Repeat variation in the human PER2 gene as a new genetic marker associated with cocaine addiction and brain dopamine D2 receptor availability

E Shumay1, JS Fowler1, G-J Wang1, J Logan1, N Alia-Klein1, RZ Goldstein1, T Maloney1, C Wong1 and ND Volkow2

Low dopamine D2 receptor (D2R) levels in the striatum are consistently reported in cocaine abusers; inter-individual variations in the degree of the decrease suggest a modulating effect of genetic makeup on vulnerability to addiction. The PER2 (Period 2) gene belongs to the clock genes family of circadian regulators; circadian oscillations of PER2 expression in the striatum was modulated by dopamine through D2Rs. Aberrant periodicity of PER2 contributes to the incidence and severity of various brain disorders, including drug addiction. Here we report a newly identified variable number tandem repeat (VNTR) polymorphism in the human PER2 gene (VNTR in the third intron). We found significant differences in the VNTR alleles prevalence across ethnic groups so that the major allele (4 repeats (4R)) is over-represented in non-African population (4R homozygosity is 88%), but not in African Americans (homozygosity 51%). We also detected a biased PER2 genotype distribution among healthy controls and cocaine-addicted individuals. In African Americans, the proportion of 4R/three repeat (3R) carriers in healthy controls is much lower than that in cocaine abusers (23% vs 39%, \( P = 0.004 \)), whereas among non-Africans most 3R/4R heterozygotes are healthy controls (10.5% vs 2.5%, \( P = 0.04 \)). Analysis of striatal D2R availability measured with positron emission tomography and \([11C]\text{raclopride} \) revealed higher levels of D2R in carriers of 4R/4R genotype \((P < 0.01)\). Taken together, these results provide preliminary evidence for the role of the PER2 gene in regulating striatal D2R availability in the human brain and in vulnerability for cocaine addiction.

Translational Psychiatry (2012) 2, e86; doi:10.1038/tp.2012.11; published online 6 March 2012

Introduction

Low striatal dopamine D2 receptor (D2R) levels have consistently been reported in cocaine abusers1–3 and animal models of addiction.4,5 In humans, low D2R density has been associated with decreased metabolism in the orbital frontal cortex.6 It was proposed that the altered dopamine (DA) signaling may lead to compulsive drug administration and, subsequently, to cocaine addiction.6–9 On the other hand, drugs of abuse, including cocaine, exert their reinforcing effects by enhancing signaling in the mesolimbic and nigrostriatal DA pathways (reviewed in ref.10), so that the chronic cocaine exposure can cause stable changes in gene transcription, mRNA translation and metabolism,11 thus further intensifying D2R loss.5

Marked differences in individual responses to both acute and chronic cocaine administration are likely to reflect an impact of individual genomic makeup that is modulated by environmental factors.12,13 Drug addiction is highly heritable (estimated heritability is about 0.72).14 As a complex non-Mendelian disorder, it is likely influenced by many genes;14 the known candidate genes cannot, however, explain this level of heritability.15 Among the most studied candidate genes for substance-use disorders16–18 is the DRD2 gene. Although the individual differences in the DRD2 expression are well established,16 it is not clear as to how the level of expression is regulated. Many human studies have focused on a single genetic variant, the DRD2/ANKK1 polymorphism (reviewed in ref.17), but inconsistency of the results of those studies18–20 creates the need to identify new genetic targets that have regulatory relationships with the DRD2.

Recent results from genetics of gene expression studies have demonstrated a role of polymorphic regulatory regions located in trans to the target gene.21 Regulation in trans by the clock genes, for example, leads to circadian oscillation of the expression of the majority of the human genes, including brain genes.22 Here we developed and tested the hypothesis that the PER2 (Period 2) clock gene might contribute to the regulation of D2R expression in the brain and, as such, might be associated with cocaine addiction, based on the following lines of evidence: (1) DA release in the striatum is subject to circadian oscillation;23 (2) PER2 modulates the reinforcing effects of cocaine in laboratory animals;24 and (3) individuals suffering from substance-use disorders25,26 have aberrant patterns of sleep and circadian rhythmicity.

In rats, the expression of clock genes and extracellular DA levels in the dorsal striatum27–29 exhibits daily oscillations. As striatal PER2 fluctuations are DA-sensitive,30 it is possible that the periodicity of DA release can mediate or be mediated by D2R–PER2 regulatory relationships.

Our understanding of the molecular mechanisms of the circadian clockworks has been largely based on the studies of...
model organisms and rodents. Even though circadian rhythms are ubiquitous, unique evolution of human lineage suggests that human clock genes might have distinct genomic features. That is, colonizing the world humans experienced highly diverse effects of new environments, notably, changes in photoperiodicity and seasonal rhythmicity. Specific populations thus had developed unique adaptations to particular climate zones and environments. Circadian periodicity is entrained by temperature and day–night cycle; those environmental inputs change in association with latitude, challenging the clock. It therefore seems likely that positive selection affected clock genes in modern humans, and, as such, these genes would have sequence characteristics and genomic features absent in ancestral lineage. Intra-species clock variations have distinct geographic pattern of distribution, suggesting that population-specific polymorphisms can enable highly adjustable regulation pattern required for population-specific adaptations.

The *PER2* locus has higher levels of inter-population genetic differentiation relative to other loci, suggesting a role for geographically restricted positive selection. Much lower than expected under a model of neutrality estimate of the coalescence age of the putatively selected *PER2*haplogroup, and relatively low FST values of the flanking polymorphic sites in both Africans vs Europeans and Africans vs Asian comparisons, both indicate that the *PER2* locus was affected by selection after the out-of-Africa expansion.

In humans, single-nucleotide polymorphisms (SNPs) in the *PER2* gene have been associated with abnormal circadian parameters, chronotypes, depression and also with enhanced alcohol intake, although no relationship with cocaine dependence was found in a case–control study that focused on several *PER2* SNPs.

The mutagenic potential of repeat regions is higher than that of single point mutations, making them more favorable for generating potentially ‘adaptable’ genetic substrates. Indeed, a correlation between a *PER2* repeat polymorphism (microsatellite) and latitude was reported in *Drosophila melanogaster*, where clinal distribution pattern of microsatellite alleles suggested that the latitudinal structure of this polymorphism may facilitate fine tuning of circadian oscillation. We postulated that putatively polymorphic repeat variation in the human *PER2* might enable environmentally influenced transcriptional regulation in a population-specific manner. Here, we report evidence supporting this hypothesis. We identified and characterized new variable number tandem repeat (VNTR) polymorphism in intron 3 of the *PER2* by comparing the prevalence of the VNTR alleles among African Americans and non-Africans, and among healthy controls and cocaine abusers. We also assessed the effect of this polymorphism on striatal D2R expression using brain imaging data (obtained with positron emission tomography (PET) using the D2R–radioligand [*11C*]raclopride). We found an association between the *PER2* genotype and striatal D2R availability, as well as significant differences in VNTR allele frequencies between cocaine abusers and healthy controls that, in combination, point out that this genetic variation can contribute to vulnerability for cocaine addiction and other disorders of behavioral control that are associated with low striatal D2R levels (such as, obesity).

Multi-allelic genetic markers, such as the *PER2* intron 3 VNTR, provide information on the evolutionary history of populations and help to identify the loci targeted by selection. Previously, signals for population-specific selection acting on the *PER2* gene were detected using a SNP-based analysis. Our VNTR-based analyses revealed significantly different patterns of *PER2* alleles and genotypes across ethnic groups; this finding, along with population-specific SNPs and FST values and local recombination rate, points to the involvement of positive selection in the evolution of this gene.

Materials and methods

Bioinformatic analyses. We used SERV server (http://www.igs.cnrs-mrs.fr/SERV/) to estimate repeats variability and suitability for genotyping. For assessment of the signs of selection, we used the Human Genome Diversity Panel browser (http://hgdpcgi-bin/gbroense/HGD/P/), which is based on the data from the Human Genome Diversity Panel CEPH and the Phase II HapMap. Population-specific SNPs were obtained using Genome Variation Server (http://gvs.gs.washington.edu/GVS/).

Participants. A population sample included 509 unrelated individuals originally recruited for different imaging studies who also agreed to participate in a genetic study approved by the IRB of Stony Brook University. Written informed consent was obtained from each subject after the study had been fully explained to them. All demographic data and clinical measures were acquired via the original imaging study. Race and ethnicity was defined by self-assignment.

DNA extraction and genotyping. DNA was extracted from the blood cells using the PAXgene kit (Qiagen, Germantown, MD, USA). The following primers (forward: 5′-TTGGGTATAGCGGTGA-3′ and reverse: 5′-CTAGGTGTCTTTCC TGA-3′) were used to amplify the region encompassing VNTR (>chr2: 239 184 578–239 185 009). The size of the polymerase chain reaction (PCR) products was established by QIAxcel system of a multi-capillary electrophoresis. Individual genotype assignment was carried out based on the PCR results, wherein the alleles were categorized as four repeat ‘4R’ (333 bp product), three repeat ‘3R’ (277 bp) and two repeat ‘2R’ (212 bp).

PET imaging data. The [*11C*]raclopride values associated with the genetic samples were retrieved from the imaging data set of the BNL Brain Imaging Center for studies performed over the period 2006–2010. All PET scans used in this study were performed on a Siemens, HR scanner. The procedures for subjects positioning and the scanning protocols were described previously and the imaging results have been reported. In this study, we used regional B_max/KO values calculated for three regions of interest: caudate, putamen and ventral striatum.

Statistical analysis. Because the genetic model for this locus is unknown, we first used four-genotype classification, where the carriers of each observed genotype formed a
genotype group. That is, four genotype groups represented three common genotypes: ‘4R/4R’, ‘4R/3R’ and ‘3R/3R’ and ‘rare’, which comprised of carriers of 2R allele. PER2 genotypes were evaluated with regard to the Hardy–Weinberg equilibrium (HWE) using the OEGE software http://www.oeges.org/software/hwe-mr-calc.shtml and popgen genetic aptplet http://www.husdyr.kvl.dk/htm/kc/popgen/genetik/applets/ktttest.htm.

All individuals participating in the imaging study were African Americans; therefore, in exploratory analyses of imaging data, we tested potential confounding effects of sex and age. Sex differences in striatal D2R binding were not significant (t-test), but age negatively correlated with D2R availability at \( P < 0.01 \) level (t-test, two-tailed) as has been reported previously.\(^{45}\) Fitting a general linear model showed low importance of the gender with respect to the D2R levels \( P = 0.342 \) and, as expected,\(^{45}\) a strong effect of age \( P < 0.001 \); hence, age was included in a model. Analysis of the relationships between the D2R brain availability and the \( \text{PER2} \) allele frequencies in the population sample \( (N = 286) \) and non-African \( (N = 223) \) subsamples. Because of the low frequency of the 2R allele, the individuals who carry 2R allele(s) were assigned to the genotype group ‘rare’.

The frequencies of the VNTR-based genotypes showed statistically significant differences between African Americans and non-Africans \( (\chi^2 = 81.1, \text{d.f.} = 3, P < 0.001) \). Minor allele frequency (3R) was 0.22 in the African Americans and only 0.056 in non-Africans. Table 2 shows the observed genotype count and population-specific genotype frequencies in percentiles.

The \( \text{PER2} \) genotype frequencies deviated from the values expected under HWE; we detected this deviation both by analyzing the sample as a whole and testing African-American and non-African subsamples separately. The results of HWE calculations \( (\chi^2\text{-test}) \) using the OEGE software (bi-allelic markers) and popgen genetic aptplet (multi-allelic markers) are shown in Table 3a and b, respectively.

A deficit of heterozygotes in relation to HWE, as it is observed in our sample, points to non-random mating or population stratification rather than to genotyping errors;\(^{46}\) nonetheless, we validated the PCR results by testing more than 300 samples as duplicates and triplicates, as well as tested half of the samples (about 250) using a different PCR system and protocols (not shown). Test–retest procedure confirmed genotyping assignment with a 100% accuracy. A large number of cocaine abusers in our sample (see below) suggested a possibility that bias distribution of the \( \text{PER2} \) genotypes was due to the disease. When we tested HWE only in healthy control groups within the ethnic subsamples, we found a good agreement between the observed and the expected frequencies (African Americans: \( \chi^2 = 1.57, P > 0.1 \); non-Africans: \( \chi^2 = 2.79, P > 0.05 \)).

Unequal distribution of VNTR genotypes in healthy controls and cocaine users. We next examined a potential effect of the VNTR polymorphism on the risk for cocaine addiction by comparing \( \text{PER2} \) genotype frequencies in healthy controls and in cocaine abusers. Considering the differences in genotype distribution patterns, we ran separate analyses for the ethnic subsamples. Table 4 shows the absolute numbers (actual count) of the genotypes in the groups of healthy controls and cocaine abusers and Figure 2 illustrates the differences in the patterns of genotype distribution between the disease groups in two populations. The relationship between the \( \text{PER2} \) genotype (four genotypes classification) and diagnosis was statistically significant in both subsamples (African Americans: \( \chi^2 = 8.63, \text{d.f.} = 3, P = 0.035 \); non-Africans: \( \chi^2 = 8.25, \text{d.f.} = 3, P = 0.041 \)).

As clearly seen from Figure 2, in African Americans the proportion of 4R/3R carriers in cocaine abusers is much higher than that in healthy controls (39% vs 23%). This difference is statistically significant: \( \chi^2 = 8.2, \text{d.f.} = 1, P = 0.004 \). It appears that the 4R/3R genotype increases a risk for cocaine addiction, but only in the American-African population. In non-Africans, in contrast, 10.1% of healthy controls but only 2.5% of cocaine abusers carry 4R/3R genotype; thus, 4R/3R heterozygotes seem to be protected. Because most non-African individuals carry two major (4R)}
alleles and the frequencies of minor alleles are very low, we applied a binomial analysis comparing the 4R/4R homozygotes with the carriers of at least one minor allele (two genotypes classification). Under this partitioning, the interaction between the PER2 genotype and diagnosis (controls vs cocaine abusers) for the whole sample was

Table 1 Demographic characteristics of the sample

|                | N     | Male       | Female     | Healthy controls | Cocaine abusers |
|----------------|-------|------------|------------|------------------|-----------------|
| African Americans | 286   | 208 (72.7%)| 76 (26.6%) | 174 (60.8%)      | 112 (39.3%)     |
| Caucasians      | 154   | 115 (74.7%)| 39 (25.3%) | 128 (83.1%)      | 26 (16.9%)      |
| Hispanics       | 61    | 39 (63.9%) | 22 (36.1%) | 51 (83.6%)       | 10 (16.4%)      |
| Asians          | 8     | 3 (37.5%)  | 5 (62.5%)  | 7 (87.5%)        | 1 (12.5%)       |
|                 | 509   |            |            |                  |                 |

Values are shown in actual numbers and percentiles (within parenthesis).
significant \( \chi^2 = 16, \text{d.f.} = 1, P < 0.001 \). Separate analysis of ethnic subsamples revealed, however, that the genotype effect remains significant only in the African-American subsample \( \chi^2 = 5.6, \text{d.f.} = 1, P = 0.002 \), and does not reach a significance level in the non-African subsample \( \chi^2 = 0.7, \text{d.f.} = 1, P = 0.4 \).

**PER2 allele frequency in different populations is likely driven by geography.** To explain the high divergence in VNTR-based allele frequencies between the populations in our sample, we explored the possibility that it might be due to geography-driven selection. Our inspection of the 100 kb genomic locus encompassing the **PER2** revealed that the highest \( F_{ST} \) score \( F_{ST} \) is commonly used as an estimate to measure the degree of genetic differentiation among populations\(^47\) is in the vicinity of intron 3 VNTR (Figure 3). Also, we noted an abrupt change in population heterozygosity that coincided with the VNTR region (circled). The degree of population differentiation can be inferred from a set of population-specific SNPs classified by their physical location and functional impact. Under the assumption of neutrality, the degrees of differentiation should be the same; any deviation from this expectation could be attributed to selection.\(^48\) Indeed, the variants leading to amino-acid changes (non-synonymous mutations) are over-represented among SNPs showing high levels of \( F_{ST} \); hence, an excess of non-synonymous SNPs in one population compared with the other points out to population-specific action of natural selection.\(^48\)

**Table 2** **PER2** genotype frequencies in subpopulations

| Genotype       | Total | African Americans | Non-Africans |
|----------------|-------|-------------------|--------------|
|                | 4R/4R | 4R/3R | 3R/3R | 4R/2R | 3R/2R | 2R/2R |
| African Americans \( N = 286 \) | 148 (51.1%) | 85 (30.1%) | 20 (7.1%) | 22 (7.8%) | 5 (1.8%) | 6 (2.1%) |
| Non-Africans \( N = 223 \) | 197 (88.2%) | 20 (8.7%) | 3 (1.3%) | 3 (1.3%) | 0 | 1 (0.4%) |

Abbreviation: **PER2**, Period 2.

**Table 3** HWE equilibrium testing

| Genotype       | Sample | African Americans | Non-Africans |
|----------------|--------|-------------------|--------------|
|                | Obs    | Exp   | Obs    | Exp   | Obs    | Exp   |
| (a) Multi-allelic model |       |       |       |       |       |       |
| 4R/4R          | 346    | 331   | 144   | 138   | 202    | 199   |
| 4R/3R          | 105    | 125   | 85    | 91    | 20     | 24    |
| 3R/3R          | 23     | 12    | 20    | 15    | 3      | 1     |
| 4R/2R          | 25     | 35    | 22    | 27    | 3      | 5     |
| 3R/2R          | 5      | 7     | 5     | 9     | 0      | 0     |
| 2R/2R          | 7      | 1     | 6     | 1     | 1      | 0     |
| \( \chi^2 \)   | 56.5   |       | 21.1  |       | 43.3   |       |

(b) Bi-allelic model

| Genotype       | Sample | African Americans | Non-Africans |
|----------------|--------|-------------------|--------------|
|                | Obs    | Exp   | Obs    | Exp   | Obs    | Exp   |
| 4R/4R          | 346    | 334.05 | 144   | 138.75 | 202    | 199.75|
| 4R/3R          | 105    | 126.9 | 85    | 93.5 | 20     | 24.5  |
| 3R/3R          | 23     | 12.05 | 20    | 15.75 | 3     | 0.75  |
| \( \chi^2 \)   | 14.08  |       | 2.05  |       | 7.58  |       |

Abbreviations: Exp, expected; HWE, Hardy–Weinberg equilibrium; Obs, observed.

HWE was calculated using popgen genetic applet (a, multi-allelic markers) and OEGE software (b, bi-allelic markers).

**Table 4** **PER2** genotypes distribution in healthy controls and cocaine abusers

| Genotype       | Total | African Americans | Non-Africans |
|----------------|-------|-------------------|--------------|
|                | 4R/4R | 4R/3R | 3R/3R | Rare |
| African Americans |       |       |       |      |
| Healthy controls | 100   | 41    | 13    | 20   | 174  |
| Cocaine abusers  | 48    | 44    | 7     | 13   | 112  |
| Count           | 148   | 85    | 20    | 148  | 286  |
| Non-Africans    |       |       |       |      |
| Healthy controls | 164   | 19    | 1     | 4    | 188  |
| Cocaine abusers  | 36    | 1     | 2     | 0    | 39   |
| Count           | 200   | 20    | 3     | 4    | 227  |

Abbreviation: **PER2**, Period 2.

significant \( \chi^2 = 16, \text{d.f.} = 1, P < 0.001 \). Separate analysis of ethnic subsamples revealed, however, that the genotype effect remains significant only in the African-American subsample \( \chi^2 = 5.6, \text{d.f.} = 1, P = 0.002 \), and does not reach a significance level in the non-African subsample \( \chi^2 = 0.7, \text{d.f.} = 1, P = 0.4 \).

**PER2 allele frequency in different populations is likely driven by geography.** To explain the high divergence in VNTR-based allele frequencies between the populations in our sample, we explored the possibility that it might be due to geography-driven selection. Our inspection of the 100 kb genomic locus encompassing the **PER2** revealed that the highest \( F_{ST} \) score \( F_{ST} \) is commonly used as an estimate to measure the degree of genetic differentiation among populations\(^47\) is in the vicinity of intron 3 VNTR (Figure 3). Also, we noted an abrupt change in population heterozygosity that coincided with the VNTR region (circled). The degree of population differentiation can be inferred from a set of population-specific SNPs classified by their physical location and functional impact. Under the assumption of neutrality, the degrees of differentiation should be the same; any deviation from this expectation could be attributed to selection.\(^48\) Indeed, the variants leading to amino-acid changes (non-synonymous mutations) are over-represented among SNPs showing high levels of \( F_{ST} \); hence, an excess of non-synonymous SNPs in one population compared with the other points out to population-specific action of natural selection. Comparison of the population-specific SNPs in the **PER2** locus (YRI vs CEU) showed an excess of non-synonymous SNPs (3 vs none) in CEU (Supplementary Table 1). This finding is quite unusual, given that the African populations show a higher level of genetic and nucleotide diversity than the European population.\(^49,50\)

Taken together, these results indicate that the **PER2** locus is under selection pressure that can act discernibly on some populations and not on the others; hence, pronounced differences in **PER2** allele frequencies among populations are rather expected.

**PER2 genotype effect on striatal DRD2 binding potential.** Finally, we assessed an effect of the **PER2** VNTR polymorphism on brain endophenotype, using the baseline measures (non-stimulated) of striatal D2R availability...
or non-displaceable binding potential (BP_{ND}) for [11C]raclopride, as a proxy for brain D2R phenotype (for simplicity, herein we refer to it as D2R availability). For statistical reasons (high prevalence of the 4R/4R genotype in non-Africans diminished the power to detect an association), we included in this analysis only African-Americans individuals. The brain imaging data were available for 52 subjects (43 males and 9 females; age: 20–51 (mean: 35 ± 8.9) years). The analysis of covariance revealed that the effect of the PER2 genotype, corrected for age, on D2R binding was significant in all striatal regions (Table 5).

Comparison of the genotype groups (four genotype classification) revealed that the 3R/4R heterozygotes' and carriers' rare allele had lower D2R binding across the brain regions relative to either 4R or 3R homozygotes (Figure 4a).

Representative PET scans of male carriers of 4R/4R and 4R/3R genotypes that participated in the same study are shown in Figure 4b.
Table 5 Striatal D2R binding in PER2 genotype groups

| PER2 genotype | 4R/4R | 4R/3R | 3R/3R | Rare | F (d.f.) | P-value | Nonicept | Observed power |
|---------------|-------|-------|-------|------|---------|---------|----------|---------------|
| Caudate       | 2.7 ± 0.06  | 2.54 ± 0.06 | 2.76 ± 0.17 | 2.14 ± 0.2 | (7.44) = 9.18 | <0.001 | 64.3 | 1 |
| Putamen       | 3.31 ± 0.07 | 3.08 ± 0.1 | 3.46 ± 0.22 | 2.66 ± 0.25 | (7.44) = 8.46 | <0.001 | 59.4 | 1 |
| Ventral striatum | 2.91 ± 0.09 | 2.55 ± 0.13 | 3.02 ± 0.25 | 2.31 ± 0.29 | (7.36) = 4.13 | 0.002 | 28.9 | 0.97 |

Abbreviations: ANCOVA, analysis of covariance; D2R, dopamine D2 receptor; d.f., degrees of freedom; PER2, Period 2.
Values of DRD2 binding in striatal regions by PER2 genotype groups are shown as model adjusted means ± s.e. based on the non-transformed DRD2 binding data. *F*-value, *P*-value (significance) and observed power (computed using *z* = 0.05) values are from ANCOVA. *η* is a nonicept parameter.

![Figure 4](image_url)  
**Figure 4** Effect of the PER2 (Period 2) genotypes on striatal D2R receptor (D2R) binding. (a) The baseline (non-stimulated) measures of striatal D2R availability in PER2 genotype groups in three striatal regions. Y axis shows non-displaceable binding potential BP_{ND}, for [11C]raclopride. Bars correspond to mean D2R availability and standard errors of means in PER2 genotype groups in caudate, putamen, and ventral striatum. (b) Representative PET scans of the carriers of PER2 4R/4R and 4R/3R genotypes. Normalized to the SPM template parametric images of the [11C]raclopride PET scans. Transaxial planes at the level of the striatum of individual with 4R/3R genotype (upper panel) and individual with 4R/4R genotype (bottom panel). Rainbow color scale indicates D2R availability: red shows region with the highest and blue with the lowest receptor levels. Note more red color on the planes 3, 4 and 5 of the right panel compared with the left one, corroborating the differences in the striatal binding between the genotype groups (Table 4 and Figure 3).

Discussion

The aim of this study was to interrogate the genomic region of the human PER2 gene for polymorphic repeats and to investigate the phenotypic effects of its putative variations on D2R expression and its association with cocaine addiction. We discovered a new VNTR polymorphism in intron 3 of the PER2 gene and found preliminary evidence that this variation is associated with striatal D2R availability, that is, heterozygous carriers of the shorter, 3R allele, or carriers of rare alleles had lower D2R availability in the striatum compared with 4R homozygotes. Comparison of the PER2 allele and genotype frequencies in healthy controls and cocaine abusers revealed significant differences in their patterns, where the shorter alleles (3R and 2R) were enriched in the group of cocaine abusers. These two findings are in line with the consistent finding of reduced striatal DRD2 availability in drug addiction.3

Observed differences between the populations in PER2 genotype by disease interactions suggest that genetic model of this polymorphism can be population-specific. It seems that effect of the 3R allele in different populations is opposing, so that African-American 3R/4R heterozygotes have higher risk for cocaine abuse, whereas in non-Africans the same allele is over-represented among healthy controls (Figure 2).

To our knowledge, this is the first report of an association between a polymorphism in a circadian gene PER2 (VNTR in intron 3) and striatal D2R availability in the human brain and with cocaine addiction. Our findings are consistent with those from prior preclinical studies,24,51 which showed that PER2 modulates the rewarding responses to cocaine in rodents. However, they differ from the results of a prior clinical study36 that failed to detect an association between PER2 polymorphisms and drug addiction. This is not a contradiction, however, as the later study was based on SNPs analysis, whereas our analysis was focused on a new VNTR polymorphism of the PER2. Furthermore, Malison and others studied heterogeneous population, where African-American individuals were disproportionately over-represented among cocaine abusers (46%) and under-represented among healthy controls (12%); that bias may have limited their ability to detect differences. Here, we accounted for the effect of ethnicity, and to avoid such bias, analyzed African-American and non-African populations separately.

Our findings provide new insight into a mechanism by which drug-induced disruptions in DA signaling and related changes in the pattern of clock genes expression might lead to the development of pathological motivational states such as drug addiction. New evidence obtained in human populations advances our current understanding of counter-regulation of...
the clock genes and DA-mediated reward processes that are based on the investigation of rodents.\textsuperscript{24,52,53} Our results can also be interpreted in a context of pathogenicity of Parkinson’s disease. In patients with Parkinson’s disease, severe DA depletion in the striatum\textsuperscript{54} was observed in association with dysregulation of clock genes expression and disruptions of daily behavioral and physiological rhythms.\textsuperscript{55,56}

An earlier study by Cruciani et al.\textsuperscript{31} reported a sign of population-specific selection acting on the \textit{PER2} gene that they detected by analysis of a set of SNPs. Our findings are also consistent with population-specific positive selection and, using a new multi-allelic genetic marker, allowed us to detect statistically significant differences in frequency of \textit{PER2} alleles and heterozygosity between populations. We noted hAT-Charlie family DNA retrotransposon (MER20) residing immediately upstream of the intron 3 VNTR. Recent theoretical and experimental studies have characterized transposable elements as potentially advantageous generators of variations, which enable the action of natural selection (reviewed in ref. 57); thus, the MER20 Eutherian-specific sequence elements play a central role in rewiring the gene-regulatory landscape.\textsuperscript{58} In light of such evidence and considering that unusual genotype patterns, homozygosity and extreme values of $F_{ST}$ are indicative of recent adaptation,\textsuperscript{59} our results support the action of positive selection on the human \textit{PER2} gene.

This study has several aims and implements different analytical tools and modalities. We predicted a new polymorphism in the \textit{PER2} gene and validated it; assessed \textit{PER2} allele distribution across ethnic groups corroborating prior evidence for geography-driven positive selection; determined differences in genotype patterns between healthy controls and cocaine users; and finally, tested a genotype effect on the levels of striatal D2R availability—a hallmark for addiction vulnerability. This approach is different from traditionally more focused studies, but we believe that our characterization of a new polymorphism is comprehensive and informative. Moreover, previous preclinical data obtained on animal models are consistent with our findings, supporting their reliability.

\textbf{Study limitations}

We want to emphasize that our analysis was not aimed to establish causative relationships between the \textit{PER2} and the D2R, as this would require a different experimental design. Our population sample is ethnically heterogeneous, rather than include carefully matched cases and controls, it comprises participants from different brain imaging studies performed in our laboratory; it does, however, adequately represent the diverse population of Long Island, NY, USA.

Because of the relatively small sample size of our imaging study (as is the case for most imaging genetic studies), we consider our finding of an association between variations in the \textit{PER2} gene and brain D2R availability as preliminary and in need of replication.

We did not include in our analysis traditional markers for the \textit{PER2} locus, as a prior study showed lack of an association with cocaine addiction.\textsuperscript{36} And lastly, considering that D2R availability is influenced by environmental exposures including drugs,\textsuperscript{60} we did not expect that the link between striatal D2R availability and cocaine addiction could be fully explained by \textit{PER2} genotype.

In summary, here we identified a new VNTR polymorphism in the \textit{PER2} gene (third intron) and showed that the \textit{PER2} variances have different prevalence across ethnic groups. We detected a link between the polymorphism and D2R brain levels and significant differences in VNTR allele frequencies between CA and HC that, in combination, point out that this genetic variation can contribute to vulnerability for cocaine addiction and, perhaps, other disorders associated with low striatal D2R levels. These findings broaden our understanding of the complex networks that regulate brain dopaminergic transmission, by considering counter-regulation between the circadian clock and D2R-mediated dopamine signaling.

\textbf{Conflict of interest}

The authors declare no conflict of interest.

\textbf{Acknowledgements}

This study was supported by National Institute on Drug Abuse (NIDA) Grants KO1 DA025280 (to ES) and R01DA023579 (to RZG). We are grateful to BNL Center for Translational Neuroimaging team for PET operation, to Dr Frank Telang, RNs Barbara Hubbard and Millard Jayne for collecting blood samples and to Karen Apelisik-Torres, AA, for protocol coordination. We also thank the individuals who volunteered to participate in this study.

1. Volkow ND, Fowler JS, Wolf AP, Schwyer D, Shuie CY, Alpert R et al. Effects of chronic cocaine abuse on postsynaptic dopamine receptors. \textit{Am J Psychiatry} 1990; 147: 719–724.
2. Volkow ND, Wang GJ, Fowler JS, Logan J, Galdef SJ, Gifford A et al. Prediction of reinforcing responses to psychostimulants in humans by brain dopamine D2 receptor levels. \textit{Am J Psychiatry} 1989; 156: 1440–1443.
3. Volkow ND, Wang GJ, Fowler JS, Tomasi D. Addiction circuitry in the human brain. \textit{Annu Rev Pharmacol Toxicol} 2011.
4. Flores G, Wood GK, Barbeau D, Quirion R, Sivastava LK. Lewis and Fischer rats: a comparison of dopamine transporter and receptors levels. \textit{Brain Res} 1998; 814: 34–40.
5. Dalley JW, Fryer TD, Richard L, Robinson ES, Theobald DE, Laane K et al. Nucleus accumbens D2R receptors predict trait impulsivity and cocaine reinforcement. \textit{Science (New York, NY)} 2007; 315: 1267–1270.
6. Volkow ND, Fowler JS, Wang GJ, Hintzemann R, Logan J, Schwyer DJ et al. Decreased dopamine D2 receptor availability is associated with reduced frontal metabolism in cocaine abusers. \textit{Synapse (New York, NY)} 1993; 14: 162–177.
7. Volkow ND, Fowler JS, Addiction, a disease of compulsion and drive: involvement of the orbitofrontal cortex. \textit{Cereb Cortex} 2000; 10: 318–325.
8. Lee B, London ED, Poldrack RA, Farah J, Nacca A, Monterosso JR et al. Striatal dopamine d2/d3 receptor availability is reduced in methamphetamine dependence and is linked to impulsivity. \textit{J Neurosci} 2009; 29: 14734–14740.
9. Volkow ND, Wang GJ, Fowler JS, Tomasi D, Telang F. Addiction: beyond dopamine reward circuitry. \textit{Proc Natl Acad Sci USA} 2011; 108: 15037–15042.
10. Wise RA. Roles for mesocorticolimbic—not just mesocorticostriatal—dopamine in reward and addiction. \textit{Trends Neurosci} 2009; 32: 517–524.
11. Schafer A, Im HJ, Vento MT, Fowler CD, Min A, Intrator A et al. Argonaute 2 in dopamine 2 receptor-expressing neurons regulates cocaine addiction. \textit{J Exp Med} 2010; 207: 1843–1851.
12. van der Kam EL, Ellenbroek BA, Cools AR. Gene–environment interactions determine the individual variability in cocaine self-administration. \textit{Neuropsychopharmacology} 2005; 30: 685–695.
13. Alia-Klein N, Pavzan MA, Wochik PA, Konova AB, Maloney T, Shumay E et al. Gene × disease interaction on orbitofrontal gray matter in cocaine addiction. \textit{Arch Gen Psychiatry} 2011; 68: 283–294.
14. Goldmann D, Onosz G, Ducci F. The genetics of addictions: uncovering the genes. \textit{Nat Rev Genet} 2005; 6: 521–532.
15. Volkow ND, Li TK. Drug addiction: the neurobiology of behaviour gone awry. \textit{Nat Rev Neurosci} 2004; 5: 963–970.
16. Moyar RA, Wang D, Papp AC, Smith RM, Duque L, Mash DC et al. Intronic polymorphisms affecting alternative splicing of human dopamine D2 receptor are associated with cocaine abuse. \textit{Neuropsychopharmacology} 2011; 36: 753–762.
17. Noble EP. D2 dopamine receptor gene in psychiatric and neurologic disorders and its phenotypes. \textit{Am J Med Genet B} 2003; 116B: 103–125.
35. Yuferov V, Bart G, Kreek MJ. Clock reset for alcoholism. *Neurosci Lett* 2010; 473: 87–91.
36. Fernandez-Castillo N, Ribases M, Roncero C, Casas M, Gonzalvo B, Combard M. Association study between the DAT1, DRD2 and BDNF genes and cocaine dependence in a Spanish sample. *Psychiatric Genet* 2010; 20: 317–323.
37. Balon N, Leroy S, Roy C, Boudrel MC, Olle JP, Charles-Nicolas A et al. Polymorphisms Tag A of the DRD2, Ball of the DRD3, exon III repeat of the DRD4, and 3′ UTR VNTR of the DAT: association with childhood ADHD in male African-Caribbean cocaine dependents? *Am J Med Genet B* 2007; 144B: 1034–1041.
38. Cheung VG, Spielman RS, Evans KG, Weber TM, Morley M, Burdick JT. Mapping determinants of human gene expression by regional and genome-wide association. *Nature* 2005; 437: 1365–1369.
39. Sukumaran S, Almon RR, Dubois DC, Jusko WJ. Circadian rhythms in gene expression: relationship to physiology, disease, drug disposition and drug action. *Adv Drug Deliv Rev* 2010; 62: 904–917.
40. Castaneda TR, de Prado BM, Prieto D, Mora F. Circadian rhythms of dopamine, glutamate and GABA in the striatum and nucleus accumbens of the awake rat: modulation by light. *J Pineal Res* 2004; 36: 177–185.
41. Abarca C, Albrecht U, Spanagel R. Cocaine sensitization and reward are under the influence of circadian genes and rhythm. *Proc Natl Acad Sci USA* 2002; 99: 9026–9030.
42. Kowatch RA, Schnall SS, Krisjy JS, Green D, Elwack RK. Electroencephalographic sleep and mood during cocaine withdrawal. *J Addict Dis* 1992; 11: 21–45.
43. Morgan PT, Pace-Schott EF, Sahul ZH, Coric V, Stockigt R, Malsain RT. Sleep architecture, circadian and visual learning. *Addiction (Alcohol. England) 2008; 103: 1344–1352.
44. Hoods S, Caddy P, Cossette MP, Weigl Y, Verwey M, Robinson B et al. Endogenous dopamine regulates the rhythm of expression of the clock protein PER2 in the rat dorsal striatum via daily activation of D2 dopamine receptors. *J Neurosci 2010; 30: 14046–14056.
45. Imbesi M, Yildiz S, Dirm Aslan A, Sharma R, Maner H, Uz T. Dopamine receptor-mediated regulation of neuronal ‘clock’ gene expression. *Neuroscience 2009; 158: 537–544.
46. Sahar S, Zocchi L, Kivoschta C, Borrelli E, Sassone-Corsi P. Regulation of BMAL1 protein stability and circadian function by GSK3beta-mediated phosphorylation. *PLoS One* 2010; 5:
47. Kyriacou CP, Peixoto AA, Sandrelli F, Costa R, Tauber E. Clones in clock genes: fine-tuning circadian rhythms to the environment. *Trends Genet* 2008; 24: 124–132.
48. Volkow ND, Wang GJ, Fowler JS, Logan J, Gatley SJ, MacGregor RR et al. Measuring age-related changes in dopamine D2 receptors with 18F- and 11C-raclopride. *Psychiatry Res* 1996; 67: 11–16.
49. Ryckman K, Williams SM. Calculation and use of the Hardy–Weinberg model in association studies. In: Haines JL et al. (eds.). *Current Protocols in Human Genetics, 57:1.18.1-1.18.11.* 2005 by John Wiley & Sons, Inc.
50. Stajich JE, Hahn MW. Disentangling the effects of demography and selection in human history. * Mol Biol Evol 2005; 22: 63–73.
51. Selin MF, Pasanuc B, Price AL. New approaches to disease mapping in admixed populations. *Nat Rev Genet 2011; 12: 523–528.
52. Miller BH, McDeammon EL, Panda S, Hayes KR, Zhang J, Andrews JL et al. Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. *Proc Natl Acad Sci USA 2007; 104: 3342–3347.
53. McClaugh CA, Nesterl J, Zachariou V. Regulation of gene expression by chronic morphine and its influence on the locus coeruleus and ventral tegmental area. *J Neurosci 2005; 25: 6005–6015.
54. Roybal K, Theobald D, Graham A, DiNieri JA, Russo SJ, Krishnan V et al. Mania-like behavior induced by disruption of CLOCK. *Proc Natl Acad Sci USA 2007; 104: 6406–6411.
55. Day M, Wang Z, Ding J, Ao X, Ingham CA, Shering AF et al. Selective elimination of glutamatergic synapses on striatopallidal neurons in Parkinson disease models. *Nat Neurosci 2006; 9: 251–259.
56. Bruguerolle B, Simon N. Biologic rhythms and Parkinson's disease: a chrono-pharmacologic approach to considering fluctuations in function. *Clin Neuropharmacol 2002; 25: 194–201.
57. Cai Y, Liu S, Sohmen RB, Xu S, Chan P. Expression of clock genes Per1 and Bmal1 in total leukocytes in health and Parkinson's disease. *Eur J Neurool 2010; 17: 550–554.
58. Oliver KR, Greene WK. Transposable elements: powerful facilitators of evolution. *BoEsaia 2009; 31: 703–714.
59. Lynch VJ, Leclerc RD, May G, Wagner GP. Transposon-mediated rewing of gene regulatory networks contributed to the evolution of pregnancy in mammals. *Nat Genet 2011; 43: 1154–1159.
60. Hawks J, Wang ET, Cochran GM, Harpending HC, Moyzis RK. Recent acceleration of human adaptive evolution. *Proc Natl Acad Sci USA 2007; 104: 20753–20758.
61. McCinty JF, Shi XD, Schwindt M, Saylor A, Toda S. Regulation of psychostimulant-induced signaling and gene expression in the striatum. *J Neurochem 2008; 104: 1440–1449.

**Translational Psychiatry** is an open-access journal published by Nature Publishing Group. This work is licensed under the Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/3.0/

Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tpp)