

ORIGINAL ARTICLE

Evidence of *CNIH3* involvement in opioid dependence

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Opioid dependence, a severe addictive disorder and major societal problem, has been demonstrated to be moderately heritable. We conducted a genome-wide association study in Comorbidity and Trauma Study data comparing opioid-dependent daily injectors ($N = 1167$) with opioid misusers who never progressed to daily injection ($N = 161$). The strongest associations, observed for *CNIH3* single-nucleotide polymorphisms (SNPs), were confirmed in two independent samples, the Yale-Penn genetic studies of opioid, cocaine and alcohol dependence and the Study of Addiction: Genetics and Environment, which both contain non-dependent opioid misusers and opioid-dependent individuals. Meta-analyses found five genome-wide significant *CNIH3* SNPs. The A allele of rs10799590, the most highly associated SNP, was robustly protective ($P = 4.30E-9$; odds ratio 0.64 (95% confidence interval 0.55–0.74)). Epigenetic annotation predicts that this SNP is functional in fetal brain. Neuroimaging data from the Duke Neurogenetics Study ($N = 312$) provide evidence of this SNP's *in vivo* functionality; rs10799590 A allele carriers displayed significantly greater right amygdala habituation to threat-related facial expressions, a phenotype associated with resilience to psychopathology. Computational genetic analyses of physical dependence on morphine across 23 mouse strains yielded significant correlations for haplotypes in *CNIH3* and functionally related genes. These convergent findings support *CNIH3* involvement in the pathophysiology of opioid dependence, complementing prior studies implicating the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate system.

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INTRODUCTION

Twin and family studies provide evidence of a genetic contribution to liability for opioid dependence with heritability estimates ranging from 40 to 60%.^{1–4} However, genetic association studies have produced few consistently replicated findings.⁵ One important factor contributing to this inconsistency is the lack of a definitive control population^{6,7} for these investigations.

Studies, for example,⁸ have used unassessed controls based on the premise that, for low prevalence disorders, this approach only modestly reduces power.⁹ However, opioids are among the most highly addictive drugs^{10,11} with high rates of progression from misuse to dependence¹² and thus the main constraint on the prevalence of opioid dependence may be the prevalence of opioid misuse. The extent to which genetic factors contribute to liability at various stages of opioid addiction (for example, initiation, regular use and dependence), and are shared between stages, is not well characterized.⁵ Thus, the use of unassessed, predominately unexposed controls might be problematic for identifying genetic effects expressed after the initiation of opioid misuse. Importantly, significant effects of common SNPs manifesting at intermediate and later stages of addiction would be missed

in comparison with unexposed controls. Similarly, comparison with assessed, unexposed controls is more useful to examine shared liability for initiation and dependence.

Analyses of candidate gene data in the current report's discovery sample, the Comorbidity and Trauma Study (CATS), showed that association findings vary substantially depending on the comparison group's substance exposure status.^{6,7} The current investigation builds on this observation and draws from genetic studies of licit drugs^{13–19} that have yielded well-replicated findings by comparing non-dependent, drug exposed to substance-dependent individuals. Unfortunately, no large samples of non-dependent opioid misusers have been collected. Our discovery^{6,7,20} and confirmation samples^{19,21–24} contain only modest numbers of non-dependent opioid misusers, but are currently the largest such samples with genome-wide association study (GWAS) data. We hypothesize that genetic polymorphisms in opioid misusers influence progression to the population's opioid dependence end point (OD_E). Our analyses of GWAS data observed the strongest association for cornichon family AMPA receptor auxiliary protein 3 (*CNIH3*) polymorphisms, findings which map nicely onto literature,^{25–29} supporting α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate system involvement in the pathophysiology of opioid dependence.

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MATERIALS AND METHODS

GWAS sample subjects and assessment

Detailed descriptions of CATS data collection have been reported.^{6,7,20} Opioid-dependent individuals, aged 18 or older, were recruited from opioid substitution therapy clinics in the greater Sydney region. Neighborhood controls, individuals with little or no lifetime opioid misuse, were recruited from socially disadvantaged neighborhoods in geographic proximity to opioid substitution therapy clinics. Written informed consent was obtained from all participants as approved by the institutional review boards of all participating institutions and clinics. Semi-structured psychiatric diagnostic interviews, a modified Semi-Structured Assessment for the Genetics of Alcoholism—Australia (SSAGA-OZ),³⁰ were completed in-person.

As 94.1% of the CATS^{6,7,20} opioid-dependent participants reported a period of daily injection, we operationalized having had such a period as the population's normative ODE. Comparisons of opioid-dependent individuals who differed on daily injection status found substantial phenotypic differences (Supplementary Table 1 and Supplementary Methods). Comparisons of dependent individuals who never injected daily to non-dependent opioid misusers revealed fewer significant differences. Our GWAS analyses compared the ODE group ($N=1167$ opioid-dependent daily injectors) with a group characterized as having opioid use with impeded progression (OUIP) that combined non-dependent opioid misusers ($N=88$; 69.3% reporting heroin use) and opioid-dependent individuals without a history of daily injection ($N=73$).

The Yale-Penn genetic studies of opioid, cocaine and alcohol dependence^{19,23,24} were recruited at five US sites. All participants gave written informed consent as approved by each site's institutional review board. We addressed design and assessment differences (Supplementary Tables 2 and 3, and Supplementary Methods) that prevented defining phenotypes identical to those in CATS by using an extreme discordant approach. We operationalized the ODE group as opioid-dependent individuals whose opioid use had been daily or near daily, included heroin and injection at least 100 times lifetime and the OUIP group as individuals reporting heroin use who met no lifetime DSM-IV opioid dependence criteria. We limited inclusion to European ancestry participants to examine confirmation in a sample of comparable ethnicity, retaining 643 ODE and 157 OUIP individuals for analysis.

The Study of Addiction: Genetics and Environment (SAGE)^{21,22} is an alcohol dependence GWAS that selected cases and controls from large investigations targeting non-opioid substance dependence. Each contributing institution's institutional review board approved the recruitment protocols. All participants provided written informed consent. As SAGE did not ascertain participants on the basis of opioid dependence, it included fewer severely dependent individuals and more participants of unclear affection status. We operationalized the ODE group as DSM-IV opioid dependence and the OUIP group as opioid misusers who met at most one dependence and no abuse criterion. Limiting inclusion to European ancestry participants, we retained 190 ODE and 319 OUIP individuals.

Genotyping and data cleaning

CATS samples were genotyped using the Illumina Human660W-Quad BeadChip at the Johns Hopkins Center for Inherited Disease Research (CIDR). For data cleaning details, see Supplementary Methods. The genotyping rate for the 470 296 SNPs that remained after data cleaning was 99.93%.

The Yale-Penn samples were genotyped on the Illumina HumanOmni1-Quad v1.0 microarray at CIDR and the Yale Center for Genome Analysis. SAGE samples were genotyped at CIDR using Illumina Human 1Mv1_C BeadChips. Genotypic data cleaning and quality control details have been reported for the Yale-Penn studies^{19,23,24} and SAGE.^{21,22}

Data analyses

Admixture. Principal component (PC) analysis was conducted using the SmartPCA program³¹ to provide additional admixture correction. Three PCs were generated via principal component (PC) analysis and included as covariates in the regression models. Similar methods were used in the Yale-Penn and SAGE data sets to generate PCs for inclusion in analyses (consistent with their prior publications).^{19,21,23,24}

SNP-based association. The genomic inflation factor for CATS data was calculated in PLINK³² based on the median χ^2 -value. Logistic regression analyses were performed in PLINK³² to examine the association between the log-additive effects of risk allele dosage and group status (ODE versus

OUIP) controlling for sex, age category and three PCs. Manhattan and quantile–quantile plots were constructed for results.

Association analyses of confirmation sample data were conducted consistent with prior reports. The Yale-Penn data were analyzed using logistic regression models embedded in generalized estimating equations to correct for correlations of data from related individuals with age, sex and three PCs included as covariates. Analyses of SAGE data were conducted in PLINK³² with contributing component study, age, sex and two PCs included as covariates.

Meta-analyses were performed using the inverse variance weighting approach of the METAL program.³³ The phenotypic variance in ODE status explained by rs10799590 was calculated³⁴ for the meta-analytic results using odds ratios, risk allele frequencies, and prevalence estimates ranging from 0.005 (population prevalence of heroin dependence)^{12,35,36} to 0.25 (approximate prevalence reported for heroin dependence among users).¹²

Epigenetic annotation

See Supplementary Methods.

Duke Neurogenetics Study (DNS)

See Supplementary Methods.

Participants

DNS participants were in good general health and provided informed written consent approved by the Duke University Medical Center institutional review board. The European ancestry subsample reported here consisted of 312 participants (age = 19.71 ± 1.23 years; 151 males; 65 with DSM-IV diagnoses).

Genotyping

DNS participants' DNA was isolated from saliva and genotyped with Illumina HumanOmniExpress BeadChips. The genotyping rate of rs10799590 was 1.0 and was within Hardy–Weinberg equilibrium $\chi^2 = 0.39$, $P = 0.53$.

DNS neuroimaging protocol blood oxygenation level-dependent functional magnetic resonance imaging (BOLD fMRI) paradigm

A widely used and reliable challenge paradigm was used to elicit amygdala reactivity. The paradigm consists of four task blocks requiring face-matching interleaved with five control blocks requiring shape-matching (see Supplementary Figure S1). All four facial expressions convey threat, ambiguity and/or novelty that robustly recruit corticolimbic circuitry that includes the amygdala.³⁷

Blood oxygenation level-dependent functional magnetic resonance imaging data analysis

Following the preprocessing steps, linear contrasts using canonical hemodynamic response functions were used to estimate amygdala habituation as the linear decrease over successive face-matching blocks (that is, block 1 > block 2 > block 3 > block 4). Follow-up analyses evaluated amygdala response differences in block 1 across genotype groups. Individual contrast images (that is, weighted sum of beta images) were used in second-level random effects models accounting for scan-to-scan and participant-to-participant variability to determine mean contrast-specific responses using one-sample t -tests. A voxel-level statistical threshold of $P < 0.05$, family-wise error corrected for multiple comparisons across the bilateral amygdala regions of interest, and a cluster-level extent threshold of 10 contiguous voxels was applied to these analyses. The bilateral amygdala regions of interest were defined using the automated anatomical labeling template. BOLD parameter estimates from maximal voxels in the right and left amygdala regions of interest exhibiting a main effect for the amygdala habituation contrast were extracted using the VOI tool in SPM8 and exported for regression analyses in SPSS (v.22). Extracting parameter estimates from clusters activated by our functional magnetic resonance imaging paradigm, rather than those specifically correlated with our independent variables of interest, precludes the possibility of any correlation coefficient inflation that may result when an explanatory covariate is used to select a region of interest.

Table 1. SNPs associated with OD_E in CATS participants (*P*-values < 1E-5)

Gene	SNP	Chr	Genomic coordinates	RA	Risk allele frequency			Odds ratio (95% confidence interval)
					OD _E N = 1167	OU _{IP} N = 161	<i>P</i> -value	
CNIH3	rs10799590	1	224822482	A	0.42	0.56	1.51E-6	0.55 (0.43–0.70)
CNIH3	rs12130499	1	224836514	T	0.42	0.56	1.15E-6	0.54 (0.43–0.70)
CNIH3	rs298733	1	224842251	A	0.42	0.57	1.53E-6	0.55 (0.43–0.70)
CNIH3	rs1436171	1	224881828	A	0.44	0.59	6.26E-7	0.54 (0.42–0.68)
CNIH3	rs1369846	1	224894095	C	0.38	0.54	9.42E-8	0.52 (0.41–0.66)
CNIH3	rs1436175	1	224908366	T	0.37	0.53	2.72E-8	0.50 (0.39–0.64)
STAB2	rs10861067	12	104038974	G	0.25	0.38	2.00E-6	0.55 (0.43–0.71)
STAB2	rs10778270	12	104045678	A	0.20	0.31	5.16E-6	0.55 (0.42–0.71)
Intergenic	rs9521590	13	110709426	A	0.05	0.11	4.31E-6	0.36 (0.23–0.55)
FUT8	rs6573615	14	66046534	G	0.36	0.49	9.68E-6	0.58 (0.46–0.74)

Abbreviations: CATS, Comorbidity and Trauma Study; OD_E, opioid dependence end point, OU_{IP}, opioid use with impeded progression.

Statistical analyses

Statistical analyses of the imaging data were conducted using linear regression in SPSS to test the association between rs10799590 A allele carrier status and amygdala habituation (that is, declining amygdala response to repeated stimuli). To maintain variability but constrain the influence of extreme outliers, all imaging variables were winsorized before analyses. Gender and psychiatric diagnosis (0,1) were entered as covariates for analyses. To determine whether rs10799590 was most strongly associated with amygdala habituation within this genomic region, SNPs ±100 kbp that had a linkage disequilibrium (LD) $r^2 \geq 0.50$ with rs10799590 within the data set were identified and binned according to LD ($r^2 \geq 0.80$). Similar analyses were then conducted to examine the association between carrier status of each SNP and amygdala habituation.

Genetic analysis of murine interstrain differences

Male mice, aged 7–8 weeks, from 23 inbred strains (Supplementary Table 5) were housed in Stanford University's animal care facilities. Experimental protocols were approved by the Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals. Details are provided in Supplementary Methods. Analysis of variance was used to calculate a *P*-value to assess the likelihood that the within-block genetic variation underlies the phenotypic distribution (that is, the mean number of jumps) observed for the inbred strains examined. Haplotype data were examined for seven genes: CNIH3, CNIH2, GRIA1, GRIA2, CACNG8, GRIP1 and DLG4. *P*-values were adjusted to control the false-discovery rate.³⁸

RESULTS

CATS GWAS

The quantile–quantile plot of association results (Supplementary Figure S2, Panel A) and the genomic inflation factor value ($\lambda = 1.01$) indicate an absence of test statistic inflation. The strongest association involves a cluster of chromosome 1 SNPs (Manhattan plot, Supplementary Figure S2, Panel B). The six most highly associated, all located in CNIH3 (Table 1), are in moderate to high LD ($r^2 = 0.35–0.97$); conditional analyses suggest they represent a single association signal (Supplementary Figure S2, Panel C). The association reached genome-wide significance (GWS) for rs1436175 ($P = 2.72E-8$; OR 0.50 (0.39–0.64)) with the risk allele halving the likelihood of progression to OD_E.

Confirmation of association findings

These analyses focused on the CNIH3 SNPs because of the substantially stronger association observed for these polymorphisms and the gene's obvious biological relevance. In the Yale–Penn data (Table 2), trend-level association was observed for three of the six SNPs examined. In the SAGE data, a stronger association was found for five of the six SNPs. In both data sets, all association signals were in the same direction as in the CATS.

Meta-analyses performed on these SNPs using data from the three samples found GWS association for five of the six CNIH3 SNPs (Table 2). The strongest meta-analytic association signal ($P = 4.30E-9$) was observed for rs10799590; the odds ratio (0.64 (95% confidence interval 0.55–0.74)) is indicative of the risk allele's robust protective effects. Rs1436175, which had the lowest *P*-value in the CATS, failed to reach meta-analytic GWS. This SNP has the lowest LD with the other CNIH3 SNPs ($r^2 = 0.35–0.54$) and the largest heterogeneity χ^2 ($P = 0.002$). The meta-analytic GWS SNPs are in high LD (Supplementary Figure S2, Panels C and D).

Epigenetic annotation

The GWS CNIH3 SNPs are intronic and not highly conserved. Although the observed associations may be due to high LD with a non-genotyped variant, no exonic SNPs in high LD were identified. Epigenetically mediated changes in gene expression, which have been reported to occur with opioid use,³⁹ are plausible mechanisms for functional associations involving intronic SNPs. Rs10799590 is located within an enhancer that is specific to fetal brain (Figure 1). It is within an H3K4me1 peak in fetal brain that DNase hypersensitivity data indicate is in an open chromatin state.^{40–42} It is predicted that rs10799590 is within the binding site of transcription factor TAL1 (which has important roles in middle brain GABAergic neuron differentiation); the G allele has significantly higher binding potential than the A allele (Supplementary Table 6).

Protective allele carrier status predicts greater amygdala habituation

The observation²⁵ that similar changes in α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) GluA1 subunits occur in the amygdala with opioid addiction²⁶ and fear conditioning,^{43–45} coupled with evidence of epigenetically mediated alterations in gene expression that ensue in both processes after environmental exposures,^{43,46} provided the rationale for examining amygdala habituation to threat-related facial expressions (a reliable intermediate phenotype linked to psychopathology).^{46,47} After accounting for sex and the presence of a DSM-IV disorder, rs10799590 A allele carrier status predicted right (stand Beta = 0.147; $\Delta F_{1,308} = 6.93$, $P < 0.009$, $\Delta R^2 = 0.022$; Figure 2), but not left (stand Beta = 0.031; $\Delta F_{1,308} = 0.302$, $P > 0.582$, $\Delta R^2 < 0.001$), amygdala habituation. G allele homozygotes ($n = 102$) had blunted right amygdala habituation (0.045 ± 0.374) relative to A allele carriers ($n = 210$; 0.164 ± 0.371). We identified three genotyped SNPs (rs1369848, rs12730234, rs1965776) in moderate LD with rs10799590 that tagged SNP blocks; however, none was more strongly associated with amygdala habituation than rs10799590 (Supplementary Table 7). Follow-up analyses revealed that genotype groups did not differ in

Table 2. Association of CNIH3 SNPs with OD_E in CATS, Yale-Penn and SAGE data, and in meta-analysis

SNP	CATS (1167 OD _E vs 161 OU _{IP})		Yale-Penn (643 OD _E vs 157 OU _{IP})		SAGE (190 OD _E vs 319 OU _{IP})		Meta-analysis		
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	Het X ² (2df) P-value
rs10799590	0.55 (0.43–0.70)	1.51E-6	0.78 (0.60–1.01)	5.76E-2	0.62 (0.47–0.82)	6.73E-4	0.64 (0.55–0.74)	4.30E-9	0.15
rs12130499	0.54 (0.43–0.70)	1.15E-6	0.78 (0.60–1.01)	6.14E-2	0.62 (0.47–0.82)	7.49E-4	0.64 (0.55–0.74)	4.31E-9	0.13
rs298733	0.55 (0.43–0.70)	1.53E-6	0.79 (0.62–1.03)	7.61E-2	0.64 (0.49–0.84)	1.25E-3	0.65 (0.56–0.75)	1.25E-8	0.12
rs1436171	0.54 (0.42–0.68)	6.26E-7	0.82 (0.64–1.04)	1.07E-1	0.65 (0.49–0.85)	2.04E-3	0.66 (0.57–0.76)	2.17E-8	0.06
rs1369846	0.52 (0.41–0.66)	9.42E-8	0.85 (0.67–1.09)	1.94E-1	0.66 (0.50–0.87)	2.96E-3	0.66 (0.57–0.77)	2.60E-8	0.02
rs1436175	0.50 (0.39–0.64)	2.72E-8	0.93 (0.73–1.18)	5.50E-1	0.77 (0.59–1.01)	6.14E-2	0.71 (0.61–0.82)	3.09E-6	0.002

Abbreviations: CI, confidence interval; CATS, Comorbidity and Trauma Study; OD_E, opioid dependence end point; OR, odds ratio; OU_{IP}, opioid use with impeded progression; SAGE, Study of Addiction: Genetics and Environment; SNP, single-nucleotide polymorphism.

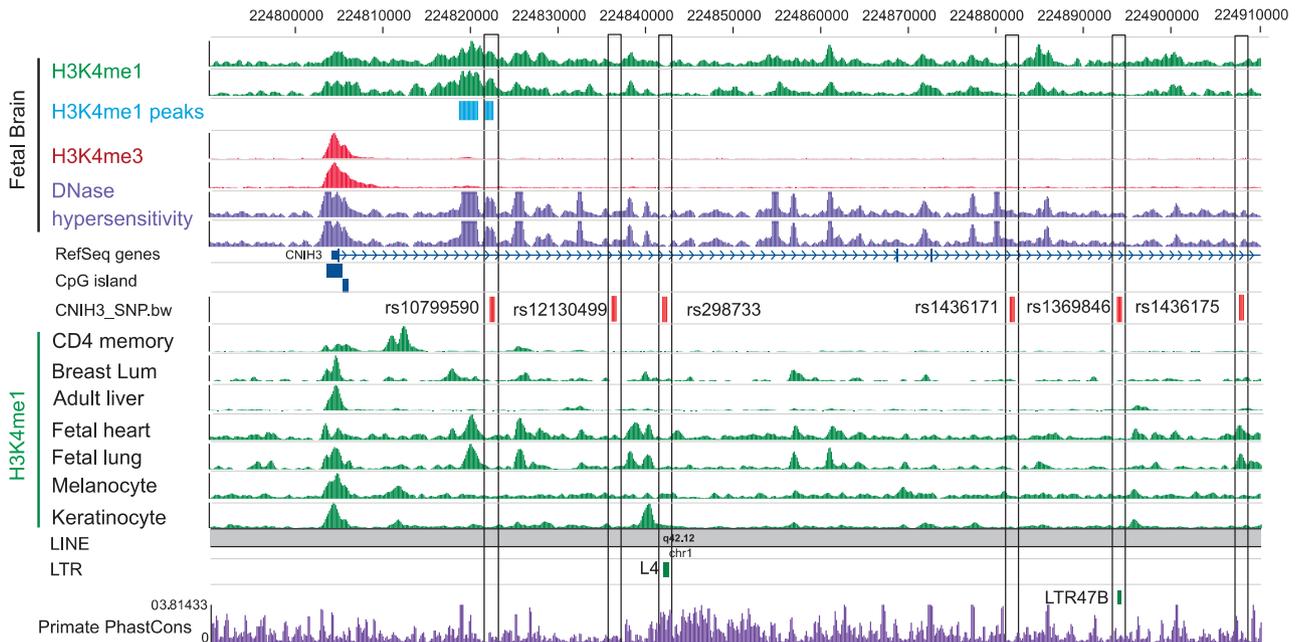


Figure 1. Epigenetic landscape of the six intronic CNIH3 SNPs. Rs1369846 and rs298733 are located within retrotransposons.^{40,41} Evidence of epigenetic functionality for rs10799590 includes the location of this SNP within a fetal brain-specific enhancer.^{46,47} Fetal brain H3K4me1 data indicate that it is within a H3K4me1 peak; DNase hypersensitivity data suggest that it is in an open chromatin state in fetal brain. This enhancer mark on rs10799590 was specific to fetal brain, but was not in CD4+ T cells, breast luminal epithelial cells, adult liver, fetal heart, fetal lung, melanocyte, or keratinocytes. The results of motif analyses (Supplementary Table 6) predict that rs10799590 is within the binding site of transcription factor Tal1 with the G allele having significantly higher binding affinity than the A allele.

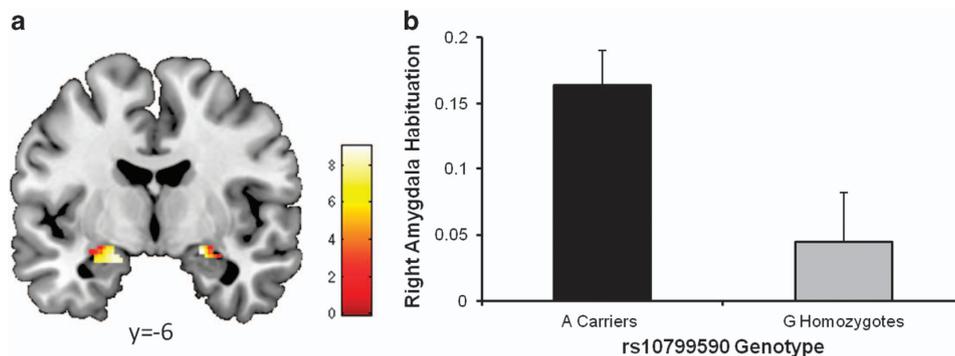


Figure 2. Amygdala habituation. (a) Statistical parametric map illustrating mean bilateral amygdala habituation across all DNS participants (left MNI coordinates: $x = -20$ $y = -8$ $z = -16$, $k_E = 144$, $t = 9.03$, $P < 0.001$; right MNI coordinates $x = 22$ $y = -6$ $z = -14$, $k_E = 83$, $t = 8.97$, $P < 0.001$). (b) A allele carriers had greater right amygdala habituation (that is, less persistent activation) relative to G allele homozygotes. The y axis indicates habituation with greater values indicating a larger decrease in activation over time. See Supplementary Figure S3 for a depiction of activation across blocks.

Table 3. Computational genetic analysis of interstrain differences in physical dependence on morphine

Gene	P-value ^a
CNIH3	4.0E-4
CACNG8	8.3E-4
GRIA1	8.1E-6
GRIP1	1.7E-5
GRIA2	7.4E-3
CNIH2	0.35
DLG4	0.35

^aCorrected for multiple testing.³⁸

initial right amygdala responses to stimuli (stand Beta = 0.067; $\Delta F_{1,308} = 1.42$, $P > 0.234$, $\Delta R^2 = 0.004$).

Genetic analysis of interstrain differences in physical dependence on morphine

To link our findings further to existing animal literature, we performed computational haplotype-based genetic mapping analyses^{48–50} of data from 23 inbred mouse strains for a robust measure of opioid physical dependence,⁴⁸ counts of jumps made by morphine-dependent mice after naloxone administration. Correlations were calculated for the distribution of the mean number of jumps per strain with known haplotype blocks across strains for *CNIH3* and genes encoding AMPAR subunits and proteins involved in alterations of AMPAR subunit composition in response to opioids. Significant correlation was observed for *CNIH3* haplotype, but not for the more widely expressed cornichon family AMPA receptor auxiliary protein 2 (*CNIH2*) (Table 3). Significant correlations were also noted for haplotypes in *GRIA1* (glutamate receptor, ionotropic, AMPA 1) *GRIA2* (glutamate receptor, ionotropic, AMPA 2), calcium channel, *CACNG8* (voltage-dependent, gamma subunit 8) and glutamate receptor-interacting protein 1, but not *DLG4* (discs, large homolog 4).

DISCUSSION

The current report provides evidence for *CNIH3* involvement in the pathophysiology of opioid dependence. *CNIH3* encodes a small, highly conserved protein. The AMPA receptor core is formed by tetramers of the GluA1–4 subunits and up to four members of three protein groups: transmembrane AMPAR regulatory proteins, cornichon homologs (*CNIH3* and *CNIH2*) and the GSG11 protein.⁵¹ The receptor's periphery contains transmembrane and other proteins (for example, post-synaptic density protein 95 (PSD-95)) that bind with core proteins and each other in the postsynaptic density.^{51–54} *CNIH2* and *CNIH3* markedly slow AMPAR deactivation and desensitization in heterologous systems.^{54–56} One study⁵⁴ suggested that the actions of *CNIH2* and *CNIH3* are selective for AMPARs containing GluA1 subunits; however, more recent reports^{51,57} do not support this specificity of binding. An investigation that focused on two hippocampal cell types with markedly different excitatory postsynaptic currents implicated *CNIH2* as largely responsible for the distinction between fast and slow excitatory postsynaptic currents. Although this report did not examine whether *CNIH3* has a similar role, prior studies, for example,⁵⁵ have found that the two proteins have comparable effects on slowing AMPAR deactivation and desensitization.

Rodent studies^{26–29} have implicated alterations in the subunit composition of brain AMPARs in diverse aspects of opioid addiction. Increased expression of GluA1-containing/GluA2-lacking AMPARs has been observed in the central nucleus of the amygdala²⁶ and the hippocampal postsynaptic density²⁸ in studies of morphine-related context-reward conditioning²⁶ and context-dependent behavioral sensitization.²⁸ The latter²⁸ found

that these changes were mediated via interactions with TARP gamma-8 and GRIP1 proteins. GluA1 knockout mice displayed impaired drug-induced state dependency after operant conditioning with morphine.²⁹ Another study²⁷ implicated downregulation of GluA2 expression in the prefrontal cortex in reinstatement of heroin self-administration after prolonged abstinence. Interestingly, *CNIH3* expression is greatest in the frontal cortex, amygdala and hippocampus in the adult human brain.⁵⁸

Our GWAS analyses found that *CNIH3* SNPs are associated with protection against progression to ODE in OUIP individuals. As this effect was observed in analyses limited to opioid misusers, it likely represents liability unrelated to that for initiation of opioid misuse. The discovery and confirmation sample OUIP groups have substantially higher risk allele frequencies than an Australian (European Ancestry) general population sample⁵⁹ (Supplementary Table 8) to which the ODE groups' risk allele frequencies are more similar. *Post hoc* SNP-based association analyses comparing the CATS OUIP group and this general population sample found substantial differences (P -values $\leq 6.4E-5$); similar comparisons with the CATS ODE group found more modest differences ($P \geq 2.3E-2$) in the opposite direction. The phenotypic variance in ODE explained by rs10799590 in our meta-analysis is estimated at 1.17 to 5.85% (Supplementary Table 9), indicative of a strong effect. Overall, our findings suggest that these *CNIH3* SNPs enable greater, but not complete control in the use of these otherwise highly addictive drugs.

An examination of human post-mortem amygdalae reported a strong positive correlation between GluA1 and PSD-95 messenger RNA expression in heroin-dependent cases, but not in controls (*CNIH3* expression was not reported).²⁵ Another study⁵⁵ observed positive correlations for these proteins with *CNIH3* (GluA1 0.43; *DLG4* 0.28) in (unexposed) mammalian brains. Thus, human²⁵ and animal²⁶ studies provide evidence of altered amygdala GluA1 expression in opioid dependence. The post-mortem report noted²⁵ that similar changes in AMPAR GluA1 subunits occur in the amygdala with associative learning of opioid reward²⁶ and fear conditioning.^{43–45} Importantly, both processes involve epigenetically mediated changes in gene expression following an environmental exposure.^{39,46} We thus examined the effects of our most strongly associated SNP using imaging genetics^{46,60} and observed significantly greater right amygdala habituation to threat-related facial expression in rs10799590 A allele carriers. Consistent with the reduced risk we observed in opioid exposed individuals, the polymorphism's protective neural effects were apparent with subsequent, but not initial, exposures to environmental stimuli. The association of this SNP with ODE likely represents a similar protective process involving greater habituation to the neural effects of opioids that impact additional opioid use.

Our focus on opioid misusers is a major strength; the modest size of our OUIP groups is an unavoidable limitation. Our assumption that SNPs can offer protection against transitions at different points of the addictive process is supported by the existing animal literature.^{26–29} We only examined confirmation of the *CNIH3* SNP associations; a broader examination might have confirmed other CATS associations. As both the epigenetic landscape and motif analysis support its potential functionality, our neuroimaging genetics study focused on rs10799590 with *post hoc* analyses confirming it as the *CNIH3* SNP most strongly associated with amygdala habituation. Future work should incorporate examination of the other GWS SNPs (for example, rs298733 also affects transcription factor binding—Supplementary Table 6) and the possibility of more highly associated, non-genotyped polymorphisms in high LD. Although similarly ascertained samples, for example,⁶¹ have comparable rates of daily opioid injection, reports in other populations have noted lower prevalence. For example,⁶² a more detailed characterization of the OUIP groups' opioid use would have been useful, but was not

obtained. We examined somewhat divergent OD_E 's in our confirmation samples because of ascertainment and assessment differences (Supplementary Table 3). Although these methodological differences are a limitation, the observed confirmation in two multi-site US-based studies supports the generalizability of our findings. The highly comorbid composition of the OD_E and OU_{IP} groups may raise concerns that intergroup differences are better attributable to another phenotype. *Post hoc* association analyses conducted in CATS to address this possibility (Supplementary Table 10) found few associations for comorbid disorders reaching nominal significance. Comorbidity pattern differences across samples (Supplementary Tables 1 and 2) further argue against this possibility. Finally, our exclusion of participants with non-European ancestry and the lack of genomic inflation ($\lambda=1.01$) suggest spurious association owing to uncorrected admixture is unlikely.

Our meta-analyses found GWS association with *CNIH3* SNPs conferring robust protective effects against OD_E , findings that map onto reports of AMPA glutamatergic involvement in opioid dependence.^{25–29} The finding of significantly greater habituation in the right amygdala rs10799590 A allele carriers supports these SNPs' *in vivo* functional effects in humans (complementing evidence of epigenetic functionality). The genetic analyses of mouse strain data support the involvement of *CNIH3*, but not the more highly expressed *CNIH2*, in murine opioid physical dependence. The significant correlations observed for genes encoding AMPAR subunits and related proteins provide additional evidence for genetic risk mediated via this pathway. These convergent findings implicate *CNIH3*'s involvement in opioid dependence and could provide a route to target glutamatergic processes for translational research, focusing on improving opioid dependence treatments and developing opioid analgesics with lower dependence risk.

CONFLICT OF INTEREST

Although unrelated to the current study, Dr Kranzler has been a consultant or advisory board member for Alkermes, Lilly, Lundbeck, Pfizer and Roche. He is also a member of the American Society of Clinical Psychopharmacology's Alcohol Clinical Trials Initiative, which is supported by Lilly, Lundbeck, Abbott and Pfizer. The remaining authors declare no conflict of interest.

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