Addiction-related Genes in Gambling Disorders: new insights from parallel human and pre-clinical models

Running Title: Addiction genes in gambling: human and rat models

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Abstract:

Neurobiological research supports the characterization of disordered gambling (DG) as a behavioral addiction. Recently, an animal model of gambling behavior was developed (rat gambling task – rGT), expanding the available tools to investigate DG neurobiology. We investigated whether rGT performance and associated risk gene expression in the rat's brain could provide cross-translational understanding of the neuromolecular mechanisms of addiction in DG. We genotyped *tag*SNPs in 38 addiction-related genes in 400 DG and 345 non-DG subjects. Genes with $p<0.1$ in the human association analyses were selected to be investigated in the animal arm to determine whether their mRNA expression in rats was associated with the rat's performance on the rGT. In humans, DG was significantly associated with *tag*SNPs in *DRD3* (rs 167771) and *CAMK2D* (rs3815072). Our results suggest that age and gender might moderate the association between *CAMK2D* and DG. Moderation effects could not be investigated due to sample power. In the animal arm, only the association between rGT performance and *Drd3* expression remained significant after Bonferroni correction for 59 brain regions. As male rats were used, gender effects could not be investigated.

Our results corroborate previous findings reporting the involvement of DRD3 receptor in addictions. To our knowledge, the use of human genetics, pre-clinical models and gene expression as a cross-translation paradigm has not previously been attempted in the field of addictions. The cross-validation of human findings in animal models is crucial for improving the translation of basic research into clinical treatments, which could accelerate neurobiological and pharmacological investigations in addictions.

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Key-words: disordered gambling, pathological gambling, rat gambling task, genetics, gene expression, rs 167771, rs3815072.

Introduction

The worldwide expansion of legalized gambling has resulted in increased gambling-related harm, as observed by higher rates of bankruptcy, divorce and suicide secondary to excessive gambling involvement [1]. It is estimated that 0.6 to 1% [1, 2] of the population in the United States and Canada meet DSM-IV criteria for pathological gambling (PG). However, individuals who do not reach the DSM-IV diagnostic threshold also experience negative consequences from their gambling behavior [3, 4]. Thus, in keeping with the recent literature [5], we will use the term disordered gambling (DG) to characterize the full spectrum of gambling-related disorders, including PG and sub-clinical PG. The term PG will be reserved for the specific DSM-IV definition of the disorder.

Neurobiological research in DG has advanced in the last two decades, supporting its characterization as a behavioral addiction [6]. Studies show that DG and substance addictions share, at least in part, dysfunctions in similar brain regions as well as performance on neuropsychological tasks [7]. For instance, similar deficits have been reported for both DG and substance addiction in assessments of inhibitory responses [8] and reflection impulsivity [9]. In particular, DG and substance dependent subjects present deficits in the Iowa Gambling Task (IGT) [8, 10, 11], which assesses decision-making mediated by the pre-frontal cortex [12]. In regards to genetic vulnerability, it is estimated that as much as 74% of the overlap between DG and alcohol dependence are accounted for by genetic factors in men [13].

Two large twin studies estimated that genetic factors account for 50 to 60% of the vulnerability for developing DG [14, 15], showing that clinical and subclinical forms of the disorder are part of a single genetic continuum. However, there has been little advance in the understanding of the molecular genetic underpinnings of DG [16]. Most molecular genetic studies in DG were performed in small samples and investigations have focused on the dopamine and

serotonin system [16, 17]. Recently the first genome-wide association study in DG was published [18], and association trends were found with metaxin, ataxin and the very low density lipoprotein receptor genes. In addition, a canonical pathway analysis revealed that several addiction-related pathways presented a higher frequency of SNPs nominally associated with DG. Finally, the authors report that SNPs associated with DG were more frequent amongst candidate genes for dopamine-agonist induced PG in Parkinson's disease.

The combination of human genetic and animal research in substance addictions has resulted in a better understanding of neural pathways involved in the development and maintenance of addiction-related processes [19, 20]. Recently, animal models of "gambling-like" behavior using a risky decision-making paradigm have been developed [21, 22]. The utility of these animal models for the understanding of DG and for pre-clinical investigation of pharmacological treatments depends on how findings in humans and animals can be replicated across species [23].

Here, we have combined a molecular genetic association study in humans with an *in situ* hybridization study in brains of rats submitted to an animal model of gambling behavior, the Rat Gambling Task (rGT) [22]. Our goal was to investigate whether this three-component paradigm could provide preliminary support for human genetic findings in DG.

Participants and Methods

Human arm

Subject recruitment: Subjects were recruited in the provinces of Ontario and Alberta, Canada between 2006 and 2010. In Alberta, subjects were invited to participate in the Leisure, Lifestyle, and Lifecycle Project (LLLP) [24] -- a population-based study that investigates the natural progression of gambling behavior. The LLLP sample recruitment strategy has been previously

described [3] (see Supplementary Information). A total of 609 adults consented to donate blood or saliva samples for genetic analyses.

In Ontario, individuals from the Greater Toronto Area who perceived their gambling as excessive were invited to participate in a genetic study. These individuals were recruited through advertisements in the community and at problem gambling treatment centers. Thus, Ontario provided an enriched sample for disordered gambling. A total of 447 adult subjects were eligible to participate in the study and donated blood for genetic analyses.

Research protocols were approved by the participating institutions research ethics boards and were in compliance with the Declaration of Helsinki. All subjects provided written informed consent to participate in the study.

Measures: Lifetime and past-year gambling behavior were assessed using a DSM-IV based interview [25] and research analysts from both sites were trained on the administration of the instrument by board certified psychiatrists.

Diagnosis of psychotic disorders (e.g., schizophrenia and bipolar disorder) and neurological illnesses (e.g., seizures, Parkinson's Disease) were considered exclusion criteria. Subjects were screened for psychiatric disorders through the Structured Clinical Interview Diagnosis in Psychiatry based on DSM-IV criteria (SCID-NP) in the Toronto (Ontario) sample and through the Composite International Diagnostic Interview (CIDI) in the Alberta sample.

Gene Selection and Genotyping: The Knowledgebase for Addiction Related Genes (KARG) pathways database [26] was our initial source for gene selection, from which 24 genes were randomly selected through a computerized random number generator. Another 16 genes were selected based on higher association reliability scores (≥ 1) in the KARG database and/ or on preliminary evidence of association from our previous studies [27-30], totaling 40 genes (384 SNP panel).

Selection of *tag*SNPs was performed using genotypic data from the International Haplotype Map Project database public release #3 (www.hapmap.org) in the Utah residents with ancestry from northern and western Europe sample (i.e., CEU population). *Tag*SNPs with minor-allele frequencies (MAF) ≥ 0.10 and with a minimum distance of 60bp between SNPs were selected using the aggressive pairwise-tagging algorithm in the software Haploview v4.2 [31].

Genomic DNA was extracted using standard methods from either blood lymphocytes or saliva (Oragene™ DNA Self-Collection Kit). Genotyping for *tag*SNPs was performed using Illumina GoldenGate® custom SNP genotyping protocols (Illumina, San Diego, California, USA). Genetic analysis was performed using an Illumina Beadstation 500G platform. Quality control metrics from GenCall v6.2.0.4 were used to exclude SNPs with poor performance: genotype calls <95%, with < 10% MAF, and not in Hardy-Weinberg equilibrium ($p<10^{-3}$) in either cases or controls. A total of 320 *tag*SNPs distributed along 38 genes were included in the analysis (Table 1).

Statistical Analysis: Power calculations were performed through QUANTO [32] and revealed that a case-control ratio of 1: 1, with 400 cases would have ~80% power to detect associations with an odds ratio of 1.5 (two-tailed *p*-values), considering a MAF of 0.10, and a population prevalence of 0.05 (mean prevalence of DG in Canada) [4, 33, 34].

We compared DG and non-DG groups in regards to gender and age, using chi-squared and *t*-tests respectively. Allelic-based genetic associations were investigated using chi-squared tests or logistic regressions as appropriate, using Golden Helix SNP and Variation Suite v7.6.11 [35](Golden Helix, Inc., Bozeman, MT) . SNP spectral decomposition[36] was used to correct association tests for multiple testing.

Animal Arm

Sample: 12 male Sprague-Dawley rats weighing 200–250 g (Charles River, Quebec) were used for all experiments. All procedures were approved by the Animal Care Committee at the Centre for Addiction and Mental Health and complied with Canadian Council on Animal Care (CCAC) and NIH standards and guidelines.

Rat Gambling Task (rGT): The Rat Gambling Task (rGT) [22], a rodent analogue of the human Iowa Gambling Task[12], was conducted in commercial five-choice chambers (Med Associates, St. Albans, VT) as described previously[22] and as recently implemented in our laboratory [37]. Each rGT option (P1-P4) was calculated as a percent of total trials per session, and an impulsive choice ratio was determined for each animal (high-risk P4 choices divided by optimal P2 choices). A high rGT impulsive choice ratio reflects persistent choice of high-risk options, which are linked to larger rewards, but ultimately result in fewer pellets earned per session (Supplementary Information).

In situ hybridization: Upon completion of the behavioural testing, brains were recovered and coronal sections were prepared Hybridization was performed with [35S]UTP labeled riboprobes complementary to the sequences of interest (Supplementary Information – Table S1). *In situ* hybridization signals on film were quantified using MCID Basic 7.0 image analysis software (Interfocus Imaging, Linton, UK) (Supplementary Information). Densitometry data for each region were averaged across brain sections for each animal. Association between the rGT choice data and mRNA density in different rat brain regions for the three genes was performed using correlation analysis.

Results

Human Arm

Sample Characteristics: 1,056 individuals were recruited for this study, of which 76% (*n*=803) reported European Caucasian, 6.8% (*n*=72) African, and 9.2% (*n*= 97) Asian ancestry. Ancestry was determined using genealogical information provided by the participants (e.g., subjects reporting a minimum of three European Caucasian grandparents were considered as Caucasians). Individuals who were adopted, reported mixed ancestry (e.g., 50% Caucasian and 50% Asian) or were unable to provide information regarding their ethnic background comprised 8% (*n*=84) of the sample and were excluded from the analyses. To further confirm ancestry, we compared the distribution of 269 SNPs[1] between self-reported Caucasians in the Toronto and Alberta samples and HapMap populations (HapMap release #3, www.hapmap.org). Results from principal component analysis (PCA) show that the majority of self-reported Caucasian subjects from our sample were clustered within the HapMap CEU sample (Supplementary Information - Figure S1). A second PCA was performed to investigate whether population stratification was present between the Toronto and Alberta samples (Supplementary Information - Figure S2). Subjects identified as outliers through PCA were excluded.

Subjects endorsing a minimum of two PG DSM-IV criteria lifetime were included in the DG group. This cut off was selected for two main reasons: (i) previous studies in Canadian samples show that reporting two or more PG symptoms is the optimal threshold for determining gambling-related harm [3, 4, 38], whereas subjects reporting 0 to 1 PG criteria are considered low-risk gamblers and do not experience gambling-related harm; (ii) heritability for DG is significantly higher in subjects presenting ≥2 PG DSM-IV criteria lifetime compared to those presenting only one criteria [14, 15]. Because different thresholds can be used to define DG in the literature, we have also performed secondary analyses using broader (≥ 1 DSM-IV criteria) and

[1] From the SNPs in our panel, 296 SNPs were available in HapMap populations and were used for PCA.

narrower (≥ 5 DSM-IV criteria) definitions of the disorder. Subjects included in the non-DG group did not endorse any DSM-IV criteria for PG in their lifetime.

Our final sample was composed of 745 Caucasian subjects (400 DG and 345 non-DG subjects). The majority of control subjects (99%) originated from the LLLP study and the majority of DG subjects originated from the Toronto sample (73.5% of DG subjects). The prevalence of pathological gambling (≥ 5 DSM-IV criteria) in the general population is 0.6 to 1% [1, 2], which is in keeping with the frequency found in the LLLP sample (1%).

The proportion of males and females was significantly different between groups (DG 37.5% females, non-DG 68.4% females, χ^2 70.8, $df = 1$, $p < 0.001$) and subjects in the DG group were significantly older (DG mean age = 43.5±13.6 years, non-DG mean age = 37.5 ±20.6, $t_{(578)} = -4.7$, $p < 0.001$). The majority of the DG group met DSM-IV criteria for PG (≥5 criteria, 68.5%, $n = 274$), with 10.5% ($n = 42$) endorsing two criteria and 21% ($n = 84$) endorsing three or four DSM-IV criteria.

Genetic Association Analyses: Because it has been recently shown that the addition of known covariates can significantly decrease power to identify new genetic variants when disease prevalence is < 15%[39] we present results of allelic association tests and of a logistic regression model including age and gender as covariates (Table 2).

Single nucleotide polymorphism spectral decomposition[36] was applied to the 320 SNPs analyzed, resulting in 185.3 independent tests. After inclusion of covariates in a logistic regression model, the association with rs167771 (*DRD3*) remained significant, and a *tag*SNP in *CAMK2D* (rs3815072) also became significant (Table 2). Secondary analyses using both broader (≥ 1 DSM-IV criteria) and narrower (≥ 5 DSM-IV criteria) definitions of DG reveal that the same SNPs in *CAMK2D* and *DRD3* (rs3815072 and rs16771, respectively) were associated

with the smallest p-values, although with different levels of significance due to changes in sample power and in the specificity of the phenotype (Supplementary Information - Table S3).

In order to investigate possible functional effects of these polymorphisms, we used *in silico* function prediction tools and searched for effects of these *tag*SNPs on *DRD3* and *CAMK2D* expression in public gene expression databases. Brain Cloud [40] data shows that rs167771does not alter *DRD3* expression in the human prefrontal cortex, whereas the GTEx eQTL database (http://www.ncbi.nlm.nih.gov/gtex/GTEX2/gtex.cgi) has no information regarding expression of rs167771 in brain tissue. Neither Brain Cloud nor GTEx eQTL databases have information regarding the expression of rs3815072. Our *in silico* analysis using RegulomeDB[41] reveals that there is minimal evidence for transcription factor binding sites in rs167771 (RegulomeDB score= 5) and in rs3815072 (RegulomeDB score= 6) in samples from peripheral tissue and embryonic stem cells.

Animal Arm

Rat Gambling Task (rGT) Performance: Individuals generally exhibited an extremely stable pattern of behavior, with small within-subject variability (Supplementary Information, Figure S3), but considerable variability in the choice distribution between animals (P2 range: 15-98%; P4 range: 0-26% of total choices per session). The average P4/P2 ratio for the group was 0.32 (SD = ± 0.61). While the majority of animals adopted the most optimal strategy, a few rats exhibited varying preferences for the higher risk options (P4) (Figure 1 – only last 6 trials shown), consistent with previous reports[22]. One animal was of particular interest, as it had a high impulsive choice ratio of 2.07.

rGT Performance and In situ hybridization Correlation Analyses: Genes with p-values <0.1 (χ^2 tests, Table 2) in the human genetic association analyses were selected to be investigated in the

animal arm. In situ hybridization was performed in eight brain regions for *Drd3*[2], 32 regions for *Camk2d* and 19 regions for *Htr2a*. The rat genes *Drd3, Htr2a,* and *Camk2a* present 85%, 91% and 90% nucleotide identity with human orthologues respectively (www.genecards.org)[42].

Both rGT performance and *in situ* hybridization data were normally distributed (Kolmogorov-Smirnov tests, *p*-values > 0.05). Pearson's *r* correlation tests are presented in Table 3 by brain region for each of the three genes of interest. Figure 2 illustrates gene expression in brains of rats with high and low impulsive choice.

After correction for multiple testing (59 brain regions tested in total) only the association between rGT performance and (mRNA)*Drd3* levels in the Islands of Calleja remained significant. Noteworthy is the fact that the correlation with rGT performance presents opposing direction in the Islands of Calleja and Islands of Calleja Major. Opposing direction of association is a common finding in animal studies using detailed regional analyses of the brain (e.g. Creed et al.[43]).

Discussion

Human and Pre-Clinical findings on Dopamine D3 gene/ receptor in addictions

A relatively small number of human genetic studies have investigated the association of *DRD3* polymorphisms with substance and behavioral addictions, with most candidate gene studies being conducted in small samples. Recently, studies on larger ethnically homogeneous samples have reported significant associations of a number of *DRD3* polymorphisms with nicotine [45, 46], opioid [47], and alcohol addiction [48].

[2] In accordance with current nomenclature standards, human genes are indicated by capital letters in italics (e.g., *DRD3*) and human gene products by capital letters only (e.g., DRD3). Rat genes are indicated by lower case letters in italics (e.g., *Drd3*) and rat gene products by lower case letters only (e.g. Drd3). When referring to both human and animal genes and gene products, we use capital letters and specify whether we are referring to genes or its products (e.g. DRD3 receptors, DRD3 gene).

In keeping with previous DG candidate gene studies [17, 49, 50], we did not find a significant association with the functional *DRD3* polymorphism rs6280 (Ser9Gly). However, we did find a significant association with *DRD3* rs167771. Possibly we were able to unveil this association because we used a larger sample and because the use of 15 *tag*SNPs allowed us to cover 98% of the variation in *DRD3*, whereas previous studies genotyped only rs6280. It should also be noted that rs6280 and rs167771 are in high linkage disequilibrium (D' = 0.92), thus either SNP (as well as other neighboring SNPs in high linkage disequilibrium) could contribute to association signals.

The function of rs167771 is unknown, but studies suggest it may have a functional role because of its association with motor side-effects secondary to risperidone use [44]. Although Brain Cloud data does not indicate a functional effect for this SNP, it is important to note Brain Cloud has no expression data for *DRD3* polymorphisms in brain areas where the *DRD3* gene is highly expressed (e.g., nucleus accumbens and Islands of Calleja). Likewise, *in silico* functional prediction analysis (RegulomeDB database) did not use brain tissue data. Thus, the effect of rs16771 on *DRD3* expression in the striatum remains to be investigated.

Approximately 85% of other impulse control disorders cases in Parkinson's disease patients are associated with the use of selective DRD3 receptor agonists[51] (e.g., pramiprexole, ropinirole, and rotigotine) . Lee et al. [52] have found *DRD3* to be associated with the occurrence of impulse control disorders (including DG) in Parkinson's disease patients receiving dopamine (DA) agonist therapy in a large Korean sample. Imaging studies have also found an association between impulse control disorders in Parkinson's disease and D2/D3 receptor availability in the striatum[54]. Similarly, a positron emission tomography study in humans reported a significant association of DRD3 receptor binding with DG severity[53]. Thus, together with our results, a small but consistent body of research supports the involvement of *DRD3* in DG.

The animal arm of our study also suggests the involvement of *Drd3* in DG, supporting our human study results. Our findings relate to *Drd3* gene expression in a very circumscribed brain area in the ventral striatum (namely the Islands of Calleja) where *Drd3* expression and Drd3 receptor binding as assessed with D3-specific ligands is particularly high [55].

Regarding animal models of gambling behavior, previous animal studies have investigated the role of D1 and D2 receptors [21], while investigations of the role of D3 receptors in this context are at an early stage. Recently, Cocker et al. [56] reported that risk aversion in rats was associated with decreased Drd2/Drd3 availability in the caudate-putamen, whereas no association was found with Drd2/Drd3 availability in the ventral striatum. Several aspects may have contributed to the discrepancy between Cocker et al.'s and our findings. Firstly, the decision-making task ("betting task') used by Cocker et al. captures biases specifically for gains by giving animals the choice between a guaranteed reward and a 50% chance of doubling that reward or receiving nothing [57]. In contrast, the rGT captures decision-making under uncertainty, where reward and punishment are randomized across five options [22]. In the betting task, lesions to the basolateral amygdala (BLA) do not affect choice preference [57], whereas in the rGT, BLA lesions increase risky decision-making behavior in animals [58]. Thus, the rGT and the betting task likely measure different decision-making constructs. Secondly, it is unclear to what extent Cocker et al.'s findings reflect Drd3-specific availability. Previous studies suggest that raclopride can provide an indirect measure of D3 receptor density [59], however raclopride has significantly higher affinity to D2 compared to D3 receptors and other ligands have been shown to be more suited for assessing D3 availability [59]. On the other hand, although mRNA measures are highly specific they might not always correlate with receptor availability since post-transcriptional mechanisms may also affect gene expression [60]. Thus, in order to better

understand the role of D3 receptors, future studies would benefit from using Drd3-specific methods across different gambling and decision-making tasks.

In substance addiction, both increased [61, 62] and decreased [63] D3 receptor levels have been associated with amphetamine-induced DA release. Recent studies report increased amphetamine-induced release of striatal DA in pathological gamblers [64] and in animals exposed to a gambling-like schedule of reinforcement [65]. While our study did not address functional mechanisms, it is conceivable that the decreased Drd3 gene expression observed in our animals reflects: (1) a compensatory mechanism for increased DA release, or (2) a vulnerability mechanism in which decreased availability of D3 receptors (which act as autoreceptors in the nucleus accumbens, inhibiting DA release [66]) leads to decreased inhibition of dopamine release. In fact, a model has been proposed in which highly impulsive individuals presenting low midbrain D2/D3 receptor density would present higher DA release in response to reward [63]. Further investigations will be necessary to determine whether or not this proposed model would apply equally to D2 and D3 receptors.

Human and Pre-Clinical findings on CAMK2D gene/kinase in addictions:

To the best of our knowledge, no other study has investigated *CAMK2D* genetic polymorphisms in association with addictions. Similarly to *DRD3* rs167771, the effect of rs3815072 on *CAMK2D* expression is yet unknown.

CAMK2D is part of a larger family of type 2 Ca^{2+}/calmodulin-dependent protein kinase genes, which are the common link between all five proposed addiction-related genetic pathways [26]. CAMK2 kinases have been found to have an important role in mediating stimulant-induced DA release [67], conditioned place preference [68], and behavioral sensitization [69], to be involved in nicotine-induced neuroplastic changes [70, 71], and to participate in processes leading to the

development of opioid tolerance and addiction [72]. In particular, pre-clinical studies have found Camk2d to be involved in the regulation of neuroplastic processes, such as embryonic axonal development and neuronal apoptosis [73], and in the recovery from traumatic brain injury [74]. Recently, *Camk2d* transcripts have been found to be up-regulated in GABAergic neurons in nucleus accumbens of rats in an animal model of nicotine self-administration, suggesting that *Camk2d* may be involved in nicotine-induced signaling changes from GABAergic projections in the nucleus accumbens to the ventral pallidum [75].

Our results indicate that sex might moderate the association between *CAMK2D* and DG. Interestingly, estrogen has been shown to modulate Camk2 activity in the brain [76]. Moreover, estrogen has been shown to regulate Camk2 activity in female rats submitted to a chronic cocaine administration treatment, which has not been observed in male rats under the same treatment regimen [77]. No studies have investigated the effect of estrogen specifically on CAMK2D kinase activity in the brain; however, pre-clinical studies have found that estrogen regulates Camk2d in cardiac tissue [78, 79]. Together, these findings suggest that sex might moderate the association between CAMK2D (kinase/ gene) and addiction – a hypothesis that deserves further investigation.

Genetic Associations in previous DG studies

Previous DG candidate gene studies reported associations with polymorphisms in *DRD1* [50, 80], *DRD2* [80, 81], *DRD4* [82], *HTR2A* [30], *MAO-A and MAO-B* [83]. Our analyses included the same polymorphisms for *DRD1, DRD2*, and *HTR2A* but our results do not replicate previously reported associations with these genes. Earlier studies included only pathological gamblers, whereas our study included subjects presenting sub-clinical forms of the disorder. However, it is unlikely that sample composition alone could account for the lack of replication because the

majority of cases in our sample (68.5%) meet DSM-IV criteria for PG. Likely, the lack of replication of results from earlier studies is related to differences in sample size (less than 150 cases per sample in earlier studies), advances in technology which now allow the genotyping of increasingly number of variants within a gene, and/ or to potential population stratification bias.

Currently only one DG genome-wide association study (GWAS) has been published [18]. The authors suggest that genes in chromosomes 9 and 12 may be associated with DG, which does not coincide with the chromosomal location of either *DRD3* or *CAMK2D.* Nevertheless, results show that SNPs nominally associated with DG are enriched within KARG and Parkinson's disease genetic pathways, which is in line with our findings and candidate gene selection. It is unclear whether our study is fully comparable to Lind et al.[18] due to the significant variation in sample composition (i.e., proportion of individuals meeting PG diagnostic criteria) and genetic methodology.

Limitations and Conclusion

As with any study, it is important to consider limitations in the interpretation of our results. Our human sample is of moderate size, thus there is a potential for false positives, which we aimed to minimize by using appropriate multiple testing correction procedures. We did not use specific ancestry informative markers; however we combined self-report ancestry information and HapMap population data to minimize possible effects of population stratification. While we have made efforts to ensure that the Toronto and Alberta samples are genetically homogeneous, because of methodological limitations (e.g., number of SNPs analyzed) it is possible that some degree of population stratification still exists. This issue could be addressed in future studies using other methodological approaches and larger samples. It is also important to note that samples of pathological gamblers (≥ 5 DSM-IV criteria) are difficult

to obtain – the prevalence of PG is estimated at 1% [1, 2] and only 7-12% of pathological gamblers seek treatment [83]. Thus, while the potential for population stratification cannot be fully excluded, combining subjects from Toronto and Alberta allowed us to obtain a sample composed by a larger proportion of pathological gamblers.

Although we used *tag*SNPs, coverage of the selected genes was partial because we used a small SNP-panel. An important strength of our sample is the large proportion of subjects that met DSM-IV criteria for PG. Considering that the heritability of DG is higher in subjects who meet DSM-IV criteria for the disorder[14, 15] and that broader phenotype definitions may result in a "dilution of the effect" of the phenotype [85], future studies could benefit from increasing the proportion of subjects more severely affected by DG in their samples.

The major limitation of our preliminary animal study is sample size. Future studies should include a larger sample, with more animals with high rGT impulsive ratios. At this time our animal study is descriptive in nature and possible functional roles of the observed alterations will need to be tested with other experimental designs using controlled brain interventions. For example, causal associations could be tested by means of local injections of D3 receptor agonists/antagonists into these brain areas. Nonetheless, we believe the current data serve as an important proof-of-concept for the use of functional animal data as an adjunct to human genetic findings.

In summary, we have found a significant association with DG and rs167771 (*DRD3)* and with rs381572 (*CAMK2D)* in humans. Most importantly, our results regarding *DRD3* were supported by results from animal experiments. We could not investigate age and gender effects in the animal arm of the study, which might account for the lack of association between (mRNA)*Camk2d* and rGT performance.

The replication of human findings in animal models is crucial for improving the translation of basic research in to clinical treatments. If replicated, our results suggest that the use of human genetics, pre-clinical models and gene expression as a cross-translation paradigm could significantly accelerate neurobiological and pharmacological investigations in DG and possibly other addictive disorders.

Conflict of Interest. The authors declare that Dr. Kennedy has received honorarium from Roche Pharma, Lilly, and Novartis in the last three years. None of the other authors have received financial compensation or support from any individual or corporate entity over the past three years for research or professional service and there are no financial holdings that could be perceived as constituting a potential conflict of interest.

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Supplementary information is available at *Molecular Psychiatry's* website

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Table 1: Genes and number of polymorphisms (*tag*SNPs) included in the analysis.

Gene Name	*Gene Symbol (number of tag***SNPs** *included in the analysis)*
Ankyrin repeat and kinase domain containing 1	*ANKK1 (4)* [1]
Calcium/calmodulin-dependent protein kinase, types 2A, 2B, 2D	*CAMK2A (17), CAMK2B (10), CAMK2D (33), CAMK2G (4)*
Cocaine- and amphetamine-regulated transcript	*CARTPT (7)*
Cannabinoid receptor, type 1 and type 2	*CNR1 (2), CNR2 (3)*
Dopamine receptor gene, types 1 2, 3, and 4	*DRD1 (3), DRD2 (7)* [1]*, DRD3 (15), DRD4 (2)*
Gonadotropin-releasing hormone, types 1 and 2	*GNRH1 (3), GNRH2 (1)*
Metabotropic glutamate receptor, types 1 and 5	*GRM1 (10), GRM5 (18)*
Serotonin receptor, types 1B, 2A, 3A, 3B, 6, and 7	*HTR1B (8) ,HTR2A (23), HTR3A (7), HTR3B (7), HTR6 (5), HTR7 (4)*
Mitogen-activated protein kinase, kinase 1, 2, and 4	*MAP2K1 (4), MAP2K2 (9), MAP2K3 (5),*
Mitogen-activated protein kinase 1	*MAPK1 (4)*
Phospholipase D, type 1 and 2	*PLD1 (5), PLD2 (6)*
Protein kinase, cAMP-dependent, catalytic, types beta and gamma	*PRKACB (9), PRKACG (2)*
Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	*SLC6A3 (5)* [2]
Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	*SLC6A4 (8)* [3]
Tachykinin receptor, types 1, 2, and 3	*TACR1 (23), TACR2 (3), TACR3 (9)*
Tyrosine hydroxylase	*TH (6)*
Tryptophan hydroxylase, type 2 (neuronal)	*TPH2 (20)*
Tetratricopeptide repeat domain 12	*TTC12 (9)* [1]

[1] ANKK1, TTC12 and DRD2 are in close physical relationship and the TaqA1 variant is now known to be located in ANKK1; [2] also known as DAT1; [3] also known as 5-HTT or SERT

Table 2: Genetic associations with Disordered Gambling (DG) in a sample of 400 DG and 345 non-DG subjects, allelic association tests and logistic regression analysis with gender and age as covariates[1].

tagSNP	Gene	A1/A2[2]	A1 Count (frequency)		χ^2	Allelic Test p-value[3]	Logistic regression p-value[4]	OR (95%CI)
			Cases	Controls				
rs167771	***DRD3***	A/**G**	626 (0.78)	593 (0.87)	14.7	**2.2E-02**	**4.0E-02**	**1.7 (1.3-2.2)**
rs7997012	*HTR2A*	G/A	483 (0.60)	354 (0.51)	12.4	7.8 E-02	8.0E-01	1.4 (1.2-1.8)
rs3815072	***CAMK2D***	**A**/G	705 (0.88)	562 (0.82)	12.1	9.2 E-02	**1.7E-02**	**1.7 (1.2-2.2)**
rs1524998	*CAMK2D*	A/G	486 (0.61)	360 (0.52)	11.1	1.6E-01	4.0E-01	1.4 (1.2-1.7)
rs9534512	*HTR2A*	A/G	462 (0.57)	346 (0.5)	7.8	7.4E-01	4.1E-01	1.4 (1.1-1.7)

[1] Top 5 results shown. Significant associations highlighted in bold.

[2] A1 refers to major allele and A2 to minor allele, significantly associated alleles highlighted in bold.

[3] SNP spectral decomposition corrected p-value, chi-squared test (no covariates).

[4] Logistic regression p-value for the genetic association with DG, including gender and age as covariates, SNP spectral decomposition corrected p-value.

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Table 3: Correlation tests between rGT impulsive choice ratio (P4/P2, mean 0.32 ±0.61) and *Htr2a, Drd3* and *Camk2a* mRNA density by brain region, Pearson's *r* [1].

Rat Gene	Brain Region	mRNA density mean (±SD)	Pearson's *r*	*p*-value	Corrected *p*-value [2]
Drd3	Islands of Calleja	44.47 (13.06)	-0.91	8.96 e-05	0.005
	Islands of Calleja Major	53.45 (11.69)	0.62	0.04	> 0.05
Camk2d	Medial Amygdaloid Nucleus	60.49 (14.19)	-0.65	0.04	> 0.05
	Lateral Amygdaloid Nucleus	15.85 (3.95)	-0.64	0.04	> 0.05
Htr2a	Cingulate Cortex	21.47 (2.76)	0.65	0.03	> 0.05
	Piriform Cortex	33.16 (5.61)	0.61	0.04	> 0.05

[1] Only correlations (Pearson's *r*) above 0.6 shown

[2] Bonferroni correction for a total of 59 brain regions.