) to non-dependent controls ($n = 1495$).

Haplotype	*Frequency*	*Odds ratio*	*P value*
GA	0.077	1.68	1.41×10^{-5}
AA	0.193	0.98	0.73
GG	0.181	1.13	0.10
AG	0.550	0.84	1.85×10^{-3}

Association analyses comparing cases to non-dependent controls for SNPs in the other opioid receptors are most noteworthy for the dearth of findings reaching even nominal significance. Of the 93 *OPRM1* SNPs, only rs10485058 attained this minimal standard [$P = 0.045$; OR = 0.86 (95%CI 0.74–1.00)]. Nominal significance was found for only 2 of the 22 *OPRK1* SNPs: rs12548098 [$P = 0.016$; OR = 0.85 (95%CI 0.74–0.97)] and rs7826614 [$P = 0.015$; OR = 0.84 (95%CI 0.74–0.97)].

The results of additional analyses comparing cases and neighborhood controls found no association for an *OPRD1* SNP that reached even nominal significance; the ORs for rs2236857 and rs2236855 were 1.09 ($P > 0.28$). For a relatively small number of *OPRM1* and *OPRK1* SNPs, associations of nominal significance were found (Supporting Information Table S3); however, none was within an order of magnitude of the *P* value threshold required to correct for multiple testing.

Neighborhood controls were divided into those who were dependent on either alcohol or a non-opioid illicit drug ($n = 256$) and those who were not ($n = 275$), consistent with the inclusion criteria for non-dependent control group. The ORs for association of rs2236857 and rs2236855 (identical due to complete LD) with heroin dependence in respective comparisons of cases to neighborhood controls, with and without substance dependence diagnoses, were 1.21 ($P = 0.08$) and 1.00 ($P = 0.98$).

DISCUSSION

Our investigation compared a large sample of heroin-dependent cases to individuals with no history of illicit drug or alcohol dependence and found a strong association of four *OPRD1* SNPs with heroin dependence. The

estimates of risk (odds ratio 1.25) for the two most highly associated SNPs, rs2236855 and rs2336857, are of similar magnitude to those observed with other complex traits. The degree of LD observed between these SNPs and most of the other nominally associated *OPRD1* polymorphisms is consistent with a single strong signal. These results replicate the findings of a prior report (Levran *et al.* 2008), which also used Illumina GoldenGate technology to genotype 1536 SNPs (including *OPRM1*, *OPRD1* and *OPRK1* polymorphisms) in similarly ascertained cases (412 heroin-dependent individuals on methadone maintenance therapy in the United States and Israel) and controls (184 individuals screened as having no substantial history of illicit drug or alcohol use). The nine SNPs that were most strongly associated with heroin dependence in their sample included three *OPRD1* SNPs, all of which were genotyped in the current report (see Supporting Information Table S4). Two (rs2236857 and rs3766951) were among the four SNPs in our sample for which corrected *P* values were significant; the third (rs2236861) reached only nominal significance. The agreement observed in the *OPRD1* findings across these reports is extremely encouraging. Our results contrast with those of another group (Zhang *et al.* 2008) who observed a significant association with rs1042114 after correction for multiple testing and no significant association involving either of our two most significantly associated SNPs (rs2236857 or rs2236855). Differences in design may have contributed to the divergence in findings. Their sample's 103 opioid-dependent cases included individuals recruited from settings other than ORT clinics including some who were dependent on opioids other than heroin. They genotyped samples using other methods (either polymerase chain reaction restriction fragment length polymorphism or the TaqMan technique). Finally, they report *P* values for case-control comparisons involving rs2236855 and rs2236857 that, while non-significant, differ substantially in magnitude, surprising given that these two SNPs are in complete LD (i.e. $r^2 = 1$) in both our sample and in all HapMap populations.

The four *OPRD1* SNPs that we found to be most strongly associated with heroin dependence are all located in the first intron of this gene. Although these SNPs are not located at a branch point and do not alter either a splice enhancer or silencer, there is some evidence supporting potential epigenetically mediated functionality. rs2236855 has a high level of evolutionary conservation (PhyloP Score = 1.14) and is located within a DNase I hypersensitivity site in many, but not all, cell types (Fujita *et al.* 2011) suggesting it could be a cell type-specific regulatory element. Both rs2236857 and rs2298897 are located within interspersed repeats: the former in a long interspersed element and the latter in a long terminal repeat (Fujita *et al.* 2011). The *post hoc* examination of the two SNP haplotype (rs2236857 and rs581111), prompted by the limited attenuation of association signal for rs581111 (also located in intron 1) in an analysis conditioned on the most strongly associated SNP, found evidence of an association stronger by more than an order of magnitude (uncorrected $P = 1.41 \times 10^{-5}$). These results are suggestive of a non-genotyped SNP with a less prevalent minor allele (the GA haplotype frequency is 0.077), associated with substantial heroin dependence risk (OR 1.68), the identification of which could likely be achieved using currently available approaches such as deep sequencing. Given that this polymorphism is apparently located in an intronic region, its effects may involve altered *OPRD1* expression (e.g. see Lomelin, Jorgenson & Risch 2010). Ongoing research examining the regulation of *OPRD1* expression (Tuusa, Leskelä & Petäjä-Repo 2010) and DOR trafficking (Bie *et al.* 2010) will also help guide further investigation.

In marked contrast to our *OPRD1* findings, our results do not provide support for association with heroin dependence involving *OPRM1* and *OPRK1* SNPs. The results of our study and multiple meta-analyses do not support an association of rs1799971 with heroin dependence. We also failed to replicate the association of two *OPRM1* and one *OPRK1* SNP reported by Levran *et al.* (2008) although our comparison with neighborhood controls found a nominal association with rs3778151. Considering the additional power provided by our considerably larger sample and the lack of consistently replicated findings for polymorphisms in these genes, our results suggest that it may be prudent to focus attention preferentially in future association studies on *OPRD1* over the other two opioid receptor genes. However, it remains possible that significant heroin dependence risk associated with *OPRM1* and *OPRK1* SNPs was not detected in the current study because of small effect size, low MAF in our sample, greater variability across populations of differing ethnicity for some of these SNPs (e.g. rs6473797, rs3778151) or our failure to genotype the specific risk-associated SNPs. Given the well-documented involvement of MORs and KORs in the effects of opioids (including important aspects of dependence, e.g. see Christie 2008) and the close functional interrelationships of these three receptors in which heterodimerization may be integrally involved (Ferré & Franco 2010; von Zastrow 2010), they remain extremely important targets for other avenues of research in order to improve current understanding of the pathophysiology of heroin dependence as well as treatment of this disorder.

Despite decades of active investigation, relatively basic questions about opioid receptors remain unanswered. The distinct pharmacologic profiles of the various opioid receptors are fairly well characterized; their molecular

basis, including whether they are homomeric or heteromeric, remains unclear (van Rijn & Whistler 2009). For example, researchers have variously proposed that the DOR1 is actually a heterodimer composed of DOR and either MOR (van Rijn & Whistler 2009) or KOR (Bhushan *et al.* 2004) subunits. Similarly, a provocative report (Yekkirala, Kalyuzhny & Portoghese 2010) found that the affinity of opiate agonists at MOR-DOR heteromers exceeded that at MOR homomers. DORS are primarily intracellular; chronic opioid use results in substantial translocation to cell membranes where they may form heteromers with MORs (von Zastrow 2010). Increased levels of MOR-DOR heteromers have been reported (Gupta *et al.* 2010) after chronic morphine administration. The formation of these MOR-DOR heteromers has recently been implicated (He *et al.* 2011) as playing a major role in opioid tolerance. DOR antagonists have been shown to block the sensitization to the conditioned rewarding effects of morphine that occurs with opioid pretreatment (Shippenberg, Chefer & Thompson 2009). A recent report (Billa, Xia & Morón 2010) found that administration of a DOR2 antagonist blocked morphine-induced CPP in rats and resulted in an increase in expression of the DOR dimer in the hippocampal postsynaptic density. These reports support a role for DORs in pathophysiology of opioid dependence, which may be at least partially mediated by altered expression. In fact, a recent report proposing the design of an opioid drug that causes reduced tolerance and dependence advocated for drug development focused on DOR/MOR heteromers (Berger & Whistler 2010). Although MORs and DORs are generally considered to have synergistic antinociceptive effects (Zhang & Pan 2010), opposing effects on other behaviors such as impulsivity have been reported in rodents (Olmstead, Ouagazzal & Kieffer 2009).

A possible alternative interpretation of our findings is suggested by a report (Zeller *et al.* 2010) that conducted genome-wide expression analyses using RNA extracted from peripheral blood monocytes of a community-based German sample in which a genome-wide association study had been completed. Among a total of 2745 expression quantitative trait loci (eqtl's) that had *P* values less than 5.78×10^{-12}, rs2236855 was found to be a cis-eqtl for two genes (*P* values shown): *PHACTR4* (7.57×10^{-27}) and *ATPIF1* (2.85×10^{-12}). Both are expressed in the brain and located in proximity (~300–600 kb) to *OPRD1* on chromosome 1. *PHACTR4* is thought to be a protein phosphatase-1 (PP-1) inhibitor (Allen *et al.* 2004). cAMP-regulated phosphoprotein (DARPP-32) mediates the action of multiple drugs of abuse (including opioids) by regulating striatal dopaminergic transmission via its actions as a inhibitor of PP-1 and protein kinase A (Svenningsson, Nairn & Greengard 2005). DARPP-32 has been implicated in maintenance of morphine-induced CPP (Narita *et al.* 2010) and suggested as a putative therapeutic target for opiate addiction (Mahajan *et al.* 2009). *ATPIF1*, a mitochondrial ATPase inhibitor, plays important and diffuse roles in energy regulation. Although the degree to which gene expression data from peripheral blood monocytes correlates with that of brain remains unclear, altered expression of *PHACTR4*, and perhaps *ATPIF1*, deserves consideration as an alternative route through which our findings could be explained.

Several limitations must be considered when interpreting our findings. Our cases were ascertained entirely from maintenance clinics in the Greater Sydney Area. Additional study may be needed to determine whether similar results would be seen in samples not currently in treatment or from other areas. Non-dependent controls were interviewed via telephone; cases and neighborhood controls completed an in-person assessment. Given the low general population prevalence and extreme severity of heroin dependence, it seems highly unlikely that telephone administration, used at QIMR for more than 25,000 interviews to date, led to a substantial number of false negative diagnoses. Although both our cases and non-dependent controls included primarily individuals of European ancestry, the groups differed somewhat in ethnic composition with more Asians found among cases. It is possible that population stratification could have contributed to the significant differences that we observed. Since we found an excess of the same alleles among cases as observed in a prior report (Levran *et al.* 2008) in a predominately Caucasian sample with some Middle Eastern contribution, we consider this possibility unlikely. We also reran analyses increasing the number of PCs to control for admixture to 10 (from four), with little effect on results. Despite its widespread use as a method to control for multiple testing, spectral decomposition may be viewed by some as inadequately conservative. It is thus important to note that when a more stringent threshold such as a Bonferroni correction (i.e. $0.05/136 = 3.68 \times 10^{-4}$) is applied, our two most highly associated SNPs remain significant. Despite the considerably larger size of our sample (more than threefold larger than most prior association studies of heroin dependence), it is possible that we may have failed to detect significant associations because of limited power (i.e. Type 2 error). Similarly, the relatively smaller size of the neighborhood control sample and any sharing of risk with related phenotypes (e.g. other substance dependence or externalizing disorders) more prevalent in this group (than the non-dependent controls) could be contributing to the lack of significant differences found in this comparison. Although not significant, the point estimates (ORs 1.09) for rs2236855 and rs2236857 in the comparison of cases to neighborhood controls were

greater than unity suggestive of shared risk. Interestingly, the respective ORs for comparisons of cases to neighborhood controls, with and without substance dependence diagnoses, were 1.21 and 1.00, respectively. Although neither value is significant, the estimate for the comparison to substance-dependent neighborhood controls is fairly similar to that found for the case-non-dependent control comparison, a finding more suggestive of specificity of risk for opioid dependence. It is possible that other factors are protective against substance dependence in non-dependent neighborhood controls.

Our investigation provides further evidence that *OPRD1* polymorphisms are associated with risk for heroin dependence. Although the strongest observed signal involves intronic SNPs, support is found for potential functionality including either epigenetically mediated mechanisms (Fujita *et al*. 2011) or via rs2236855's status as an eqtl for other genes (Zeller *et al*. 2010). Our *post hoc* finding that greater risk is associated with the less-prevalent rs2236857-rs581111 haplotype suggests the importance of additional genotyping to determine the identity of an underlying functional polymorphism. The lack of significant association observed for any SNPs in *OPRM1* and *OPRK1* provides a striking contrast to the *OPRD1* findings. Overall, our results support prioritizing research aimed at increasing current understanding of the important role played by *OPRD1* in the pathophysiology of heroin dependence including ongoing efforts focusing on improved opioid drug design (Berger & Whistler 2010).

Acknowledgements

This work was supported by the National Institute on Drug Abuse (R01 DA017305 to ECN); support was also received from the National Drug and Alcohol Research Centre and the Australian National Health and Medical Research Council (to LD). The authors would like to thank Anthony Caracella for his work in sample receipt and preparation, Megan Campbell for project coordination and Lisa Bowdler and Sara Smith for their efforts in sample genotyping.

Conflicts of Interest

None of the authors have a financial or personal conflict of interest.

Authors Contribution

ECN, MTL, ACH, LD, NGM and GWM were responsible for the study concept and design. NW, AA, AJS assisted with data analysis and interpretation of findings. ECN drafted the manuscript. MTL, ACH, NW, AA, FLS, AKH, LW, AAT, AJS, PAFM, LD, NGM and GWM provided critical revision of the manuscript and contributed to its intellectual content. All authors critically reviewed content and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 Linkage disequilibrium analysis of *OPRM1* SNPs (r^2 values are shown).

Figure S2 Linkage disequilibrium analysis of *OPRK1* SNPs (r^2 values are shown).

Figure S3 Scatterplots depicting distributions of cases (diamonds) and non-dependent controls (squares) for combinations of PCs as follows: (a) panel1 PC1 (x axis) versus PC2 (y axis); panel 2 PC1 vs PC3; (b) panel 1 PC2 vs. PC3; panel 2 PC1 vs PC4. (c) panel 1 PC2 vs. PC4; panel 2 PC3 vs PC4. Outliers on the various combinations of PCs 1,2, and 3 are likely of Asian ancestry; PC4 appears to identify a Northern European ancestry subgroup.

Table S1 *OPRM1* SNP features.

Table S2 *OPRK1* SNP features.

Table S3 Association of *OPRM1* and *OPRK1* SNPs with heroin dependence in a comparison of cases with neighborhood controls.

Table S4 Comparison of association studies results for *OPRD1* SNPs and opioid dependence.