Research Article
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Pharmacogenomics

Dopaminergic pathway polymorphisms and heroin addiction: further support for association of *CSNK1E* variants

Background & aim: The dopaminergic pathways have been implicated in the etiology of drug addictions. The aim of this study was to determine if variants in dopaminergic genes are associated with heroin addiction. **Materials & methods:** The study includes 828 former heroin addicts and 232 healthy controls, of predominantly European ancestry. Ninety seven SNPs (13 genes) were analyzed. **Results:** Nine nominally significant associations were observed at *CSNK1E*, *ANKK1*, *DRD2* and *DRD3*. **Conclusion:** The results support our previous report of association of *CSNK1E* SNP rs1534891 with protection from heroin addiction. *CSNK1E* interacts with circadian rhythms and DARPP-32 and has been implicated in negative regulation of sensitivity to opioids in rodents. It may be a target for drug addiction treatment.

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Keywords: casein kinase 1ε • circadian rhythms • dopaminergic reward pathways • heroin addiction • SNP rs1534891

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Drug addiction is a major public health and social issue. Heroin addiction is a chronic brain disease caused by genetic, environmental and drug-induced factors. The nonmedical abuse of subscription opioids is a growing epidemic.

Opioid drugs mediate their reinforcing effects by dopamine-dependent and dopamine-independent mechanisms. The dopaminergic mesocorticolimbic reward pathways have been implicated in the etiology of drug addictions [1–3]. Addictive drugs transiently increase extracellular dopamine in the ventral striatum inducing abnormal learning process and promoting compulsive drug abuse [4]. Positron emission tomography (PET) studies showed reduced striatal dopamine $D_{2/3}$ receptor availability and presynaptic dopamine release in heroin-dependent subjects compared with healthy controls [5,6].

Polymorphisms in genes of the dopamine pathway are candidates for drug addiction vulnerability. This study examined polymorphisms in the following genes encoding receptors, transporters and metabolizing enzymes of dopamine; L-DOPA, the precursor to dopamine, is produced by the enzyme TH and is catalyzed to dopamine by DDC. Dopamine is degraded by COMT. Dopamine is the primary endogenous ligand for two classes of G protein-coupled dopamine receptors; the D-like receptors (D1 and D5) and the D2-like receptors (D2, D3, and D4). The dopamine transporter (DAT1, *SLC6A3*) mediates the active reuptake of dopamine from the synapse and is a principal regulator of dopaminergic neurotransmission. ANKK1 is a RIP Ser/Thr protein kinase that may play a role in signal transduction [7] and is activated by the dopaminergic agonist apomorphine [8]. *ANKK1* is located 10 kb downstream of *DRD2,* and includes SNP rs1800497 that was originally called '*DRD2* TaqIA.' DARPP-32 (PPP1R1B, dopamine- and cAMP-regulated phosphoprotein, 32 kDa), is a key regulatory molecule in the dopaminergic signaling pathway [9]. The casein kinase 1 epsilon isoform (encoded by *CSNK1E*) is a serine/threonine-

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selective phosphotransferase that regulates diverse cellular processes, including circadian rhythms and dopaminergic signaling through DARPP-32 [10]. DBH converts dopamine to norepinephrine thus regulating the ratio of the two neurotransmitters.

Several studies examined for association between the dopaminergic pathway genes and heroin addiction or abuse. We have reported a tentative association of the *CSNK1E* gene SNP rs1534891 with heroin addiction in subjects with European descent [11]. SNP rs135745, in the 3′ region flanking *CSNK1E,* was associated with heroin addiction in Han Chinese [12]. Several *ANKK1* SNPs were associated with heroin addiction in European, Australian and Chinese populations [13–17]. Several *DRD2* SNPs and haplotypes were associated with heroin addiction in various populations [18–22]. *DRD1* SNP was associated with heroin abuse [22]. *DBH* SNP was associated with a more progressive nature of heroin addiction in an injection subgroup [23].

This study was designed to determine whether variations in dopamine pathway-related genes account for the heritable factors in susceptibility to heroin addiction and attempted to corroborate our previous results that were obtained in a smaller sample.

Materials & methods

Subjects

The study included 828 cases (32% female; mean age 40 ± 12) and 232 controls (50% female; mean age 42 ± 16). This study was a major expansion of our previous study [11]. The current study included a majority of the samples from the original study as well as 465 new cases (230 Americans and 235 Israeli) and 89 new controls (59 Americans and 30 Israeli) that were recruited at the same clinics. Ninety samples (49 cases and 41 controls) from the original study were excluded from this study based on stricter filtering criteria for ancestry and phenotype, relative in the study, DNA availability and DNA quality. All subjects were self-identified as having European and/or Middle-Eastern ancestry. Ancestry was verified by STRUCTURE analysis, and specific inclusion criteria were employed to increase homogeneity. To be included an individual had to show at least a 75% European, Middle-Eastern or combined ancestry contributions (see below).

The case subjects were former heroin addicts with a history of at least one year of daily multiple uses of heroin, treated at a methadone maintenance treatment program. The case subjects were recruited at the Rockefeller University Hospital, the Manhattan campus of the VA NY Harbor Health Care System and the Dr Miriam and Sheldon G Adelson Clinics for Drug Abuse Treatment and Research in Las Vegas and Israel. The control sample was mainly from New York City with 30 samples from Israel. Ascertainment of cases and controls was made by personal interview performed in a similar manner at the recruiting places, using several instruments: the Addiction Severity Index [24], Kreek-McHugh-Schluger-Kellogg Scale (KMSK) [25] and Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV). The following exclusion criteria from the healthy control category were used: at least one instance of drinking to intoxication or any illicit drug use in the previous 30 days; a history of alcohol drinking to intoxication or illicit drug use, more than twice a week, for more than six consecutive months and cannabis use for more than 12 days in the previous 30 days or past cannabis use for more than twice a week for more than 4 years. Subjects with active DSM-IV axis I disorder were excluded from the study. All subjects completed a family history questionnaire. The Institutional Review Boards of the Rockefeller University Hospital, the VA New York Harbor Healthcare System and the Tel Aviv Sourasky Medical Center (Helsinki Committee) approved the study. All subjects signed informed consent for genetic studies.

Genes/SNPs selection & array design

Thirteen genes were selected based on their known function in the dopaminergic pathway (Table 1 & Supplementary Table 1; see online at: www.futuremedicine.com/doi/suppl/10.2217/pgs.14.145). A new custom array (GS0013101-OPA) was designed based on the 'addiction' array (GS0007064-OPA; Illumina, San Diego, CA, USA) [26] that was used in our previous studies [11,27], with some modifications. The original design included tagging SNPs with minor allele frequency (MAF) >0.005 that capture the full haplotype information, as well as potentially functional coding SNPs and SNPs within splice sites. Fourteen SNPs, in these genes were excluded from the new array based on low quality in the original array, and 19 SNPs were excluded based on low MAF in this population. Nine SNPs were added based on reports of association with related phenotypes or functionality, including three SNPs in the *ANKK1* gene that were not included in the original array (Supplementary Table 1).

Genotyping

Blood samples were taken and DNA was extracted and quantified using standard methods. DNA (700 ng) was precipitated as described [11]. Genotyping was performed on a 1536-plex GoldenGate Custom Panel at the Rockefeller University Genomics Resource Center according to the manufacturer's protocol. Eighteen samples were genotyped in duplicate with 0.003%

Table 1. Dopaminergic pathway genes analyzed in the study.

Symbol	Name
ANKK1	Ankyrin repeat and kinase domain containing 1
COMT	Catechol-*O*-methyltransferase
CSNK1E	Casein kinase 1, epsilon
DBH	Dopamine beta-hydroxylase
DDC	DOPA decarboxylase
DRD1	Dopamine receptor D1
DRD2	Dopamine receptor D2
DRD3	Dopamine receptor D3
DRD4	Dopamine receptor D4
DRD5	Dopamine receptor D5
PPP1R1B	Protein phosphatase 1, regulatory (inhibitor) subunit 1B (DARPP-32)
SLC6A3	Solute carrier family 6 member 3 (dopamine transporter, DAT)
TH	Tyrosine hydroxylase

error rate. Analysis was performed with BeadStudio software v2.3.43 (Illumina). The genotype data for all SNPs were visually inspected.

Ancestry contribution

Biographic Ancestry Scores (e.g., fractions of affiliation of an individual in each cluster) were estimated by STRUCTURE 2.2 with seven clusters (K) using data from 155 ancestry informative markers (AIMs) with high quality. Each subject was anchored against genotypes of 1051 samples from 51 worldwide populations represented in the Human Genome Diversity Cell Line Panel, as described [28]. To be included in the study, an individual had to show at least a 75% European, Middle-Eastern or both ancestry contribution. The decision to include both European and Middle-Eastern clusters was based on their low population differentiation [29,30]. From the original cohort of subjects who self-identified as having European ancestry, 57 subjects were excluded because they did not meet the inclusion criteria. Seven subjects were excluded because STRUCTURE results were in conflict with their self-identified European ancestry. The average European ancestry was 0.76 ± 0.28 in the control group and 0.73 ± 0.32 in the case group. The average Middle-Eastern ancestry was 0.17 ± 0.26 in the control group and 0.21 ± 0.31 in the case group (Supplementary Table 2).

Potential regulatory function analysis

The USCS browser was used for visualizing ENCODE (Encyclopedia of DNA Elements) data [31], which are indicative of regulatory function, for the *CSNK1E* gene.

Statistical analysis

Pairwise linkage disequilibrium (LD; D′ and r^2) was estimated using Haploview 4.2. LD blocks were identified based on 'Solid Spine of LD' algorithm with a minimum D′ value of 0.8. Exact tests for deviation from Hardy–Weinberg equilibrium (HWE) were performed with the PLINK program, with SNPs to be rejected based on threshold of $p \leq 0.001$ in controls. Association analyses were conducted using PLINK for each SNP separately by logistic regression, under dominant, and recessive model assumptions. The direction of the regression coefficient represents the minor allele. The re-analysis of the three *CSNK1E* SNPs consisted of the 465 cases that were not included in the original study and 232 control samples (including the old and new samples). Genotype patterns analysis of *CSNK1E* SNP was performed using χ^2. Correction for two levels of multiple testing were carried out with the MAXSTAT program [32] as follows: for each SNP, testing under dominant and recessive model assumptions was done as a maximum test, that is, the larger of the two χ^2 served as the test statistic, whose associated significance level was evaluated in 40,000 permutation samples. Testing 97 SNPs was allowed for by computing the p-value associated with the largest of the SNP-specific maximum test statistics, which was done in the same 40,000 permutation samples.

Results

Analysis was conducted in 1060 subjects (828 cases and 232 controls). The ancestry of all subjects was verified as predominantly European using STRUCTURE analysis of 155 AIMs. There was no evidence for population substructure ($\lambda = 0.93$). One hundred and eighteen SNPs from 13 genes related to the dopa-

minergic pathway were genotyped (Table 1 & Supplementary Table 1). Twenty SNPs were excluded based on low quality. One SNP (*DDC* rs4947535) was excluded based on significant deviation from HWE in controls (p = 0.00032). The remaining 97 SNPs were analyzed for association with heroin addiction. Analysis of LD suggested at least 69 related SNPs (D′ > 0.75), of which 25 SNPs are highly correlated (r^2 > 0.75; Supplementary Figures 1 & 2, & Supplementary Table 3).

Nine SNPs in four genes showed nominally significant association (p < 0.05) of genotype with heroin addiction, under two different models of inheritance (Dom/Rec; Table 2). The top signals are from the following genes: *CSNK1E, ANKK1, DRD2* and *DRD3.* None of the signals survived correction for multiple testing. The association signal of the *CSNK1E* SNPs was driven by higher MAF in the control group than in the case group indicating protective effect. Two of the *DRD3* SNPs (rs9288993 and rs2654754) are relatively rare (MAF 0.032 in cases and 0.006 in controls) so their signal may not reflect true association. From the top signals, SNP pairs *DRD3* rs9288993/rs2654754 and SNP triplets *DRD2* rs1076563/ *DRD2* rs2587548/ *ANKK1* rs2734849 and *CSNK1E* rs1534891/rs6001093/rs135757 are in strong LD (r^2 > 0.6; Supplementary Figures 1 & 2, & Supplementary Table 3). These results suggest four major independent association signals.

Haploview analysis of *CSNK1E* SNPs predicts one haplotype block #1 (Figure 1) with 'yin-yang' haplotypes comprised of the reference haplotype (GTC) and the variant haplotype (AGT), in addition to several rare haplotypes. Table 3 lists the genotype patterns of the three SNPs that include two major patterns (#1 and 4), and three minor patterns (#2, 3 and 5), with all the other patterns included in the 'other' group. There was significant difference in genotype patterns between cases and controls (χ^2 = 17.62, degrees of freedom = 5, overall p = 0.0035).

Re-analyses

Re-analysis of the three *CSNK1E* SNPs that were associated with protection from heroin addiction was performed in a subgroup consisting of the 465 cases that were recruited after the conclusion of the original study [11] and 232 controls, including the original and the new samples. The three *CSNK1E* SNPs rs1534891, rs6001093 and rs135757 were associated with protection from heroin addiction under the dominant model (p = 0.0006, 0.0094, 0.0138, respectively), corroborating the original results.

To insure that the association signals are not due to population substructure, the data for the associated SNPs was re-analyzed in the US sample only (cases, n = 542, controls, n = 202). The results were similar and in the same direction but the two *DRD2* SNPs did not reach significance (p = 0.08; Supplementary Table 4). In addition, the MAF of the associated SNPs were compared among cases and controls with and without the Israeli subjects (Supplementary Table 5). The difference in MAF between cases and controls did not change significantly when the Israeli subjects are excluded.

Since the three *CSNK1E* SNPs showed protection from heroin addiction based on higher MAF in the control group compared with the case group, the MAF

Table 2. Details of SNPs with nominally significant associations with heroin addiction (p < 0.05).

Gene	Chr.	SNP	Gene position	Major allele	Minor allele	Test	p-value†	OR (95% CI)‡
CSNK1E	22	rs1534891§	Intron	G	A	Dom	0.0015	0.59 (0.43–0.80)
	22	rs6001093§	Intron	A	G	Dom	0.0197	0.69 (0.51–0.92)
	22	rs135757§	Intron	C	T	Dom	0.0280	0.69 (0.51–0.92)
ANKK1	11	rs2734849¶	His490Arg	A	G#	Rec	0.0147	0.67 (0.48–0.93)
DRD2	11	rs2587548¶	Intron	G	C	Dom	0.0393	1.43 (1.06–1.93)
	11	rs1076563¶	Intron	G	T	Dom	0.0311	1.47 (1.07–1.95)
DRD3	3	rs2654754††	Intron	T	C	Dom	0.0007	5.24 (1.62–16.94)
	3	rs9288993††	Intron	A	G	Dom	0.0025	4.51 (1.39–14.64)
	3	rs1486009	Intron	T	C	Dom	0.0056	0.56 (0.37–0.85)

†Association analyses were conducted under two models of inheritance of the minor allele (Dom and Rec). Only the lowest p-value is listed.
‡OR < 1 represents protective effect of the minor allele, OR > 1 represents risk effect of the minor allele.
§The three SNPs are in moderate to strong LD (r^2 > 0.7; see Figure 1).
¶SNPs in strong LD (r^2 > 0.6).
#Alleles frequencies are almost equal. The variant G allele was the minor allele in cases and the minor allele in controls.
††SNPs in strong LD (r^2 > 0.8).
Chr.: Chromosome; Dom: Dominant; LD: Linkage disequilibrium; OR: Odds ratio; Rec: Recessive.

of the control sample was compared with the average of five reference European populations based on the 1000 Genomes database (Supplementary Table 6). The average difference in MAF was ± 0.02 (standard deviation [SD] ± 0.02). The MAF of these SNPs was higher in the control group than the reference European populations (+5.5–7.5%).

Potential regulatory function of *CSNK1E* SNPs rs1534891

ENCODE ChIP-seq assay data for the *CSNK1E* gene suggest that SNP rs1534891 is located in a regulatory region. This suggestion is based on evidence of enrichment in histone marks (H3K4me1 and H3K27ac) in human primary cell line from epidermal keratinocytes that are signature of enhancer (Supplementary Figure 3).

Discussion

CSNK1E & the dopamine pathway

The study detected nominally significant associations of three *CSNK1E* SNPs and haplotype with protection from heroin addiction under the dominant model of inheritance. The MAF of these SNPs was higher in the control group than in the case group or reference European populations. It is unlikely that the results were affected by population substructure, selection bias of the control sample or technical error.

The most significant result is of the intronic SNP rs1534891. The function of this SNP is still unknown but it is located in a region of potential regulatory function based on histone marks. This result corroborated our previous study [11], and is in line with reports of association of *CSNK1E* flanking SNP rs135745 with heroin addiction in Han Chinese [12], and with increased sensitivity to D-amphetamine [33]. HapMap data show strong LD between SNPs rs135745 and rs1534891 ($D' = 0.8$, $r^2 = 0.01$ in Han Chinese and $D' = 0.9$, $r^2 = 0.1$, in subjects with European ancestry). SNP rs135745 was not included in our analysis.

The casein kinase 1 (CSNK1, CK1) family consists of evolutionarily conserved serine-threonine kinases with seven isoforms in mammals that are involved in the regulation of diverse cellular processes. The closely related δ and ε isoforms are involved in the generation of psychostimulant-induced behaviors by phosphorylation of DARPP-32, leading to an inhibition of protein phosphatase I and subsequent phosphorylation of various targets [9]. They also modulate the activation of glycogen synthase kinase-3, a downstream target of DARPP-32, which is important in stimulant drug response [34]. DARPP-32 mediates the actions of multiple drugs of abuse and these post-translational modifications could lead to synaptic plasticity within the dopaminergic system. Studies from our laboratory and others have shown

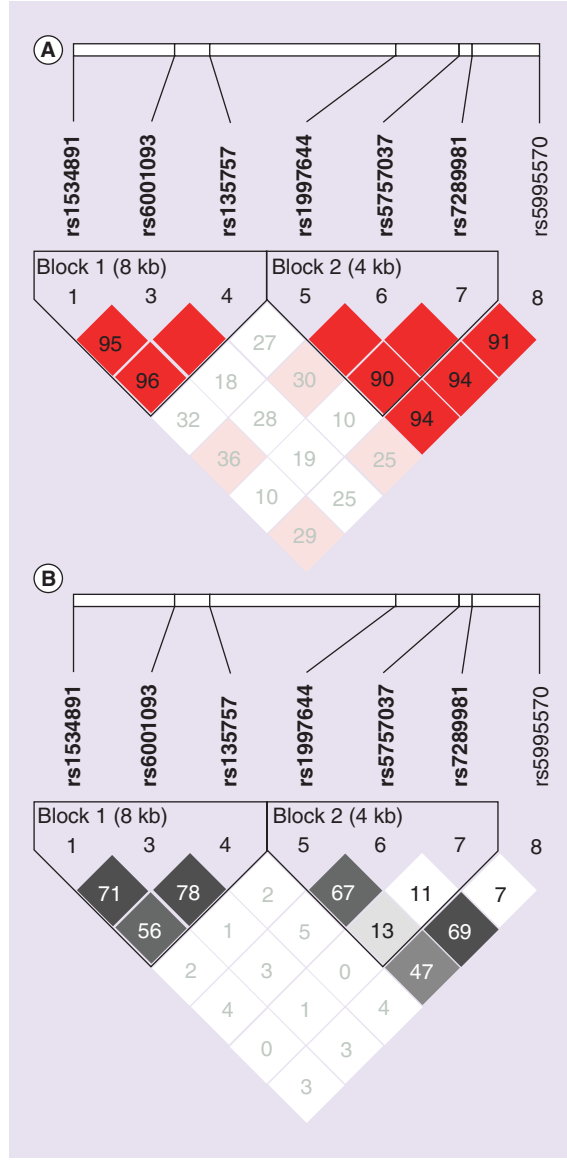

Figure 1. *CSNK1E* pairwise linkage disequilibrium. The pairwise correlation between SNPs was measured as **(A)** D′ and **(B)** r^2. The values are shown (×100) in each box. The color scheme indicates the magnitude of the value. When the value is equal to 1.0 no number is given. SNP rs5995565 (#2) was excluded based on minor allele frequency = 0.002. Blocks were defined according to 'Solid Spine of Linkage Disequilibrium' algorithm with a minimum D′ value of 0.8.

that Ser130A-DARPP-32 mutant mice self-administered more cocaine than wild-type mice and showed attenuated cocaine induced increases in dopamine levels in the dorsal striatum [35]. Csnk1ε is crucial for the locomotor stimulant response to methamphetamine [36–38].

CSNK1E & circadian rhythms

Circadian clocks are based on rhythmic synthesis of transcriptional repressors that repress their own tran-

Table 3. *CSNK1E* genotype patterns of haplotype block 1.

	Pattern†	Controls	f	Cases	f
GG-AA-CC	1	107	0.46	456	0.55
GG-A**G**-C**T**	2	21	0.09	90	0.11
GG-AA-C**T**	3	17	0.07	60	0.07
G**A**-A**G**-C**T**	4	72	0.31	155	0.19
AA-GG-TT	5	7	0.03	24	0.03
Others	6	9	0.04	43	0.05

The variant minor alleles are bolded.
χ^2 = 17.62, degrees of freedom = 5, overall p-value = 0.0035.
†SNPs rs1534891, rs6001093 and rs135757.
f: Frequency.

scription (negative feedback loops) [39]. The δ and ε casein kinase 1 isoforms are involved in post-translational regulation of the circadian rhythm by phosphorylating several clock proteins, including BMAL1, CRY, PER1 and PER2 [40]. CSNK1 is the human homolog of the Drosophila double-time [41]. The circadian system has substantial influence on regulating reward processing, learning and memory [42]. Drug addiction has been linked to disruptions in circadian rhythms [43–45] and several circadian rhythm genes were associated with drug addiction [46,47]. Abstinent heroin addicts showed a persistent disruption in clock genes expression in PBMC [48].

Several studies in rodents showed that CSNK1 inhibitors modulate behavioral abnormalities, including addiction [37–38,49–51] suggesting that CSNK1 may be a target for intervention in the treatment of drug addiction. CSNK1E inhibitors may prevent drug craving and relapse behavior through circadian clock stabilization and/or DARPP-32-PP1 signaling pathway modulation. Casein kinase 1 is being targeted for drug development for the treatment of several diseases and these medications may be useful for the treatment of heroin addiction [52].

Dopamine receptors D2 & D3

There are no previous reports on association of the two *DRD2* intronic SNPs indicated in this study, but the missense *ANKK1* SNP rs2734849 (His490Arg) that is in relatively strong LD with these SNPs in this population, was previously shown to yield differential suppression of NF-κB-regulated gene expression *in vitro,* and may affect DRD2 expression [53]. The A1 allele of SNP rs1800497 (*DRD2* TaqIA) that is located in *ANKK1*, has been associated with a higher heroin consumption and poor response to methadone treatment [17]. Although this SNP was excluded from this study because of low quality, it is in perfect LD, in the HapMap CEU population, with SNP rs7118900 that was included in the study, but did not show significant association with heroin addiction. SNP rs1079597 (also called 'TaqIB') has been previously associated with heroin dependence [20]. Although this SNP was not included in the study, it is in perfect LD, in the HapMap CEU, with SNP rs2471857 that was included in the study, but did not show significant association with heroin addiction. The functional synonymous *DRD2* rs6277 that was associated with alcohol and nicotine dependence [54,55], was not included the study, and will be included in further analysis. The putative functional *DRD2* SNP rs1076560, which was shown to shift splicing from the short isoform to the long isoform, has been recently associated with opioid dependence in European and African–Americans [18]. We have not observed significant association of this SNP with heroin addiction in this study. Of note, the cohort used by Clarke *et al.* includes an unknown number of DNA samples from our laboratory that were submitted to the NIDA Center for Genetic Studies DNA Repository and are probably included in our study.

Previous association studies of *DRD3* and heroin addiction were inconclusive [56–58]. A recent study suggests an association of a *DRD3* haplotype block, specifically with early-onset heroin addiction, in Han Chinese [59]. There are no previous reports on the two *DRD3* intronic SNPs indicated in this study and the p-values for two of the SNPs may be affected by their low MAF.

Conclusion

The study identified associations of nine SNPs located in four dopamine pathway-related genes (*CSNK1E, ANKK1, DRD2* and *DRD3*). These SNPs include a potentially functional nonsynonymous *ANKK1* SNP, and several SNPs in LD with SNPs that were previously associated with similar phenotypes in similar and different populations. An important result is of an intronic *CSNK1E* SNP in a potential regulatory region that corroborates our previous study [11] and supports

human and rodent studies suggesting CSNK1E as a target for treatment of drug addiction.

Future perspective

Future studies are required to replicate the results of this study in independent cohorts as well as in different populations and other drug addictions. Future research should also look at other types of genetic variations and functionality, as well as combinations of polymorphisms and environmental effects. CSNK1E inhibitors may be useful in future treatment of heroin addiction.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- Drug addiction is a major public health and social issue.
- The dopaminergic mesocorticolimbic reward pathways have been implicated in the etiology of drug addictions.
- Polymorphisms in genes of the dopamine pathway are candidates for drug addiction vulnerability.

Sample

- This hypothesis-driven candidate gene association study included 828 cases (former heroin addicts in methadone maintenance treatment program) and 232 controls.
- STRUCTURE analysis of ancestry informative markers (AIMs) confirmed >75% European/Middle-Eastern ancestry.

Results

- Nine SNPs in four genes (*CSNK1E*, *ANKK1*, *DRD2* and *DRD3*) showed nominally significant association of genotype with vulnerability to or protection from heroin addiction but none of the signals survived correction for multiple testing.
- The results include potentially functional nonsynonymous *ANKK1* SNP and corroborate previous finding of association of *CSNK1E* SNPs with heroin addiction.

Discussion

- CSNK1E phosphorylates several clock proteins and is involved in regulation of the circadian rhythm. It also phosphorylates DARPP-32 that mediates the actions of multiple drugs of abuse.

Conclusion

- CSNK1E inhibitors may prevent drug craving and relapse behavior through circadian clock stabilization and/or DARPP-32-PP1 signaling pathway modulation.

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