Investigation of Selected Signalling System Genes in Pathological Gambling

Final Report

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Table of Contents:

	Page
Acknowledgements	5
Abstract	6
1. Introduction	7
1.1 Literature Review	7
2. Research Design and Methodological Approach	13
2.1 Sample	13
2.2 Gene and Polymorphism (SNP) Selection	14
2.3 Statistical Analysis	16
2.4 Sequencing of DRD4 exon III region	16
3. Results	16
3.1 PG subjects x Control Subjects	16
3.2 PG subjects with alcohol and/or drug abuse x Control Subjects	17
3.3 PG subjects with alcohol and/or drug abuse – PG-ADA (n =99) x PG subjects without substance abuse	18
3.4. DRD4 sequencing analysis	18
4. Limitations and Changes to Original Proposal	19
5. Discussion	20
6. References	25

List of Tables

	Page
Table 1: List of genes and SNPs selected for genotyping within their respective chromosomes	32
Table 2: Haplotype comparisons between problem gamblers (PG) with healthy control subjects	36
Table 3: Haplotype comparisons between problem gamblers with alcohol and/or drug abuse (PG-ADA) with control subjects	39
Table 4: Haplotype comparisons between problem gamblers with alcohol and/or drug abuse (PG-ADA) with PG subjects without substance abuse history	40

List of Figures

	Page
Explanation of symbols in figures 1-5	42
Figure 1: Neuroactive ligand receptor interaction pathway	43
Figure 2: Long-term potentiation pathway	44
Figure 3: GnRH signalling pathway	45
Figure 4: MAPK signalling pathway	46
Figure 5: Gap junction pathway	47
Figure 6: Diagrammatic Representation of the DRD4 gene and DRD4 exon III VNTR	48
Figure 7: Dopamine D4 receptor graphical representation	49
Figure 8: Screenshot of the sequence alignment (DRD4 exon III)	50
Figure 9: Screenshot of the deletion on DRD4 exon III from one subject in our sample	51

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Abstract:

Background: Several studies have provided evidence for a biological basis for problem gambling (PG): first-degree relatives (FDR) of PG subjects present a higher than expected chance to develop PG and a large twin study has reported that genetic factors account for 52% of the variance of PG diagnosis. The increased risk for developing substance addiction in FDR of PG subjects, the high comorbidity between substance dependence and PG, and a common underlying genetic vulnerability for PG and alcohol dependence provide converging evidence that a common neurobiological system underlies both substance addiction and PG. **Objectives:** 1. to continue the investigation of the genetic basis of problem gambling (PG), through the analysis of genes involved in brain's signalling system that underlies addictive disorders; 2. to further investigate how the variations in the sequence of the 2 and 7-repeat alleles of the D4 receptor gene (DRD4) are associated with PG severity (from our previous results on the association of DRD4 and PG). **Methods:** DNAs and clinical data from 514 PG subjects and 214 controls assessed during our previously funded OPGRC projects. *Hypotheses:* 1. genes underlying common genetic pathways in substance addiction will also be associated with increased vulnerability for PG; 2. variations in the sequence of the 2 and 7-repeat alleles of the D4 receptor gene will better predict PG severity. *Gene selection:* 1. based on our previous association of DRD4 2 and 7-repeat alleles with PG severity, we will proceed to sequence these gene variants; 2. addiction-related genes were selected through the Knowledgebase for Addiction Related Genes; 3. variants within each gene were selected using *tag* SNPs in order to provide maximum gene coverage. *Statistical analysis:* Three groups were used for analysis: 1. PG subjects x Control subjects; 2.PG subjects with alcohol and/or drug abuse x PG subjects without history of substance misuse and PG subjects with substance misuse compared with healthy control subjects. Genetic analysis was performed for haplotypes (blocks of variants) using modified qui-squared tests implemented in the software Golden Helix SNP & variation suite version 7.Correction for multiple testing was performed through False Discovery Rate tests (FDR). **Results:** Haplotypes in CAMK2D, HTR2A, PRKACB, and PLD2 were significantly associated with PG. No significant associations were found in the comparison between PG with alcohol and/or drug abuse and controls, and between PG without substance abuse and PG with alcohol and/or drug abuse. Nominal associations were found with CAMK2D, dopamine D2, serotonin 2A, and glutamate receptor genes. Sequencing of DRD4 exon III VNTR revealed a new deletion on the 7-repeat allele sequence that may alter gene expression. Our associations suggest that, similarly to substance addiction, PG is associated with genes involved in neuronal plasticity (CAMK2D), signal transduction (PRKCAB), and serotonin signalling (HTR2A). PLD2A is regulated by G-coupled receptors (dopamine, glutamate), suggesting that altered dopamine release reported in PG may be linked to down-stream alterations in dopamine signalling pathways. Our findings corroborate the view of PG as an addiction and suggest that further investigation of these signalling pathways should provide a better understanding of the neurobiology of PG. **Application of findings:** Our results have provided further insight in the neurobiological processes underlying PG. The reported associations with genes that synthesize protein kinases (CAMK2A, PRKCAB) open the possibility of testing protein-kinases inhibitors for the treatment of PG.

Key words:pathological gambling, problem gambling, genetics, addiction

1.Introduction:

Goals:

- Goal #1: To continue the investigation of the genetic basis of problem gambling (PG), through the analysis of genes involved in brain's signalling systems that underlie addictive disorders, building on our readily available sample of DNAs from PG subjects and controls and on the newly available technology and evidence.
- Goal #2: In our previous research we have found that the rare 7-repeat allele of the dopamine receptor type 4 gene polymorphism (DRD4 exon III VNTR) was significantly associated with PG and that the 2-repeat allele of DRD4 exon III VNTR showed a trend of association with PG. It is known that there is variation within the sequence that composes the 2- and 7-repeat alleles, but there has been little research on how these variations might impact associations of these alleles to complex traits (e.g., Novelty Seeking, PG). Our objective is to further investigate if the variations in the sequence of the 2 and 7-repeat alleles of the D4 receptor gene are associated with PG severity.

1.1 Literature Review:

Pathological gambling is defined as a persistent and recurrent maladaptive behaviour that disrupts personal, professional, and family relationships and is not better explained by a manic episode (DSM-IV, APA). Evidence from several studies suggests that PG presents a biological basis. Family studies have shown that first-degree relatives (FDR) of PG subjects present a higher than expected chance to develop PG and a large twin study has reported that the **genetic factors account for approximately 50% of the variance of PG diagnosis in men** (Eisen, et al., 1998; Eisen, et al., 2001) **and women** (Slutske, Zhu, Meier, & Martin, 2010).

Since Marks (Marks, 1990) proposed that PG should be considered as an addictive disorder (i.e. as a behavioural addiction), studies have investigated the relationship between substance and behavioural addictions (Blanco, Moreyra, Nunes, Saiz-Ruiz, & Ibanez, 2001; Cardinal & Everitt, 2004). Currently, several lines of investigation provide converging evidence that a common neurobiological system underlies both substance addiction and PG:

1. Lifetime Comorbidity: Individuals diagnosed as pathological gamblers present a significantly higher risk to develop (at any point in life) substance dependence compared to the general population (Black & Moyer, 1998; Bland, Newman, Orn, & Stebelsky, 1993; Crockford & el-Guebaly, 1998; Cunningham-Williams, Cottler, Compton, & Spitznagel, 1998; Lesieur & Heineman, 1988; Lynch, Maciejewski, & Potenza, 2004; Maccallum & Blaszczynski, 2002; Petry, Stinson, & Grant, 2005;

Ross, Glaser, & Germanson, 1988; Roy, et al., 1988; Vitaro, Brendgen, Ladouceur, & Tremblay, 2001). Likewise, a person who had a diagnosis of substance dependence (at any point in life) has a greater risk of being diagnosed with PG (Daghestani, Elenz, & Crayton, 1996; Feigelman, Wallisch, & Lesieur, 1998; Lesieur, Blume, & Zoppa, 1986; Petry, 2001; Ross, et al., 1988; Shaffer & Korn, 2002; Spunt, Lesieur, Hunt, & Cahill, 1995; Welte, Barnes, Wieczorek, Tidwell, & Parker, 2001). For instance, approximately 70% of PG subjects present nicotine dependence (Crockford & el-Guebaly, 1998) and 50 to 70% present alcohol abuse or dependence (McCormick, Russo, Ramirez, & Taber, 1984; Petry, et al., 2005). Pathological gambling rates has also been reported to be increased among subjects under methadone maintenance (7 – 52%) (Feigelman, Kleinman, Lesieur, Millman, & Lesser, 1995; Ledgerwood & Downey, 2002; Spunt, 2002; Spunt, et al., 1995; Weinstock, Blanco, & Petry, 2006), and among cocaine dependent subjects (8 – 12%) (Hall, et al., 2000; Kausch, 2003; Toneatto & Brennan, 2002).

2 Family History: FDR of PG subjects have significantly increased risk to develop substance dependence (Jacobs, 1989; Ramirez, McCormick, Russo, & Taber, 1984; Roy, et al., 1988) compared to the general population. FDR of substance dependent individuals are also at a higher than expected risk to develop PG (Herzog, Keller, Lavori, Kenny, & Sacks, 1992; Jacobs, 1989; Lesieur & Heineman, 1988; Ramirez, et al., 1984).

3 Genetics Research: In general, twin studies have provided evidence of a shared common vulnerability for any addiction, regardless of the substance used (Karkowski, Prescott, & Kendler, 2000; Kendler, Jacobson, Prescott, & Neale, 2003; Tsuang, et al., 1998). PG is reported to share 12 to 20% of its genetic risk with the risk for alcohol dependence (Slutske, et al., 2000), and research suggest that this shared vulnerability extends to the pathological use of natural rewards (i.e., food and sex) and to substance dependence (Kelley, 1999; Pelchat, 2002; Shaffer, et al., 2004).

4 Clinical Research: Clinical studies have shown that opioid antagonists, such as naltrexone and nalmefene, can be effective in the treatment of both PG (Grant, Kim, & Hartman, 2008; Grant, et al., 2006; Kim, Grant, Adson, & Shin, 2001) and alcohol dependence (Mason, Salvato, Williams, Ritvo, & Cutler, 1999; Volpicelli, Alterman, Hayashida, & O'Brien, 1992), suggesting that a common biological pathway is involved in the response to these medications in both conditions.

The dopaminergic system[1] has a well established role in the development of drug priming through the release of dopamine in the brain’s reward system, more specifically in the nucleus accumbens (Kalivas

[1] Please note that the dopaminergic system closely interacts with other neurotransmitter systems also involved in substance addiction (glutamate, serotonin), which will not be described here in respect to space limitations.

& Volkow, 2005). The nucleus accumbens works in close relationship with other areas of the brain that compose the brain's reward system: ventral tegmental area, prefrontal cortex, locus coeruleous, amygdala, and hippocampus. Research studies have implicated also the brain's reward system in the pathophysiology of behavioural addictions (Chambers & Potenza, 2003; Chambers, Taylor, & Potenza, 2003; Potenza, 2001). Imaging studies on PG subjects have shown that: 1. when exposed to gambling-related cues, PG subjects (but not controls) present increased activation of the dorsolateral pre-frontal cortex (DLPFC) (Crockford, Goodyear, Edwards, Quickfall, & el-Guebaly, 2005) and a relatively decreased activity in brain regions implicated in impulse regulation (frontal and orbitofrontal cortex, caudate/basal ganglia, and thalamus) compared with controls (Potenza, et al., 2003); 2. PG is related to response perseveration and diminished reward and punishment sensitivity as indicated by hypoactivation of the ventrolateral prefrontal cortex (VLPFC) when money is gained and lost (de Ruiter, et al., 2009); as well as to heightened limbic and sensory activation when betting for money (Hollander, et al., 2005). In summary, research has shown that an overlap exists between brain regions associated with substance addiction and those associated with PG.

Throughout the years, researchers have described different signalling pathways involved in substance addiction. For instance, the MAPK signalling pathway has been suggested to have a role in regulating synaptic plasticity related to long-lasting changes in memory associated with substance addiction (Wang, Fibuch, & Mao, 2007), and the long-term potentiation pathway has been linked to adaptations in glutamatergic transmission and synapse plasticity induced by substance addiction (Jones & Bonci, 2005). Recently, a group of researchers has integrated all the available research data (between 1976 and 2006) regarding genetic and biological pathways in addiction and developed a database of addiction related genes placed within five common signalling pathways for substance addiction (C. Y. Li, Mao, & Wei, 2008). Below are brief descriptions of each pathway. Figures 1-5 provide a diagrammatic representation of each pathway. It is important to note that these pathways are not isolated and members (genes, proteins) of one pathway may also play a role in another pathway.

A. Neuroactive-ligand receptor interaction pathway:

The neuroactive-ligand pathway is one of the pathways involved in processing information from the environment through signalling molecules, such as neurotransmitters. G-coupled protein receptors, such as dopamine and metabotrobic glutamate family receptors, are part of this pathway. Both

dopamine receptors and genes, and metabotropic glutamate receptors have been significantly associated with addictions. Several other peptides, proteins, and hormones are part of this pathway.

B. Long-term potentiation:

Long-term potentiation is a pathway specific to the nervous system. Hippocampal long-term potentiation (LTP), a ***long-lasting increase in synaptic efficacy, is the molecular basis for learning and memory***. Stimulation of neuronal afferents in the hippocampus induces glutamate release and activation of glutamate receptors in neuronal dendrites. A large increase in Ca^{2+} intake resulting from influx through NMDA receptors leads to constitutive activation of CaM kinase II (CaMKII). ***It is hypothesized that postsynaptic Ca^{2+} increases generated through NMDA receptors activate several signal transduction pathways including the MAPK and cAMP regulatory pathways***.

C. Gonadotropin Releasing Hormone (GnRH) signalling pathway:

The GnRH pathway is an endocrine pathway involved in hormone secretion. Gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus acts upon its receptor in the anterior pituitary to regulate the production and release of the gonadotropins, LH and FSH. The GnRHreceptor is coupled to proteins that activate phospholipase C which transmits its signal to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG activates the intracellular protein kinase C (PKC) pathway and IP3 ***stimulates release of intracellular calcium***. ***Signalling downstream of protein kinase C (PKC) leads to transactivation of the epidermal growth factor (EGF) receptor and activation of mitogen-activated protein kinases (MAPKs).*** Active MAPKs translocate to the nucleus, resulting in activation of transcription factors and rapid induction of genes.

D. Mitogen-activated protein kinase (MAPK) signalling pathway:

The MAPK pathway is also one of the pathways involved in processing information from the environment through signal transduction, by regulating neuronal plasticity associated with memory function and addictive properties of substances. The MAPK cascade is a highly conserved module that is involved in various cellular functions in various animal species, including cell proliferation, differentiation and migration. Mammals express at least four distinctly regulated groups of MAPKs, extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins

(p38alpha/beta/gamma/delta) and ERK5 that are activated by specific MAPK-kinases (MAPKK): MEK1/2 for ERK1/2, MKK3/6 for the p38, MKK4/7 (JNKK1/2) for the JNKs, and MEK5 for ERK5. Each MAPK kinase, however, can be activated by more than one MAPK kinase-kinase (a kinase that is activated by another kinase), increasing the complexity and diversity of MAPK signalling. Presumably each MAPKKK confers responsiveness to distinct stimuli.

E. Gap junction pathway:

The Gap junction pathway is involved in cell (including neuronal) communication. Gap junctions contain intercellular channels that allow direct communication between the cytosolic compartments of adjacent cells. Each gap junction channel is formed by docking of two 'hemichannels', each containing six connexins, contributed by each neighbouring cell. These channels permit the direct transfer of small molecules including ions, amino acids, nucleotides, second messengers and other metabolites between adjacent cells. Gap junctional communication is essential for many physiological events, including embryonic development, electrical coupling, metabolic transport, apoptosis, and tissue homeostasis. Communication through Gap Junction is sensitive to a variety of stimuli, including changes in the level of ***intracellular Ca2+,*** pH, transjunctional applied voltage and phosphorylation/ dephosphorylation processes.

As it can be inferred from the descriptions above, these pathways have points at which they merge or interact with one another, at times sharing gene products (proteins, enzymes, hormones) and at times activating another pathway. Through the construction of the KARG database, it was observed that positive feedback loops interlinked the pathways with each other through CAMKII. Two of these positive feedback loops involved signal transduction and would be considered ''fast'' loops, whereas the other two loops involved transcription and translation and would be considered ''slow'' loops (C. Y. Li, et al., 2008). Previous research had found that coupled fast and slow positive feedback loops could create a switch that was inducible and resistant to noise and played key roles in discontinuous and irreversible biological process, features characteristic of addiction (Abrieu, Doree, & Fisher, 2001; Brandman, Ferrell, Li, & Meyer, 2005). Activation of CAMKII has also been reported to play fundamental roles in the development and maintenance of addiction states (Noda & Nabeshima, 2004; Tang, Shukla, Wang, & Wang, 2006). Disruption of CaMKII translation in neurons (dendrites) impaired the stabilization of synaptic plasticity and memory consolidation (Miller, et al., 2002; Valjent, Corbille, Bertran-Gonzalez, Herve, & Girault, 2006). Taking this evidence together, the KERG

database authors have suggested that the fast and slow positive feedback loops interlinked through CAMKII may be essential for the development and consolidation of addiction and may provide a systems-level explanation for some of the characteristics of addictive disorders (C. Y. Li, et al., 2008).

In summary, based on research evidence suggesting common biological pathways for substance addictions and PG and on the recently compiled database of addiction related genetic pathways (KARG), we ***have hypothesized that genes underlying signalling pathways in substance addiction will also be associated with increased vulnerability for PG. More specifically, we expect that genes located on chromosomes 4, 5, 9, 10, 11 and 17 will be associated with PG***, since these chromosomes have the strongest evidence for harbouring susceptibility genes for addictions (M. D. Li & Burmeister, 2009).

Aside from investigating the aforementioned hypothesis, we have also proposed to investigate whether the variations in the sequence of the 2 and 7-repeat alleles of the D4 receptor gene (DRD4 exon III VNTR – see Figure 6) are associated with PG severity. This investigation is based on our previous results showing that DRD4 (exonIII) 7-repeat allele is associated with severity of PG, and that this association is not moderated by sex or age. We also found a trend of association of the 2-repeat allele with PG. The discovery of a functional polymorphism in the dopamine D4 receptor gene (DRD4, OMIM *126452) (Van Tol, et al., 1992) and its higher expression in the prefrontal cortex and temporo-limbic regions (Mulcrone & Kerwin, 1997) made DRD4 one of the most investigated genes in behavior and psychiatry. Several studies reported associations of this polymorphism with impulsive personality traits (Becker, Laucht, El-Faddagh, & Schmidt, 2005; LaHoste, et al., 1996; Laucht, Becker, Blomeyer, & Schmidt, 2007), addictions (Hill, Zezza, Wipprecht, Xu, & Neiswanger, 1999), and impulse control disorders (Comings, et al., 1999; Perez de Castro, Ibanez, Torres, Saiz-Ruiz, & Fernandez-Piqueras, 1997) with conflicting results. Thus far, the most consistent findings were reported for attention-deficit hyperactivity disorder (LaHoste, et al., 1996), as confirmed by meta-analyses (Bobb, Castellanos, Addington, & Rapoport, 2005; D. Li, Sham, Owen, & He, 2006; Thapar, Langley, Owen, & O'Donovan M, 2007).

In the previous decade, there was preliminary evidence of an inverse relationship between length of repeats, clozapine biding affinity, and gene expression levels (Asghari, et al., 1995; Jovanovic, Guan, & Van Tol, 1999; Schoots & Van Tol, 2003), and various studies compared the presence of the 7-repeat allele against short allelic variants of this polymorphism. There is evidence that transcripts from 7 and 2-repeat alleles have a lower potency when coupling with adenylyl-cyclase compared with transcripts from 4 and 10-repeat alleles respectively (Asghari, et al., 1995; Jovanovic, et

al., 1999). *In vitro* experiments suggest that the 2 and the 7 forms of the D4 receptor (see Figure 7) present higher rates of degradation due to their structure rigidity and length, respectively (Van Craenenbroeck, et al., 2005). These findings challenge the initial view that the length of the repeats is inversely associated with gene function. Another factor that increases the complexity in the study of DRD4 exon III VNTR is the fact that there is variation in the sequences within the alleles (Ding, et al., 2002), i.e. *not all individuals with the 7-repeat allele will have the exact same sequence within the allele.* Thus, since our initial results indicate an association between PG severity and the 7-repeat allele, and a marginal association between PG severity and the 2-repeat allele, **we hypothesize that variations in the sequence of the 2- and 7-repeat alleles of DRD4 exon III VNTR polymorphism will better predict PG severity**.

2.Research Design and Methodological Approach:

2.1 Sample:

Subjects were assessed for PG diagnosis through the South Oaks Gambling Screen (SOGS) (Lesieur & Blume, 1987) or the Problem Gambling Severity Index (PGSI) (Ferris & Wynne, 2001), resulting in a sample of 541 PG subjects (highest lifetime PGSI or SOGS ≥3; with either positive or negative screening for lifetime substance misuse) and 214 control subjects (highest lifetime PGSI or SOGS =0, negative screening for lifetime substance misuse). Clinical data on this sample was obtained in our previous OPGRC funded projects. All subjects were assessed through the Structured Clinical Interview Diagnosis in Psychiatry based on DSM-IV criteria (SCID-NP). The SCID is a well tested, reliable and widely used instrument in psychiatric research. It provides an assessment of lifetime diagnosis of major psychiatric disorders (mood disorders, psychotic disorders, anxiety disorders and substance addiction). The SCID is used worldwide (Amaral & Malbergiera, 2004; Chung, Tso, Cheung, & Wong, 2008; Healey, Kneebone, Carroll, & Anderson, 2008; Hodgins, Dufour, & Armstrong, 2000; Nilsson & Svedin, 2006; Rueda-Jaimes, et al., 2007; Torrens, Serrano, Astals, Perez-Dominguez, & Martin-Santos, 2004; Whelan-Goodinson, Ponsford, & Schonberger, 2008) and is considered as the gold-standard for diagnosis in psychiatric research, thus also being used to validate other diagnostic instruments (Cassidy, Schmitz, & Malla, 2008; Healey, et al., 2008.; Sanchez-Villegas, et al., 2008).

Subjects were considered to present substance misuse if they answer positively to all three SCID/NP screening questions for alcohol abuse and/or substance dependence. Noteworthy is the fact that tobacco dependence was not investigated, as it was not part of SCID-NP.

2.2 Gene and Polymorphism (SNP) Selection:

The main KARG database pathways described above were the initial source for gene selection. Genes in key positions in the pathways or genes that had been previously associated with PG in our studies were selected. Each gene was then entered into the International Haplotype Map Project database (www.hapmap.org) and available genotypic data for each gene was downloaded and analyzed for detection of haplotypes.

Haplotype detection was performed through the software Haploview version 4.2 (Barrett, Fry, Maller, & Daly, 2005). After haplotypes were detected within a gene, we proceeded to the selection of tagSNPs. TagSNPs are SNPs (variants, polymorphisms) that are in high linkage disequilibrium (LD)[2] with other SNPs within a haplotype block and are chosen in order to reduce genotyping requirements by eliminating redundancy in the information provided by SNPs in high LD. Haploview software program finds the smallest set of tag SNPs that meets the requirements regarding minor allele frequency (MAF), minimum value of r^2 (linkage disequilibrium), and minimum distance between SNPs. We selected tagSNPs with a minimum r^2 of 0.8, MAF of 0.15 and a minimum distance between SNPs of 60 base-pairs was required for accurate assay design.

After determining tagSNPs for all selected genes, we proceeded to test genetic assays for each tagSNP in order to ensure that the assays would perform adequately. During this process, some of the selected genes and tagSNPs had to be eliminated and substituted because it was not possible to design an assay. Our final list included 40 genes and a total of 384 tagSNPs (Table 1) that were genotyped using an Illumina® platform.

Figures 1-5 represent the five signalling pathways as described by Li et al. (2008). Below is a list of genes that were selected for our study from each pathway. Please note that several of these genes are present in more than one of the addiction-related pathways.

Genes selected from the Gap-junction pathway:

- dopamine receptor genes types 1 and 2 (DRD1, DRD2)
- metabotropic glutamate receptor genes 1 and 5 (GRM1, GRM5)
- serotonin receptor type 2 (HTR2A, HTR2C)

[2] Linkage disequilibrium is a measure of nonrandom association between two or more alleles such that certain combinations of alleles are more likely to occur together on a chromosome than other combinations of alleles, i.e. the degree to which knowing the alleles of one SNP will accurately predict alleles of other SNPs. It is often parameterized as the squared correlation or r^2 measure of linkage disequilibrium between two loci.

Genes selected from the MAPK pathway:

- mitogen-activated protein kinase kinase 1, 2, and 3 (MAP2K1, MAP2K2, MAP2K3)
- protein kinase, cAMP-dependent, catalytic – PKA family (PRKAC beta, alpha, and gamma)
- tumor necrosis factor receptor subfamily, member 1b – TNF family(TNFRSF1/ NBL1)

Genes selected from the GnRH pathway:

- gonadotropin-releasing hormone (GnRH1, GnRH2)
- phospholipase d, phosphatidylcholine-specific (PLD1, PLD2)
- protein kinase, cAMP-dependent, catalytic – PKA family (PRKAC beta, alpha, and gamma)
- insulin gene (INS)

Genes selected from Long-term potentiation pathway:

- calcium/calmodulin-dependent protein kinase (CAMK2A, CAMK2B, CAMK2D, CAMK2G),
- protein kinase, cAMP-dependent, catalytic – PKA family (PRKAC beta, alpha, and gamma),
- mitogen-activated protein kinase 1 (MAPK1 or ERK2).

Genes selected from Neuroactive-ligand pathway:

- dopamine receptor genes (DRD1, DRD2, DRD3, DRD4);
- metabotropic glutamate receptor genes (GRM1, GRM5),
- cannabinoid receptor gene (CNR1, CNR2),
- serotonin receptor genes (HTR1B, HTR2A, HTR3A, HTR3B, HTR6, HTR7).

Other genes that were selected based on our preliminary results:

- serotonin transporter gene (HTT/ SLC6A4),
- dopamine transporter gene (DAT/ SLC6A3),
- tyrosine hydroxylase gene (TH),
- tryptophan hydroxylase 2 gene (TPH2),
- cocaine- and amphetamine- regulated transcript (CARTPT)

- ankyrin repeat and kinase domain containing 1 gene (ANKK1) and tetratricopeptide repeat domain 12 gene (TTC12). Due to their proximity DRD2, TTC12 and ANKK1 can be considered as a cluster of genes.

2.3 Statistical Analysis:

Power calculations were performed through QUANTO power calculator (Gauderman & Morrison, 2006) and revealed that a case-control ratio of 1: 0.5, with 500 cases would have 80% power to detect associations with an odds ratio of 1.5, considering a minor allele frequency of 0.15, and a population prevalence of 0.04.

Statistical significance was set at $\alpha=0.001$. Haplotype analysis was performed through Golden-Helix SNP and Variation Suite version 7 (SVS v7). False Discovery Rate was applied for multiple-testing correction.

The minimum requirements to include a tagSNP in the pool of markers to be analyzed were: genotype calls >75%, HWE $p \geq 0.01$, and haplotypes with a minimum 5% estimated haplotype frequency for both cases and controls.

2.4 Sequencing of DRD4 exon III region:

Sequencing of the DRD4 exon III VNTR locus was performed at the Centre for Applied Genomics at the Hospital for Sick Kids and in our laboratory using the ABI-Avant 3130 Genetic Analyzer for Sequencing Analysis. All sequencing results were analyzed using SeqScape v.2.5. The results were then reviewed for quality assurance and samples that presented dubious results were sent for re-sequencing.

3. Results:

In our initial proposal, subjects could be considered as controls if their past year SOGS/PGSI scores were = 0. However, several controls were re-assessed during out project and presented **lifetime** SOGS/PGSI scores of 1 to 2. Clinically, these subjects would be considered as low-risk gamblers and had a lower likelihood of progressing to moderate and high-risk gambling categories. However, in respect to their biological risk, it has been shown that genetic vulnerability for PG increases in gamblers who have presented at least one of the DSM criteria for pathological gambling in comparison to subjects who never met any DSM criteria for PG (Eisen, et al., 2001). Moreover, all controls that presented a positive screening for alcohol abuse and/ or drug abuse (lifetime) were also excluded from the analysis. Thus, our sample of control subjects was reduced to 214 subjects. Conversely, we were

able to identify 514 subjects that were considered as problem gamblers (minimum SOGS/ PGSI lifetime scores ≥3). Amongst our cases, we also identified 99 PG subjects who also presented positive screening for alcohol abuse and/ or drug abuse.

3.1 PG subjects (n=514) x Control Subjects (n=214)

For the comparison between our total sample of PG and control subjects, a total of 307 haplotypes distributed along 40 genes were initially selected, based on our inclusion criteria of a minimum of 75% genotype calls and MAF ≥ 0.15 (Table 1). Haplotype blocks for analysis were defined using the confidence bounds method as described by Gabriel et al. (Gabriel, et al., 2002), in which 95% confidence bounds on D'[3] are generated and each comparison is called "strong LD", "inconclusive" or "strong recombination". A block is created if 95% of informative (i.e. non-inconclusive) comparisons are "strong LD". This definition allows for many overlapping blocks to be valid. The next step is to sort the list of all possible blocks, start with the largest block and keep adding blocks as long as they don't overlap with an already declared block. Only haplotype blocks with an estimated frequency >5% were included in the analysis, so that the final selection comprised 303 tagSNPs distributed along 39 genes (gene excluded: GnRH2).

Uncorrected p-values (≤0.001) show that haplotypes distributed across 13 genes were nominally associated with PG (Table 2). ***After FDR correction for multiple testing*** (α=0.001), two haplotypes on **CAMK2D**, four haplotypes on **HTR2A**, one haplotype on **PLD2**, and one haplotype on **PRKACB** were significantly associated with PG. ***FDR corrected p-values (between 0.002 and 0.01)*** revealed ***association trends*** with DRD3 (dopamine receptor gene, subtype 3), GRM1 (metabotropic glutamate receptor gene, subtype 1), HTR3A (serotonin receptor gene, subtype 3A), HTT/ SLC6A4 (serotonin transporter gene), TTC12 (part of the ANKK1-DRD2-TTC12 cluster of genes), and CAMK2A (calcium/calmodulin-dependent protein kinase, subtype 2A).

3.2 PG subjects with alcohol and/or drug abuse – PG-ADA (n=99) x Control Subjects (n=214)

For the comparison between PG subjects with positive screening for alcohol and/or substance abuse (PG-ADA), a total of 298 tagSNPs across 39 genes (gene excluded: GnRH2) met our selection criteria as described above.

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[3] D prime (D') is a scaled measure of the difference in frequency between observed number of haplotype pairs and the expected number. This measure is an estimator of linkage disequilibrium (LD).

Uncorrected p-values (≤0.001) show that 1 haplotype in: **CAMK2D** is nominally associated with PG-ADA (Table 3). The CAMK2D haplotype **rs2158196 _GCA** is inversely associated with PG-ADA; i.e. the haplotype might be protective, since its frequency is significantly (uncorrected p-value) lower in PG-ADA subjects compared to healthy controls. None of these associations remained significant after FDR correction for multiple testing.

3.3 PG subjects with alcohol and/or drug abuse – PG-ADA (n =99) x PG subjects without substance abuse (n =257)

For the comparison between PG- ADA subjects and PG subjects without alcohol and/or drug abuse history, a total of 298 tagSNPs across 39 genes (gene excluded: GnRH2) met our selection criteria as described in item 3.1

Uncorrected p-values (≤0.001) show that **GRM1, CAMK2D, and DRD2** (one haplotype on each gene) genes were nominally associated with the group of PG subjects without substance abuse history, (Table 4). None of these associations remained significant after FDR correction for multiple testing.

As we first hypothesized, we found genetic associations with genes located on chromosomes 4 (CAMK2D), 5 (CAMK2A), and 17 (PLD2, HTR2A), which are chromosomes that have strong evidence for harbouring susceptibility genes for addictions (M. D. Li & Burmeister, 2009), and on chromosome 1 (PRKACB). We found nominal associations with genes located on chromosome 6 (GRM1), chromosome 11 (TTC12, DRD2), and chromosome 17 (HTT / SLC6A4). **Noteworthy is the fact that across all group comparisons significant associations were found with CAMK2D**, **which is part of the pathway that interlinks the other 4 pathways**.

3.4. DRD4 sequencing analysis

The DRD4 exon III VNTR locus was sequenced for 170 of our PG subjects. ***We discovered a novel single-nucleotide deletion*** in two out of eleven individuals homozygous for the 7-repeat allele in our problem gambling sample (Figure 8). The novel single-nucleotide deletion variant was found in the first repeat unit for one subject and in the third repeat unit for the second subject. In both cases the deletion was of the “A” nucleotide. Although the deletions are located in two different repeat units the deletions occur at the same position within the repeat units.

In silico analysis showed that this deletion would give rise to a shift in the reading frame, causing a premature stop codon and truncated predicted amino acid sequence (Figure 9). This deletion was found in two out of eleven subjects (18%) who had the 7-7 genotype. Both subjects were pathological gamblers; however, due to the low frequency of the 7-7 genotype and of the deletion, we were not able to verify whether the deletion was associated with PG severity. No deletions were found in any of the subjects who had the 2-2 or 4-4 genotype.

4. Limitations and Changes to Original Proposal:

The main change to our original proposal is in regard to sample composition. As described in item 3 (page 14), in order to maintain a more strictly defined phenotype, the number of control subjects in our sample was decreased (initially 400 controls, actual sample: 214), while we were able to increase the number of cases (PG subjects – initially 400 subjects, actual sample: 514). **This change decreased our power to detect associations with very small odds ratio (< 1.5). Nevertheless, we were still able to detect associations that remained significant after correction for multiple testing.**

We were granted a 6-month extension for our project. Our project was delayed initially due to problems in importing genetic assays from the United States. Another important source of delay was the legal process of authorizing DNA transfer between institutions, since part of our genotyping and sequencing was done at another genetic laboratory in the University of Toronto. Finally, technical difficulties with our sequencer delayed DRD4 sequency.

In regards to other limitations, we acknowledge that the sample size of our group of PG-ADA has limited the interpretation of our results. At this point, we can say that the nominal associations found (uncorrected p-values ≤ 0.001) can be regarded only as preliminary data. In this specific case, preliminary data is important since this was the first study to investigate genetic associations with PG subjects with positive screening for substance abuse.

The number of genes and tagSNPs selected could also be a source of concern. Our choice of genes reflected the current knowledge on neuropathways involved in addictions *per se* or in brain systems that are closely related to PG and addictions. Each of these systems is composed of many parts, which in turn are synthesized by a number of genes. **In fact, our choice of genes was parsimonious compared to genome-wide association studies and reflects our concern with type I error. We opted for a conservative α level (≤ 0.001) and applied a multiple-testing correction that is considered as conservative for candidate gene studies.**

Despite limitations, this investigation presents many methodological advantages (sample size, substantial rational for the choice of genes) compared to previously published PG genetic association studies.

We are aware of the fact that different addictions (substance or behavioural) will not completely share genetic vulnerability factors and that there is a significant environmental component in the vulnerability for addiction disorders. We recognize that we investigated only a portion of the genetic architecture of PG that overlaps with substance addiction, and that we selected only genes that are considered as common vulnerability factors for addiction across different substances of abuse. It was not our objective to investigate any environmental factors involved in PG.

As mentioned on page 16, the low frequency of the deletion found in subjects homozygous for the 7-7 genotype, prevented the investigation of a possible association of this deletion with PG. It was not possible to know beforehand if we were going to find a new deletion and its frequency. Nevertheless, the finding is valid and will be reported to the scientific community through publication on a peer-reviewed scientific journal.

5. Discussion:

Is PG an addiction? If so, can a behavioural addiction affect the same brain systems as substances of abuse? These questions have been the focus of attention of many researchers in the gambling field. Recently, the Diagnostic and Statistical Manual for psychiatric disorders has acknowledged that clinical and research data support the construct of PG as an addiction, thus incorporating PG under addictive disorders in its next edition.

As outlined in section 1 (Introduction), imaging research has provided important evidence suggesting that PG affects areas of the brains in a very similar fashion as addictive substances. However, to the best of our knowledge, there has been no investigation on whether PG can induce changes in the brain signalling pathways that have been traditionally regarded as induced by substances of abuse. In order to provide initial data to answer this question, we have conducted a PG genetic association study on whether genes on addiction-related signalling pathways were also associated with PG. For this project, we were able to genotype 40 out of 396 genes involved in common addiction-related signalling pathways.

In summary, our results show that haplotypes on CAMK2D, PRKACB, HTR2A, and PLD2 were significantly associated with PG when compared to control subjects in a sample of 514 PG subjects and

214 control subjects. Nominal associations (uncorrected p-values) were also found for other genes that have been traditionally considered as the main candidate genes for PG: DRD3, GRM1, HTR3A, HTT/ SLC6A4, TTC12 (part of the ANKK1-DRD2-TTC12 cluster of genes).

In regards to our comparisons between PG-ADA and controls or PG subjects without substance abuse history, we found also nominal associations with genes traditionally considered as the main candidate genes for PG and addictions (DRD2, HTR2A, and GRM1), as well as with genes that had never been investigated in regards to gambling behaviour: CAMK2A and CAMK2D.

Below we will provide an overview of the role of these genes in addictive processes and discuss the implications of our findings in regards to advancing knowledge and to future research on the neurobiology of PG. Finally, we will also discuss our findings regarding sequencing of the DRD4 exon III VNTR.

A. Calcium- and calmodulin- dependent protein kinase genes (CAMK2A and CAMK2D)

- **CAMK2A located on chromosome 5**
- **CAMK2D located on chromosome 4**

The CAMK2 family of genes is responsible for the production of calcium- and calmodulin-dependent protein kinases. Protein Kinases act as regulators of cell function. Genes involved in protein kinases translation (i.e., production) constitute one of the largest and most functionally diverse gene families. Currently 518 human protein kinases have been indentified, with the vast majority of them belonging to one superfamily. Each superfamily can be clustered into groups, families and sub-families, of increasing sequence similarity and biochemical function. CAMK2 kinases (and thus CAMK2 genes) are a subfamily of the CASK family, which is part of the larger CAMK group. By adding phosphate groups to substrate proteins, they direct the activity, localization and overall function of many proteins, and serve to orchestrate the activity of almost all cellular processes. Kinases are predominantly important in signal transduction and co-ordination of complex cellular functions (Manning, Whyte, Martinez, Hunter, & Sudarsanam, 2002). CAMK2 is involved in several aspects of neuronal function, including neuroplasticity, gene expression, and neurotransmitter synthesis and release (Lee & Messing, 2008).

CAMK2A and CAMK2D are part of the long-term potentiation and GnRH signalling pathways (Figures 2 and 3, respectively). Figure 2 shows that CAMK2 genes directly regulate ionotropic glutamate receptor (AMPAR/ GRIN) cell cycle, thus affecting the Neuroactive-ligand receptor

interaction pathway, i.e. CAMK2 modulation affects how AMPAR will respond to glutamate. CAMK2 also modulates D1 receptor cell cycle (not represented in Figure 2).

Figure 3 illustrates how CAMK2 genes ultimately regulate gene expression and secretion of gonadotropins, thus directly affecting GnRHR1 and 2 genes on the Neuroactive-ligand receptor interaction pathway. ***Ultimately, the 5 common pathways for addiction are integrated with each other through fast and slow positive feedbacks loops that are all interlinked through CAMK2*** (C. Y. Li, et al., 2008).

CAMK2 has been reported to be involved in several addictive processes: sensitization, drug tolerance and self-administration, withdrawal and relapse (Lee & Messing, 2008).

B. Serotonin receptor 2A gene (HTR2A):

- **HTR2A located on chromosome 13**

Serotonin (5-HT) activity is associated with several behaviours such as behavioral inhibition (Coccaro, et al., 1989; Stein, Hollander, & Liebowitz, 1993), sensory reactivity (Sheard & Aghajanian, 1968) , sleep, sexual behaviour, and cognitive function (Patterson, et al., 1996; Ressler & Nemeroff, 2000). At least 14 subtypes of 5-HT receptors have been cloned and identified. The excitatory 5- HT2 receptor class is predominantly found on postsynaptic neurons, and activates phospholipase C. As can be seen on figure 5, phospholipase C (PLC) activates protein kinase C (PKC), also involved in addictive processes (see item C below).

Serotonin does not directly participate in motivation-reward, but exerts influence through its effects on the dopamine system. Application of 5-HT onto dopaminergic neurons from the VTA (part of the brain's reward system) increased their firing rate in vitro, an effect that was attributed to action of 5-HT on HTR2 receptors (Pessia, Jiang, North, & Johnson, 1994). As shown in figures 1-5, serotonin receptors are part of the neuroligand-receptor interaction (figure 1) and gap junction pathways (figure 5). Even though it has become common knowledge that 5-HT receptors are involved in behavioural inhibition and cognitive functions, previous candidate gene studies had focused on the investigation of the serotonin transporter (HTT). This is the first candidate gene study in PG to investigate and report an association with serotonin receptor gene 2A (HTR2A).

C. Phospholipase D2 gene (PLD2):

- **PLD2 located on chromosome 17**

There are two types of phospholipase genes: PLD1 and PLD2. Both genes synthesize pospholipase D, an enzyme that produces phosphatidic acid (PA) through hydrolyzation processes. PA is further metabolized into diacylglycerol (DAG), which regulates certain types of protein kinase C (PKC). Thus, PLD genes regulate PKC activity through the production of PA and DAG. Activation of PKC by calcium ions and the second messenger diacylglycerol is thought to ***play a central role in the induction of cellular responses to a variety of ligand-receptor systems (glutamate ionotropic receptors, dopamine D2 receptor, canabinoid receptor type 1, and nicotinic cholinergic receptors) and in the regulation of cellular responsiveness to external stimuli.***

The prefrontal cortex (PFC) is a brain region that regulates thought, behavior, and emotion using representational knowledge, operations often referred to as working memory. Birnbaum et al. (Birnbaum, et al., 2004) have tested the influence of PKC on intracellular signaling on PFC cognitive function and showed that high levels of PKC activity in prefrontal cortex (for instance, induced by stress) result in significant impairment of measures of working memory. These data suggest that ***excessive PKC activation can disrupt PFC regulation of behavior and thought, possibly contributing to signs of distractibility, impaired judgment, impulsivity, and thought disorder***. Distractibility, impaired judgment, and impulsivity are well recognized features of PG (Cavedini, Riboldi, Keller, D'Annucci, & Bellodi, 2002; Fuentes, Tavares, Artes, & Gorenstein, 2006; Goudriaan, Oosterlaan, de Beurs, & van den Brink, 2005; Petry, 2001), and ***this finding raises the possibility that working memory deficits in PG and substance addiction occur through the same pathway (PLD inducing PKC activation; PKC regulation cellular response to dopamine, glutamate receptors)***.

Similarly to CAMK2, PKC has also been involved in sensitization, drug tolerance, and drug self-administration and withdrawal (Lee & Messing, 2008).

D. Protein kinase, cAMP-dependent, catalytic, beta gene (PRKACB)_

- **PRKACB located on chromosome 1**

The PRKACB gene synthesizes the β-catalytic isoform of the c-AMP dependent protein kinase A (PKA). PKA is inactive in its natural state, and is activated when c-AMP (produced by adenylyl cyclase) binds to its regulatory region (Lee & Messing, 2008). ***It has been shown that addictive***

substances promote an acute increase in extracellular levels of dopamine in the nucleus accumbens, which stimulates adenylyl cyclase (producer of c-AMP) and PKA via D1 receptors (dopamine receptor type 1).

Substances that act as agonists in ***the D1 receptor together with PKA*** activation, increase the ***cell surface expression of glutamate receptor 1*** (ionotropic glutamate receptor 1, AMPA 1) in the nucleus accumbens and hippocampus of rats (Gao, Sun, & Wolf, 2006). Increasing the expression of the glutamate receptor increases synaptic strength. Thus, ***PKA activation may provide a mechanism for substance-induced neuroplasticity***. Similarly to CAMK2 and PKC, animal models have demonstrated PKA's involvement in sensitization, drug tolerance, and drug self-administration and withdrawal (Lee & Messing, 2008).

E. DRD4 exon III VNTR sequencing:

Our sequencing efforts on DRD4 have revealed a deletion in the 7-repeat allele of DRD4 exon III VNTR. Moreover, our results suggest that this deletion can interrupt the synthesis of the receptor, which could result in a "defective" D4 receptors (on 7-7 allele carriers) or in a decreased production of D4 receptors. ***This is the first time that a deletion that would alter gene transcription has been described for the 7-repeat allele of DRD4 exon III VNTR.***

The relatively low frequency of the 7-7 genotype in the general population (3%) highlights the need for heterozygotes to be sequenced. As of yet, PG subjects who are heterozygous for the 7-repeat allele (2-7, 4-7) were not sequenced due to cost limitations. Our future plans include sequencing 7-repeat heterozygotes and cloning sequences that present this deletion. After cloning, *in vitro* functional studies would provide experimental evidence of the effect of this deletion on the D4 receptor.

In conclusion, this is the largest genetic association study conducted on PG until now, and the first to investigate the association between PG and genes involved in addiction signalling pathways. Our original proposal indicated two major applications for this investigation: 1. to provide better understanding of the neurobiology of PG, and 2. to provide information in regards to processes that are amenable to pharmacological intervention. As discussed above, our results provide further insight into the neurobiology of PG. By taking a systems approach in selecting candidate genes we were able to identify new genes that had not been previously investigated in PG and that provide clues regarding shared genetic pathways between PG and substance addictions. ***As discussed above, CAMK2A, PRKACB, HTR2A, and PLD2 genes have important roles in synthesizing and or regulating proteins,***

enzymes, and second messengers that interact with each other and induce addiction-related neurochemical changes. These genetic findings will likely inform future neurobiological investigation into shared protein and enzymatic pathways between PG and substance addictions.

It is important to acknowledge that our results do not invalidate previous genetic associations with PG and substance addiction, since our sample had the power to detect genes with higher effect sizes. Genes, for which nominal associations were found, especially in the comparisons with PG-ADA, deserve further investigation in larger samples where comorbidity with substance use disorders has been documented.

Our findings can also help interpret results from previous genetic association studies in PG. For instance, our group had previously reported an association of PG with the DRD1 gene in a family sample (da Silva Lobo, et al., 2007), and trends for association on the DRD2/ ANKK1 TaqIA[4]/rs1800497 polymorphism (p=0.01) and the haplotype flanking DRD2 (G/C/A rs11604671/rs4938015/rs2303380) (Lobo, et al., 2010). Other studies had reported also associations with DRD2/ ANKK1 TaqIA in smaller samples of PG (Comings, et al., 1997; Comings, et al., 1996; da Silva Lobo, et al., 2007). Our results show that genes that regulate or are regulated by DRD1 and DRD2 are significantly associated with PG. This suggests that, although previously reported associations with DRD1 and DRD2 are valid, genes involved in up-stream and down-stream dopamine signalling have a greater effect (have a stronger association) on PG. The same can be said in regards to other neurotransmitter receptor genes such as glutamate receptor genes.

Also, and perhaps more importantly, our results have provided information in regards to processes that are amenable to pharmacological intervention. ***The association of PG with CAMK2D and PRKACB and PLD2 (directly or indirectly involved in protein kinases synthesis) indicates that protein-kinase inhibitors could potentially be used in the treatment of PG***. The recent availability of an animal model for gambling behaviour allows pre-clinical testing of protein-kinase inhibitors that are currently under development, which would be the appropriate next step in the investigation of our findings.

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Table 1: List of genes and SNPs selected for genotyping within their respective chromosomes.

Chromosome	Gene Symbol	SNP
1	CNR2	rs12733278
1	CNR2	rs2502993
1	HTR6	rs4912138
1	HTR6	rs6699866
1	HTR6	rs9659997
1	LOC646330/ TNFRSF1	rs4285653
1	NBL1/ TNFRSF1	rs2294630
1	PRKACB	rs11163905
1	PRKACB	rs12129674
1	PRKACB	rs12404263
1	PRKACB	rs12724598
1	PRKACB	rs2812452
1	PRKACB	rs603939
1	PRKACB	rs6661411
1	PRKACB	rs7520283
1	PRKACB	rs7547892
1	TMCO4	rs3813987
2	TACR1	rs10865408
2	TACR1	rs11680998
2	TACR1	rs11688000
2	TACR1	rs12477554
2	TACR1	rs12713837
2	TACR1	rs13428269
2	TACR1	rs17564182
2	TACR1	rs2216307
2	TACR1	rs2422148
2	TACR1	rs3729565
2	TACR1	rs3755457
2	TACR1	rs3755460
2	TACR1	rs3771827
2	TACR1	rs3771830
2	TACR1	rs3771836
2	TACR1	rs3771847
2	TACR1	rs3771856
2	TACR1	rs4439987
2	TACR1	rs4519549
2	TACR1	rs4853112
2	TACR1	rs4853119
2	TACR1	rs6546952
2	TACR1	rs6709928
2	TACR1	rs6733933
2	TACR1	rs759588
2	TACR1	rs7604270
3	DRD3	rs10934256
3	DRD3	rs11721264
3	DRD3	rs167770
3	DRD3	rs167771
3	DRD3	rs1800828
3	DRD3	rs2134655
3	DRD3	rs226082
3	DRD3	rs324022
3	DRD3	rs324029
3	DRD3	rs324030
3	DRD3	rs3773679
3	DRD3	rs6280
3	DRD3	rs7625282
3	DRD3	rs7633291
3	DRD3	rs963468
3	PLD1	rs1076504
3	PLD1	rs2178533
3	PLD1	rs3821745
3	PLD1	rs4256166
3	PLD1	rs9846460
4	CAMK2D	rs10003275
4	CAMK2D	rs10009286
4	CAMK2D	rs10012931
4	CAMK2D	rs10018022
4	CAMK2D	rs10025791
4	CAMK2D	rs10027475
4	CAMK2D	rs1011973
4	CAMK2D	rs1047187
4	CAMK2D	rs10488894
4	CAMK2D	rs11098195
4	CAMK2D	rs11098198
4	CAMK2D	rs13107662
4	CAMK2D	rs13133676
4	CAMK2D	rs13144613
4	CAMK2D	rs1524998
4	CAMK2D	rs1525000
4	CAMK2D	rs17446418
4	CAMK2D	rs17531026
4	CAMK2D	rs17531554
4	CAMK2D	rs2158196
4	CAMK2D	rs2189364
4	CAMK2D	rs2285704
4	CAMK2D	rs2293323
4	CAMK2D	rs3815072

Table 1 (cont'd List of genes and SNPs selected for genotyping within their respective chromosomes.

Chromosome	Gene Symbol	SNP	Chromosome	Gene Symbol	SNP
4	CAMK2D	rs4834349	5	CARTPT	rs4991862
4	CAMK2D	rs4834354	5	CARTPT	rs6894758
4	CAMK2D	rs6533690	5	DRD1	rs265976
4	CAMK2D	rs6533709	5	DRD1	rs265981
4	CAMK2D	rs6836139	5	DRD1	rs4532
4	CAMK2D	rs6842886	5	DRD1	rs686
4	CAMK2D	rs7438925	5	SLC6A3	rs1042098
4	CAMK2D	rs757173	5	SLC6A3	rs11564758
4	CAMK2D	rs7660775	5	SLC6A3	rs2042449
4	CAMK2D	rs7697831	5	SLC6A3	rs403636
4	CAMK2D	rs9307393	5	SLC6A3	rs463379
4	TACR3	rs10516505	5	SLC6A3	rs6347
4	TACR3	rs11097822	5	SLC6A3	rs6869645
4	TACR3	rs12641703	6	CNR1	rs4707436
4	TACR3	rs233976	6	CNR1	rs806368
4	TACR3	rs2765	6	FLJ37060	rs10457057
4	TACR3	rs3796972	6	GRM1	rs1125462
4	TACR3	rs3822292	6	GRM1	rs362835
4	TACR3	rs6831087	6	GRM1	rs362854
4	TACR3	rs7665881	6	GRM1	rs362871
5	CAMK2A	rs10066581	6	GRM1	rs3819836
5	CAMK2A	rs12514354	6	GRM1	rs6570764
5	CAMK2A	rs13357922	6	GRM1	rs7755078
5	CAMK2A	rs13360565	6	GRM1	rs9390385
5	CAMK2A	rs2053053	6	GRM1	rs960385
5	CAMK2A	rs2241695	6	HTR1B	rs11568817
5	CAMK2A	rs3756577	6	HTR1B	rs1213371
5	CAMK2A	rs3756578	6	HTR1B	rs130056
5	CAMK2A	rs3776825	6	HTR1B	rs130058
5	CAMK2A	rs3797617	6	HTR1B	rs130060
5	CAMK2A	rs3822607	6	HTR1B	rs13212041
5	CAMK2A	rs4958443	6	HTR1B	rs2000292
5	CAMK2A	rs4958445	6	HTR1B	rs4140535
5	CAMK2A	rs4958456	6	HTR1B	rs6296
5	CAMK2A	rs6869634	6	HTR1B	rs6297
5	CAMK2A	rs6896910	6	HTR1B	rs9352481
5	CAMK2A	rs7711562	6	HTR1B	rs9359271
5	CAMK2A	rs7718715	6	PARK2	rs9364654
5	CAMK2A	rs919740	7	CAMK2B	rs1003573
5	CAMK2A	rs919741	7	CAMK2B	rs10230538
5	CARTPT	rs10515114	7	CAMK2B	rs10231886
5	CARTPT	rs10515115	7	CAMK2B	rs13246801
5	CARTPT	rs10515116	7	CAMK2B	rs2075069
5	CARTPT	rs11575893	7	CAMK2B	rs2075074
5	CARTPT	rs16871471	7	CAMK2B	rs35397936
5	CARTPT	rs3763153	7	CAMK2B	rs4642534
5	CARTPT	rs3846658	7	CAMK2B	rs4724298
5	CARTPT	rs3857384	7	CAMK2B	rs6968826
5	CARTPT	rs4704172	7	CAMK2B	rs9918696

Table 1 (cont'd List of genes and SNPs selected for genotyping within their respective chromosomes.

Chromosome	Gene Symbol	SNP	Chromosome	Gene Symbol	SNP
8	GNRH1	rs1567126	11	GRM5	rs594561
8	GNRH1	rs1876281	11	GRM5	rs596370
8	GNRH1	rs6185	11	GRM5	rs6483414
9	PRKACG	rs3730386	11	GRM5	rs672981
9	PRKACG	rs3812538	11	GRM5	rs7483940
10	CAMK2G	rs17631059	11	HTR3A	rs10789980
10	CAMK2G	rs2664282	11	HTR3A	rs11214800
10	CAMK2G	rs7080350	11	HTR3A	rs1176713
10	CAMK2G	rs7098573	11	HTR3A	rs1176719
10	HTR7	rs10785973	11	HTR3A	rs1379170
10	HTR7	rs11186300	11	HTR3A	rs2276302
10	HTR7	rs11597471	11	HTR3A	rs3832782
10	HTR7	rs11599921	11	HTR3A	rs7126511
10	HTR7	rs7916403	11	HTR3B	rs10502180
10	TACR2	rs10998765	11	HTR3B	rs10789970
10	TACR2	rs2278746	11	HTR3B	rs11606194
10	TACR2	rs4644560	11	HTR3B	rs1176744
10	TACR2	rs986525	11	HTR3B	rs1176761
11	ANKK1	rs12360992	11	HTR3B	rs1185027
11	ANKK1	rs17115439	11	HTR3B	rs17116121
11	ANKK1	rs1800497	11	HTR3B	rs17116138
11	ANKK1	rs2734849	11	HTR3B	rs2276307
11	ANKK1	rs4938014	11	HTR3B	rs3758987
11	ANKK1	rs7123797	11	HTR3B	rs3782025
11	DRD2	rs12364051	11	HTR3B	rs7942029
11	DRD2	rs17529477	11	INS	rs10840447
11	DRD2	rs1800498	11	INS	rs3842756
11	DRD2	rs4648318	11	TH	rs10770141
11	DRD2	rs4936271	11	TH	rs11042978
11	DRD2	rs6277	11	TH	rs2070762
11	DRD2	rs7131056	11	TH	rs6356
11	DRD4	rs1800443	11	TH	rs7483056
11	DRD4	rs3758653	11	TTC12	rs10891539
11	DRD4	rs916457	11	TTC12	rs2236709
11	DRD4	rs936460	11	TTC12	rs2303380
11	GRM5	rs1024117	11	TTC12	rs4370966
11	GRM5	rs10430815	11	TTC12	rs635358
11	GRM5	rs10765724	11	TTC12	rs7130072
11	GRM5	rs10830200	11	TTC12	rs723077
11	GRM5	rs11021034	11	TTC12	rs7927508
11	GRM5	rs12806683	11	TTC12	rs7942392
11	GRM5	rs1504093	12	TPH2	rs10506645
11	GRM5	rs189769	12	TPH2	rs10784941
11	GRM5	rs2133395	12	TPH2	rs10879354
11	GRM5	rs2648640	12	TPH2	rs12229394
11	GRM5	rs308787	12	TPH2	rs1352250
11	GRM5	rs308884	12	TPH2	rs1386485
11	GRM5	rs308893	12	TPH2	rs1386486
11	GRM5	rs316096	12	TPH2	rs1386493
11	GRM5	rs4753106	12	TPH2	rs1386494

Table 1 (cont'd List of genes and SNPs selected for genotyping within their respective chromosomes.

Chromosome	Gene Symbol	SNP	Chromosome	Gene Symbol	SNP
12	TPH2	rs1487275	17	PLD2	rs1052751
12	TPH2	rs1487279	17	PLD2	rs2286670
12	TPH2	rs1487280	17	PLD2	rs2286671
12	TPH2	rs1843809	17	PLD2	rs2875840
12	TPH2	rs1872824	17	PLD2	rs3764899
12	TPH2	rs2171363	17	PLD2	rs7212512
12	TPH2	rs4565946	17	SLC6A4	rs1042173
12	TPH2	rs4570625	17	SLC6A4	rs12150214
12	TPH2	rs4760750	17	SLC6A4	rs140700
12	TPH2	rs4760816	17	SLC6A4	rs2020930
12	TPH2	rs6582078	17	SLC6A4	rs2020936
12	TPH2	rs9325202	17	SLC6A4	rs2020939
13	HTR2A	rs1002513	17	SLC6A4	rs2066713
13	HTR2A	rs1328684	17	SLC6A4	rs28914832
13	HTR2A	rs1885884	17	SLC6A4	rs4251417
13	HTR2A	rs1923885	17	SLC6A4	rs6354
13	HTR2A	rs1923886	17	SLC6A4	rs6355
13	HTR2A	rs2149434	19	MAP2K2	rs11666488
13	HTR2A	rs2296972	19	MAP2K2	rs12459484
13	HTR2A	rs2296973	19	MAP2K2	rs1823059
13	HTR2A	rs2770298	19	MAP2K2	rs350891
13	HTR2A	rs2770304	19	MAP2K2	rs350895
13	HTR2A	rs4941573	19	MAP2K2	rs350896
13	HTR2A	rs4942577	19	MAP2K2	rs350897
13	HTR2A	rs582854	19	MAP2K2	rs350911
13	HTR2A	rs6313	19	MAP2K2	rs350916
13	HTR2A	rs6314	19	MAP2K2	rs7258366
13	HTR2A	rs7997012	20	GNRH2	rs6051545
13	HTR2A	rs927544	20	GNRH2	rs676749
13	HTR2A	rs9534495	22	MAPK1	rs2876981
13	HTR2A	rs9534496	22	MAPK1	rs5755099
13	HTR2A	rs9534511	22	MAPK1	rs9607272
13	HTR2A	rs9534512	22	MAPK1	rs9610375
13	HTR2A	rs9567733			
13	HTR2A	rs977003			
13	HTR2A	rs985933			
14	FSCB	rs1118997			
15	MAP2K1	rs11854215			
15	MAP2K1	rs1549854			
15	MAP2K1	rs17200970			
15	MAP2K1	rs7181936			
17	BLMH	rs1050565			
17	BLMH	rs7214991			
17	MAP2K3	rs11650053			
17	MAP2K3	rs2363226			
17	MAP2K3	rs3760201			
17	MAP2K3	rs3892032			
17	MAP2K3	rs4114291			
17	MAP2K3	rs8074866			
17	MAP2K3	rs9899521			

Table 2: Haplotype comparisons between problem gamblers (PG) with healthy control subjects, modified qui-squared tests, Golden Helix, SVS v. 7. Only results with p>0.001 are shown [1].

First Marker	Gene	Haplotype	Haplotype Frequency PG	Haplotype Frequency Controls	χ2	Nominal p-value	Corrected p-valued	Odds Ratio	Odds Ratio 95% CI	
rs919741	***CAMK2A***	***GG***	***0.25***	***0.17***	***14.0***	***1.9E-04***	***0.0072***	***1.73***	***1.29***	***2.31***
rs12514354	***CAMK2A***	***GA***	***0.61***	***0.72***	***13.9***	***1.9E-04***	***0.0070***	***0.63***	***0.50***	***0.81***
rs12514354	***CAMK2A***	***GG***	***0.12***	***0.05***	***13.5***	***2.4E-04***	***0.0084***	***2.30***	***1.46***	***3.62***
rs919740	***CAMK2A***	***AC***	***0.53***	***0.63***	***12.8***	***3.4E-04***	***0.0104***	***0.66***	***0.52***	***0.83***
rs2241695	CAMK2A	GG	0.14	0.08	10.5	1.2E-03	0.0234	1.89	1.28	2.80
rs2293323	***CAMK2D***	***CA***	***0.48***	***0.33***	***29.0***	***7.4E-08***	***0.0001***	***1.89***	***1.50***	***2.39***
rs1524998	***CAMK2D***	***AA***	***0.24***	***0.14***	***20.0***	***7.9E-06***	***0.0010***	***1.99***	***1.47***	***2.71***
rs10003275	***CAMK2D***	***TA***	***0.29***	***0.40***	***17.0***	***3.8E-05***	***0.0028***	***0.61***	***0.49***	***0.77***
rs4834354	***CAMK2D***	***AA***	***0.32***	***0.42***	***14.6***	***1.3E-04***	***0.0061***	***0.64***	***0.51***	***0.80***
rs10003275	***CAMK2D***	***AG***	***0.25***	***0.16***	***14.4***	***1.4E-04***	***0.0066***	***1.76***	***1.31***	***2.36***
rs4834349	***CAMK2D***	***GA***	***0.25***	***0.34***	***14.0***	***1.8E-04***	***0.0078***	***0.63***	***0.49***	***0.80***
rs17446418	***CAMK2D***	***AA***	***0.33***	***0.23***	***13.9***	***1.9E-04***	***0.0071***	***1.63***	***1.26***	***2.11***
rs1524998	***CAMK2D***	***GA***	***0.36***	***0.46***	***13.4***	***2.5E-04***	***0.0084***	***0.65***	***0.52***	***0.82***
rs17531026	***CAMK2D***	***AG***	***0.24***	***0.33***	***12.8***	***3.4E-04***	***0.0102***	***0.64***	***0.50***	***0.82***
rs2293323	***CAMK2D***	***CG***	***0.37***	***0.47***	***12.7***	***3.6E-04***	***0.0106***	***0.66***	***0.53***	***0.83***
rs6836139	***CAMK2D***	***AA***	***0.36***	***0.46***	***12.4***	***4.2E-04***	***0.0116***	***0.67***	***0.53***	***0.84***
rs7660775	***CAMK2D***	***AT***	***0.69***	***0.78***	***12.0***	***5.2E-04***	***0.0130***	***0.63***	***0.48***	***0.82***
rs10515115	CARTPT	AG	0.24	0.32	10.5	1.2E-03	0.0226	0.67	0.52	0.85
rs2134655	***DRD3***	***GG***	***0.43***	***0.31***	***17.9***	***2.3E-05***	***0.0022***	***1.66***	***1.31***	***2.11***
rs7633291	***DRD3***	***AA***	***0.58***	***0.68***	***11.6***	***6.7E-04***	***0.0155***	***0.67***	***0.53***	***0.84***
rs6280	***DRD3***	***AC***	***0.58***	***0.68***	***11.6***	***6.7E-04***	***0.0151***	***0.67***	***0.53***	***0.84***
rs7755078	***GRM1***	***CC***	***0.27***	***0.17***	***15.5***	***8.3E-05***	***0.0051***	***1.76***	***1.33***	***2.34***
rs362854	***GRM1***	***TC***	***0.24***	***0.16***	***11.9***	***5.6E-04***	***0.0134***	***1.67***	***1.24***	***2.23***
rs2648640	GRM5	AC	0.31	0.40	10.3	1.3E-03	0.0239	0.68	0.54	0.86
rs672981	GRM5	CG	0.32	0.41	10.3	1.3E-03	0.0240	0.69	0.54	0.86

[1] Genes highlighted in yellow were significant after FDR correction (p ≤0.001). Genes in bold and italicized were significant at uncorrected p ≤0.001.

Table 2 (Cont'd): Haplotype comparisons between problem gamblers (PG) with healthy control subjects, modified qui-squared tests, Golden Helix, SVS v. 7. Only results with p>0.001 are shown[1].

First Marker	Gene	Haplotype	Haplotype Frequency PG	Haplotype Frequency Controls	χ2	Nominal p-value	Corrected p-valued	Odds Ratio	Odds Ratio	95% CI
rs6314	***HTR2A***	***GA***	***0.36***	***0.50***	***25.9***	***3.5E-07***	***0.0002***	***0.56***	***0.44***	***0.70***
rs1002513	***HTR2A***	***AA***	***0.15***	***0.26***	***25.6***	***4.2E-07***	***0.0001***	***0.50***	***0.38***	***0.66***
rs7997012	***HTR2A***	***AA***	***0.33***	***0.47***	***23.4***	***1.3E-06***	***0.0003***	***0.57***	***0.46***	***0.72***
rs2770298	***HTR2A***	***AA***	***0.14***	***0.23***	***19.9***	***8.4E-06***	***0.0010***	***0.53***	***0.40***	***0.70***
rs2296973	***HTR2A***	***CA***	***0.16***	***0.26***	***18.3***	***1.9E-05***	***0.0020***	***0.56***	***0.42***	***0.73***
rs6314	***HTR2A***	***GG***	***0.55***	***0.43***	***17.5***	***2.9E-05***	***0.0025***	***1.61***	***1.29***	***2.02***
rs6313	***HTR2A***	***GA***	***0.38***	***0.49***	***16.4***	***5.1E-05***	***0.0035***	***0.63***	***0.50***	***0.79***
rs9534512	***HTR2A***	***AC***	***0.13***	***0.21***	***15.2***	***9.6E-05***	***0.0050***	***0.56***	***0.42***	***0.75***
rs6313	***HTR2A***	***GC***	***0.20***	***0.12***	***14.8***	***1.2E-04***	***0.0059***	***1.90***	***1.36***	***2.64***
rs9534512	***HTR2A***	***CA***	***0.20***	***0.12***	***12.2***	***4.7E-04***	***0.0123***	***1.79***	***1.29***	***2.48***
rs1923885	***HTR2A***	***AG***	***0.42***	***0.52***	***11.9***	***5.5E-04***	***0.0133***	***0.67***	***0.54***	***0.84***
rs4942577	***HTR2A***	***GA***	***0.28***	***0.37***	***10.8***	***1.0E-03***	***0.0222***	***0.67***	***0.53***	***0.85***
rs7997012	HTR2A	GA	0.28	0.20	10.5	1.2E-03	0.0229	1.57	1.19	2.06
rs582854	HTR2A	AA	0.14	0.21	10.5	1.2E-03	0.0229	0.62	0.47	0.83
rs1379170	HTR3A	AA	0.24	0.15	14.0	1.8E-04	0.0074	1.77	1.31	2.39
rs4285653	CNR2	AA	0.32	0.40	10.2	1.4E-03	0.0247	0.69	0.54	0.87
rs2286670	***PLD2***	***CG***	***0.49***	***0.36***	***23.0***	***1.6E-06***	***0.0003***	***1.75***	***1.39***	***2.21***
rs2286670	***PLD2***	***CA***	***0.39***	***0.49***	***12.1***	***5.1E-04***	***0.0130***	***0.67***	***0.54***	***0.84***
rs2286671	***PLD2***	***GA***	***0.15***	***0.08***	***11.0***	***9.3E-04***	***0.0207***	***1.89***	***1.29***	***2.76***
rs12404263	***PRKACB***	***GA***	***0.36***	***0.24***	***20.2***	***7.1E-06***	***0.0011***	***1.78***	***1.38***	***2.29***
rs7547892	***PRKACB***	***GA***	***0.42***	***0.31***	***15.7***	***7.5E-05***	***0.0049***	***1.61***	***1.27***	***2.05***
rs6661411	***PRKACB***	***AA***	***0.15***	***0.08***	***15.4***	***8.7E-05***	***0.0051***	***2.16***	***1.46***	***3.20***
rs7520283	***PRKACB***	***AG***	***0.34***	***0.23***	***15.4***	***8.9E-05***	***0.0049***	***1.67***	***1.29***	***2.16***
rs12404263	***PRKACB***	***AG***	***0.40***	***0.51***	***14.0***	***1.8E-04***	***0.0077***	***0.65***	***0.52***	***0.82***
rs7520283	***PRKACB***	***GG***	***0.41***	***0.51***	***12.4***	***4.3E-04***	***0.0115***	***0.67***	***0.53***	***0.84***

[1] Genes highlighted in yellow were significant after FDR correction (p ≤0.001). Genes in bold and italicized were significant at uncorrected p ≤0.001.

Table 2 (Cont'd): Haplotype comparisons between problem gamblers (PG) with healthy control subjects, modified qui-squared tests, Golden Helix, SVS v. 7. Only results with p>0.001 are shown[1].

First Marker	Gene	Haplotype	Haplotype Frequency PG	Haplotype Frequency Controls	χ2	Nominal p-value	Corrected p-valued	Odds Ratio	Odds Ratio 95% CI	
rs140700	***SLC6A4***	***GC***	***0.12***	***0.06***	***13.0***	***3.0E-04***	***0.0099***	***2.19***	***1.42***	***3.39***
rs17564182	***TACR1***	***CA***	***0.16***	***0.23***	***10.8***	***1.0E-03***	***0.0222***	***0.63***	***0.48***	***0.83***
rs11688000	TACR1	GC	0.16	0.24	10.6	1.1E-03	0.0230	0.63	0.48	0.83
rs7604270	TACR1	GA	0.22	0.15	10.3	1.3E-03	0.0242	1.64	1.21	2.22
rs2303380	***TTC12***	***AG***	***0.25***	***0.17***	***12.9***	***3.3E-04***	***0.0104***	***1.69***	***1.27***	***2.25***

[1] Genes highlighted in yellow were significant after FDR correction (p ≤0.001). Genes in bold and italicized were significant at uncorrected p ≤0.001.

Table 3: Haplotype comparisons between problem gamblers with alcohol and/or drug abuse (PG-ADA) with control subjects; modified qui-squared tests, Golden Helix, SVS v. 7. Association trends with uncorrected p ≤0.01 are shown[1].

First Marker	Gene	Haplotype	Haplotype Frequency PG	Haplotype Frequency Controls	χ2	Nominal p-value	Corrected p-valued	Odds Ratio	Odds Ratio 95% CI	
rs2158196	***CAMK2D***	***GCA***	***0.16***	***0.29***	***10.1***	***0.001***	***0.20***	***0.48***	***0.30***	***0.76***
rs9534511	HTR2A	AGC	0.17	0.08	9.9	0.002	0.18	2.47	1.39	4.37
rs7520283	PRKACB	AGC	0.33	0.20	9.9	0.002	0.17	1.94	1.28	2.93
rs1002513	HTR2A	AAG	0.14	0.26	9.4	0.002	0.20	0.48	0.30	0.77
rs2053053	CAMK2A	GGG	0.12	0.05	9.3	0.002	0.20	2.89	1.43	5.85
rs2770298	HTR2A	AGG	0.14	0.06	9.1	0.003	0.21	2.63	1.38	5.01
rs4941573	HTR2A	AGA	0.09	0.03	9.1	0.003	0.20	3.30	1.46	7.45
rs985933	HTR2A	GAA	0.15	0.26	8.7	0.003	0.23	0.50	0.31	0.79
rs6313	HTR2A	GCA	0.20	0.11	8.5	0.004	0.24	2.12	1.27	3.53
rs1524998	CAMK2D	AGA	0.17	0.28	8.3	0.004	0.26	0.51	0.33	0.81
rs1524998	CAMK2D	AAA	0.25	0.15	8.3	0.004	0.25	1.95	1.23	3.08
rs2293323	CAMK2D	CAA	0.25	0.15	7.9	0.005	0.28	1.91	1.21	3.01
rs4285653	CNR2	AGA	0.28	0.18	7.8	0.005	0.29	1.86	1.20	2.87
rs233976	TACR3	ACA	0.11	0.05	7.6	0.006	0.30	2.58	1.29	5.17
rs4958445	CAMK2A	AAC	0.22	0.34	7.6	0.006	0.30	0.56	0.37	0.85
rs7665881	TACR3	AGC	0.36	0.25	7.3	0.007	0.34	1.73	1.16	2.57
rs2241695	CAMK2A	GGG	0.12	0.05	7.0	0.008	0.37	2.42	1.24	4.73
rs7927508	TTC12	AAA	0.08	0.16	6.9	0.008	0.37	0.45	0.25	0.83
rs12641703	TACR3	GCA	0.36	0.25	6.9	0.009	0.36	1.70	1.14	2.52
rs7942392	TTC12	AAT	0.08	0.16	6.9	0.009	0.35	0.45	0.25	0.83
rs2812452	PRKACB	GCG	0.25	0.16	6.9	0.009	0.35	1.82	1.16	2.85
rs2770298	HTR2A	AAA	0.14	0.23	6.7	0.010	0.37	0.53	0.32	0.86
rs582854	HTR2A	AAA	0.14	0.23	6.7	0.010	0.36	0.52	0.32	0.86
rs7130072	TTC12	GAA	0.08	0.16	6.6	0.010	0.38	0.46	0.25	0.84

[1]. Genes in bold and italicized were significant at uncorrected p ≤0.001

Table 4: Haplotype comparisons between problem gamblers with alcohol and/or drug abuse (PG-ADA) with PG subjects without substance abuse history; modified qui-squared tests, Golden Helix, SVS v. 7. Association trends with uncorrected p ≤0.01 are shown[1].

First Marker	Gene	Haplotype	Haplotype Frequency PG	Haplotype Frequency Controls	χ2	Nominal p-value	Corrected p-valued	Odds Ratio	Odds Ratio 95% CI	
rs7660775	***CAMK2D***	***CAG***	***0.11***	***0.24***	***12.7***	***0.0004***	***0.23***	***0.39***	***0.23***	***0.66***
rs1011973	CAMK2D	GCA	0.09	0.19	9.9	0.002	0.51	0.39	0.22	0.71
rs3815072	CAMK2D	AAA	0.42	0.57	9.8	0.002	0.38	0.54	0.37	0.80
rs2285704	CAMK2D	AAA	0.26	0.39	9.2	0.002	0.43	0.53	0.35	0.80
rs11098195	CAMK2D	AAA	0.24	0.37	8.6	0.003	0.36	0.53	0.35	0.81
rs17531026	CAMK2D	AAA	0.42	0.55	8.5	0.003	0.34	0.56	0.38	0.83
rs3815072	CAMK2D	GAG	0.16	0.08	7.8	0.005	0.42	2.34	1.27	4.31
rs6533690	CAMK2D	GGA	0.16	0.08	7.6	0.006	0.44	2.29	1.25	4.18
rs17531554	CAMK2D	ACG	0.19	0.10	7.4	0.007	0.41	2.14	1.23	3.75
rs10009286	CAMK2D	ACA	0.19	0.10	7.3	0.007	0.41	2.13	1.22	3.72
rs2293323	CAMK2D	CGA	0.42	0.30	7.1	0.008	0.44	1.72	1.15	2.56
rs2285704	CAMK2D	GAA	0.20	0.10	7.1	0.008	0.41	2.09	1.20	3.62
rs17446418	CAMK2D	AGG	0.16	0.08	7.0	0.008	0.39	2.22	1.22	4.04
rs11098198	CAMK2D	AGC	0.05	0.12	6.9	0.009	0.37	0.38	0.18	0.80
rs13144613	CAMK2D	CAC	0.19	0.10	6.7	0.009	0.37	2.07	1.19	3.63
rs2189364	CAMK2D	AAG	0.13	0.05	6.7	0.010	0.37	2.47	1.22	5.00
rs686	DRD1	GA	0.03	0.08	6.6	0.010	0.36	0.29	0.11	0.78
rs4648318	***DRD2***	***AGA***	***0.06***	***0.17***	***10.8***	***0.001***	***0.42***	***0.33***	***0.17***	***0.66***

[1] Genes in bold and italicized were significant at uncorrected p ≤0.001

Table 4 (Cont'd): Haplotype comparisons between problem gamblers with alcohol and/or drug abuse (PG-ADA) with PG subjects without substance abuse history; modified qui-squared tests, Golden Helix, SVS v. 7. Association trends with uncorrected p ≤0.01 are shown[1].

First Marker	Gene	Haplotype	Haplotype Frequency PG	Haplotype Frequency Controls	$\chi 2$	Nominal p-value	Corrected p-valued	Odds Ratio	Odds Ratio 95% CI	
rs362854	***GRM1***	***TCC***	***0.07***	***0.20***	***12.9***	***0.0003***	***0.42***	***0.33***	***0.18***	***0.62***
rs7755078	GRM1	CCG	0.14	0.26	9.2	0.002	0.38	0.47	0.28	0.77
rs1125462	GRM1	ACG	0.04	0.11	8.2	0.004	0.35	0.30	0.13	0.71
rs7755078	GRM1	ACG	0.33	0.23	7.0	0.008	0.41	1.79	1.16	2.75
rs672981	GRM5	AGG	0.08	0.19	9.8	0.002	0.43	0.39	0.21	0.71
rs2648640	GRM5	GAG	0.08	0.17	8.4	0.004	0.34	0.41	0.22	0.76
rs11021034	GRM5	GAA	0.07	0.15	7.5	0.006	0.41	0.41	0.21	0.79
rs1923885	HTR2A	AAC	0.10	0.18	6.8	0.009	0.37	0.47	0.26	0.84
rs9607272	MAPK1	ACA	0.47	0.60	6.9	0.008	0.39	0.60	0.41	0.88
rs9610375	MAPK1	CA	0.47	0.60	6.9	0.008	0.38	0.60	0.41	0.88
rs5755099	MAPK1	CCA	0.27	0.17	6.6	0.010	0.36	1.82	1.15	2.89
rs2294630	NBL1	AGC	0.35	0.24	7.5	0.006	0.43	1.80	1.18	2.73
rs3771827	TACR1	AAA	0.04	0.11	7.1	0.008	0.42	0.34	0.15	0.78
rs12641703	TACR3	AAA	0.34	0.46	6.9	0.009	0.37	0.59	0.40	0.88
rs3822292	TACR3	ACA	0.12	0.22	6.7	0.010	0.37	0.51	0.30	0.85
rs10770141	TH	ACA	0.02	0.08	8.8	0.003	0.35	0.20	0.06	0.65
rs7927508	TTC12	AAA	0.08	0.18	9.2	0.002	0.34	0.40	0.21	0.73
rs7130072	TTC12	GAA	0.08	0.18	8.8	0.003	0.39	0.41	0.22	0.75

[1] Genes in bold and italicized were significant at uncorrected p ≤0.001

Figures 1-5: The diagrams on figures 1-5 should be read considering that the network was constructed manually based on the common pathways identified in Li et al. (Li, Mao, & Wei, 2008) and protein interaction data. **Addiction-related genes** were represented as **white boxes** while **neurotransmitters and secondary messengers were highlighted in purple**. Related functional modules such as ''regulation of cytoskeleton'', ''regulation of cell cycle'', ''regulation of gap junction'', and ''gene expression and secretion of gonadotropins'' were highlighted in carmine boxes. Notation in the maps were used in accordance to the KEGG Orthology-Based Annotation System (Mao, X., Cai, T., Olyarchuk, J. G., & Wei, L. (2005). Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. *Bioinformatics, 21*(19), 3787-3793).

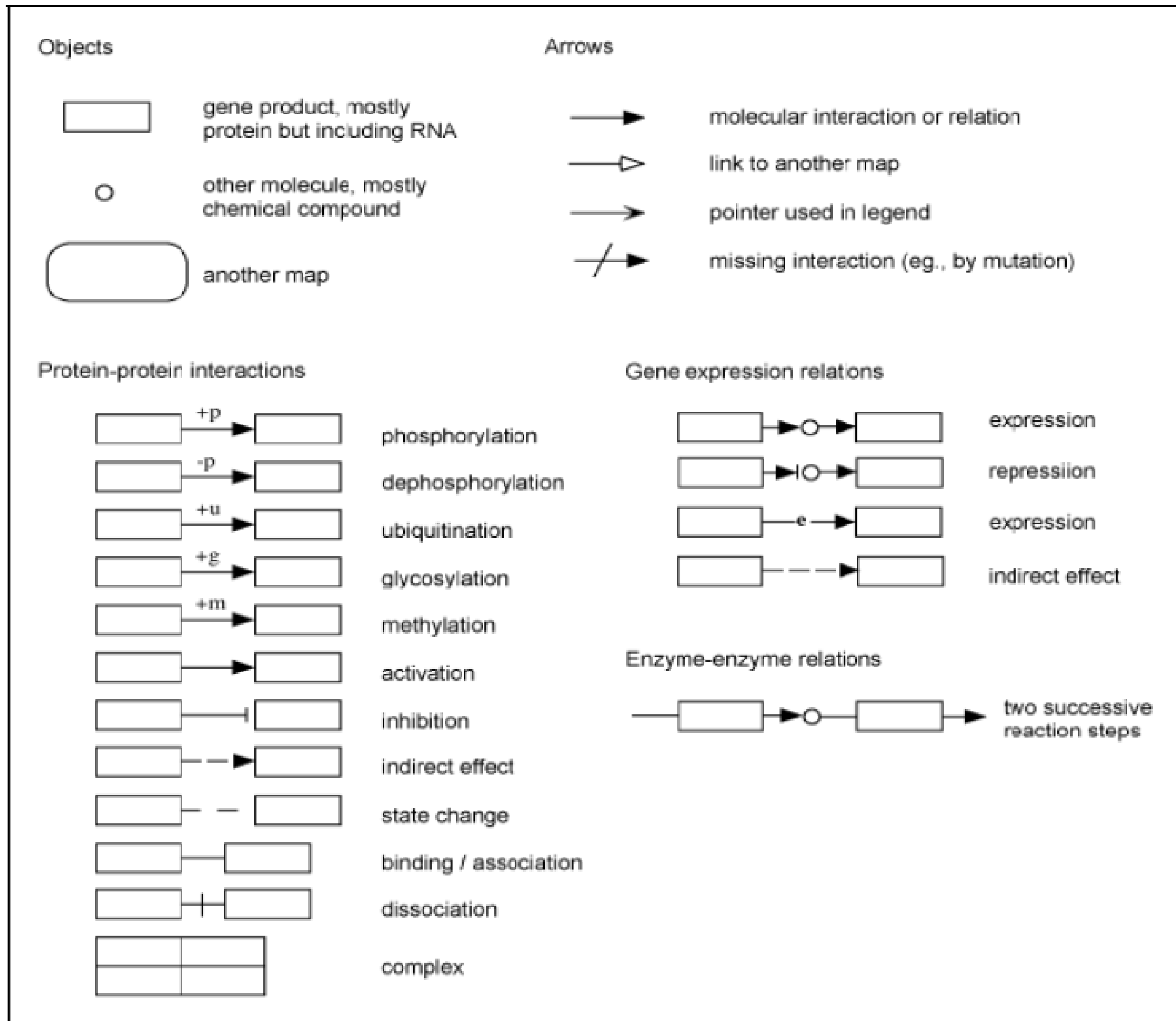


Figure 1: Neuroactive ligand receptor interaction pathway (reproduced with permission from KARG database)

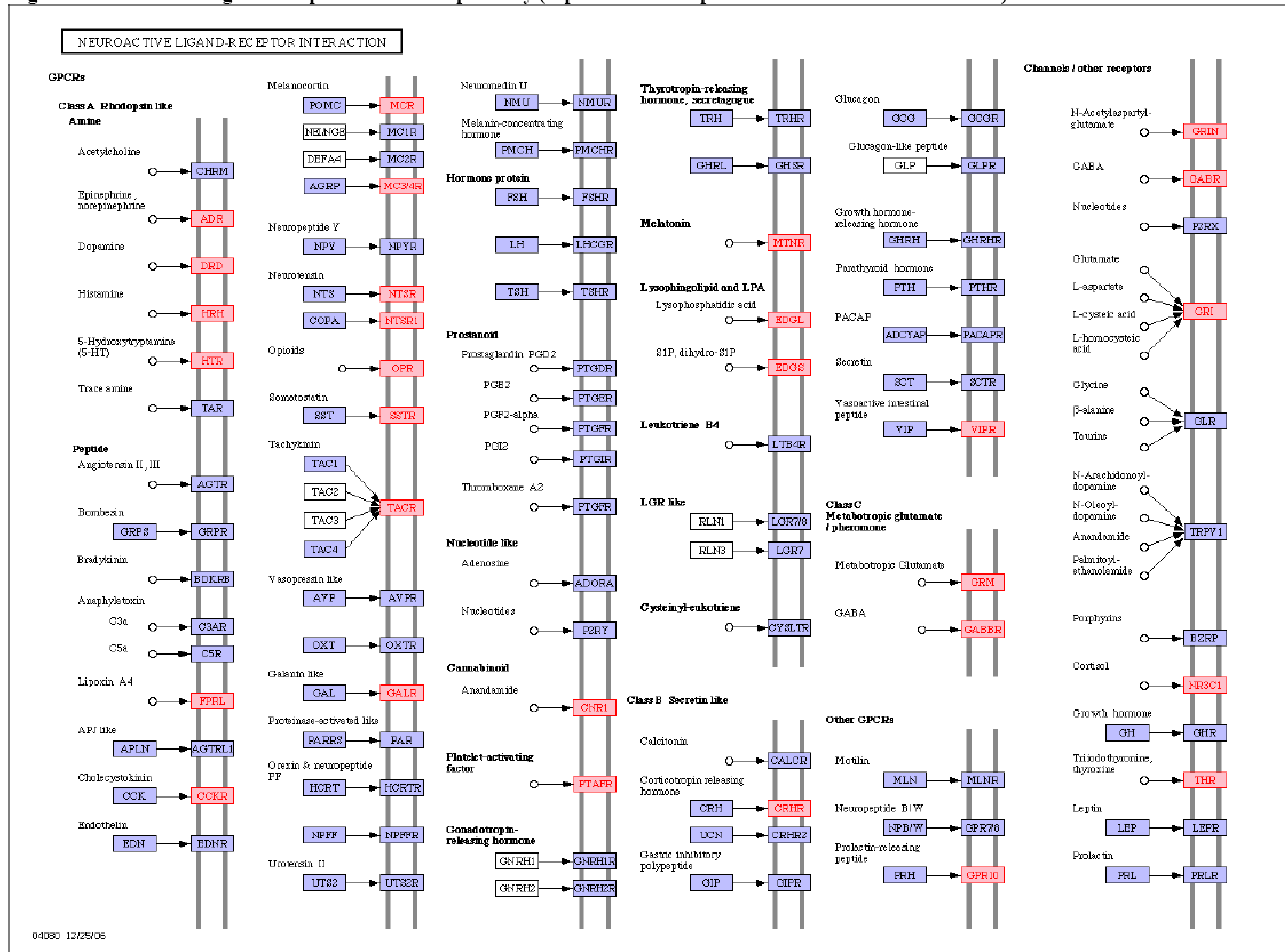


Figure 2: Long-term Potentiation Pathway (reproduced with permission from KARG database)

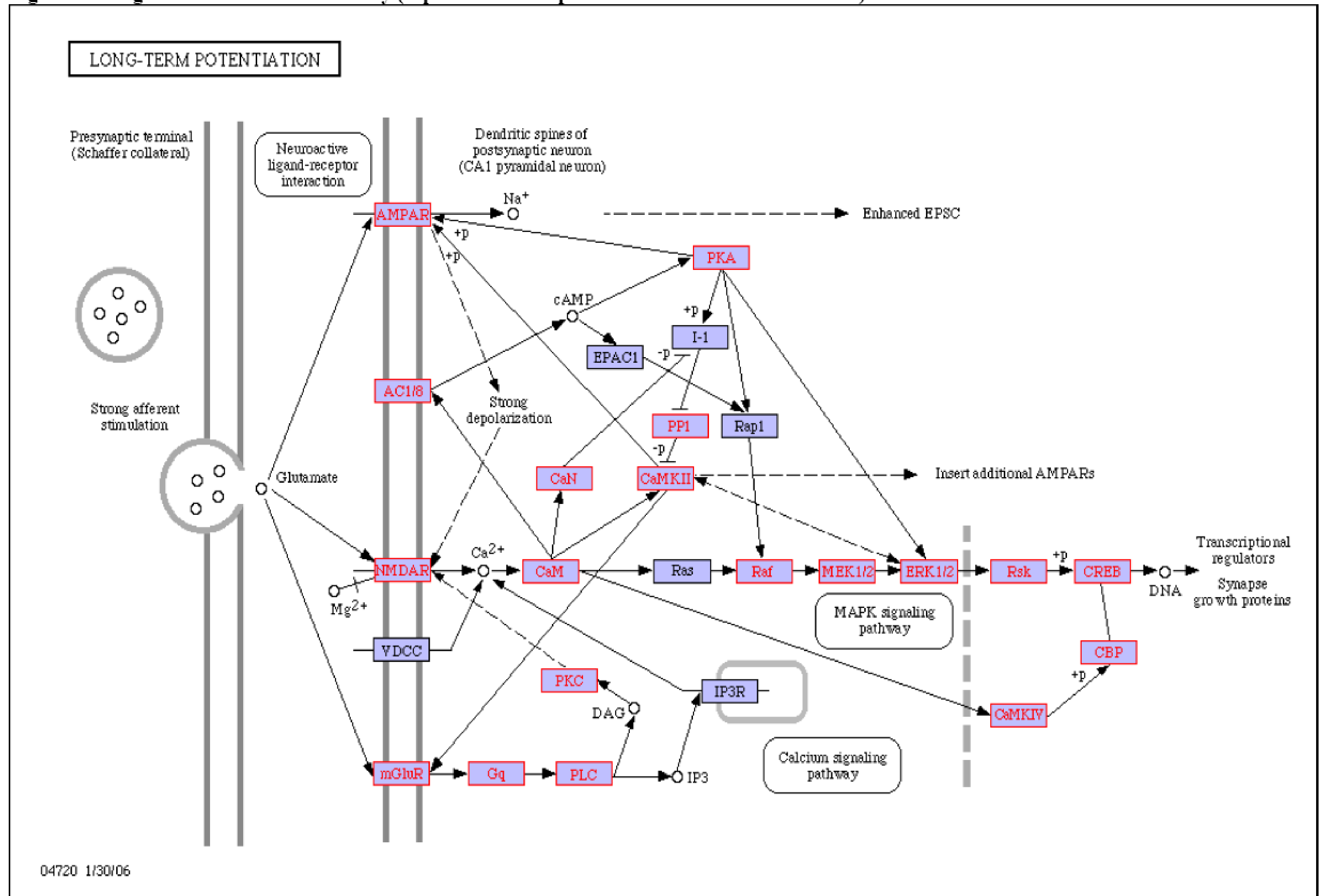


Figure 3: GnRH signaling pathway (reproduced with permission from KARG database).

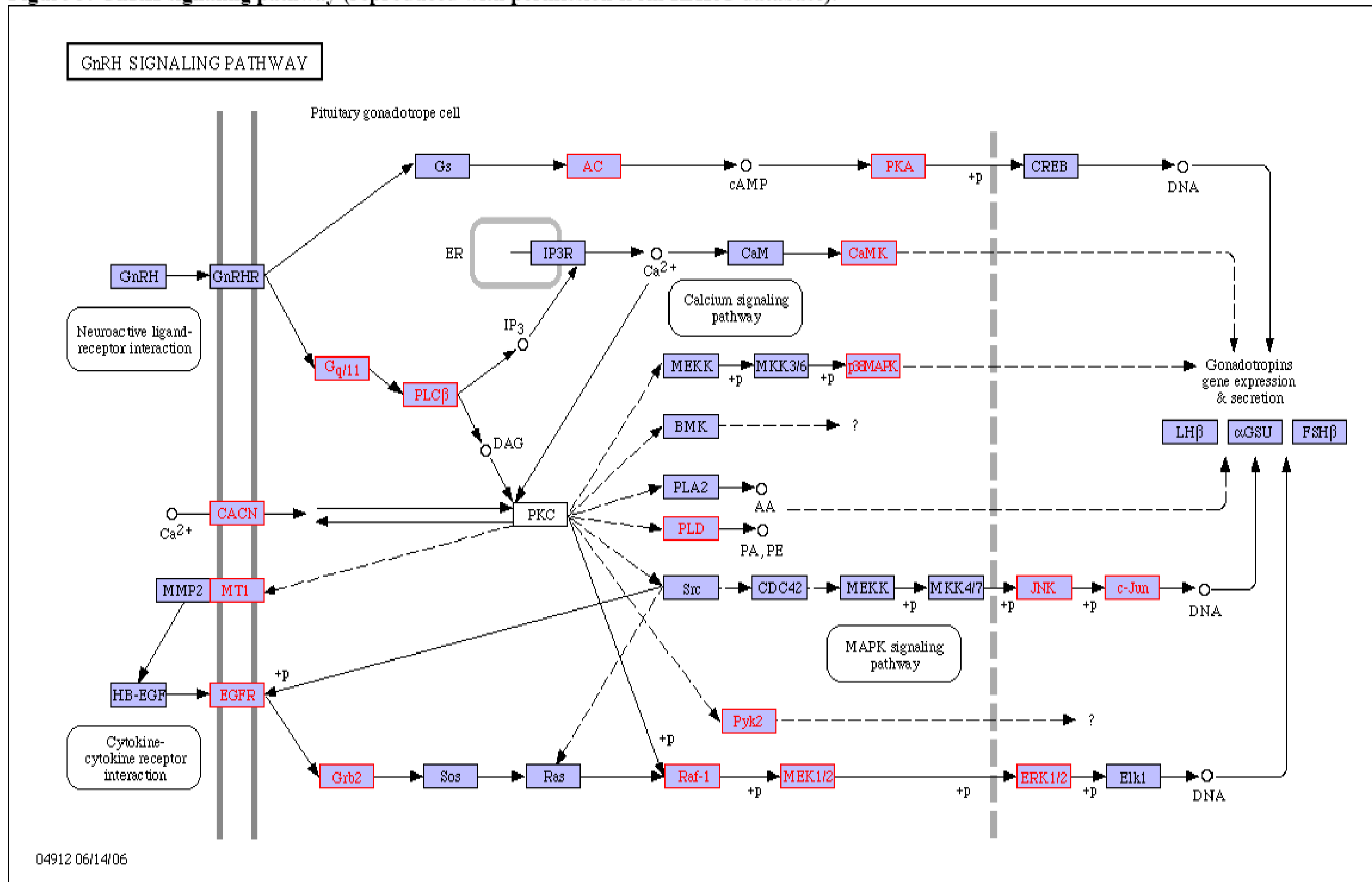


Figure 4: MAPK signaling pathway (with permission, from KARG database)

MAPK SIGNALING PATHWAY

Classical MAP kinase pathway

JNK and p38 MAP kinase pathway

ERK5 pathway

Heterotrimeric G-protein

Phosphatidylinositol signaling system

IP3
cAMP
DAG
Ca2+

CACN
NGF
BDNF
NT3/4
TrkA/B
EGF
EGFR
FGF
FGFR
PDGF
PDGFR
GRB2
SOS
Ras
RasGRF
RasGRP
CNrasGEF
PKC
PKA
Rap1
RafB
Raf1
Mos
G12
Gap1m
NF1
p120GAP
Scaffold
MEK1
MEK2
MP1
ERK
PTP
MKP
NIK
IKK
NFkB
Tau
STMN1
cPLA2
MNK1/2
RSK2
CREB
Elk-1
Sap1a
c-Myc
SRF
c-fos
DNA
PPP3C
NFAT-2
NFAT-4

Proliferation, inflammation anti-apoptosis

Proliferation, differentiation

Serum, cytotoxic drugs, irradiation, heat shock, reactive oxygen species, lipopolysaccharide, and other stress

GLK
HGK
HPK1
Cdc42/Rac
PAK1/2
CASP
MST1/2
GCK
Tpl2/Cot
MEKK1
MLK3
MEKK2/3
LZK
MUK
MLTK
ASK2
ASK1
TAK1
TAB1
TAB2
ECSIT
TRAF2
TRAF6
DAXX
GADD45
MEKK4
TAO1/2
FLNA
JIP3
ARRB
CrkII
GSTπ
HSP72
Evi1
MKK4
MKK7
JIP1/2
JNK
c-JUN
JunD
PP2CA
AKT
MKK3
MKK6
PP2CB
PP5
p38
ATF-2
Elk-1
p53
Sap1a
GADD153
MAX
MEF2C
PRAK
MAPKAPK
MSK1/2
Cdc25B
HSP27
CREB
NLK
TNF
TNFR
IL1
IL1R
FASL
FAS
TGFB
TGFBR
LPS
CD14
DNA damage

Alzheimer's disease

Proliferation, differentiation, inflammation

Apoptosis

Cell cycle

Wnt signaling pathway

Serum, EGF, reactive oxygen species, or Src tyrosinkinase downstream

MEK5
ERK5
Nur77

MAPKKKK
MAPKKK
MAPKK
MAPK
Transcription factor

04010 1/19/07

Figure 5: Gap junction pathway (reproduced with permission from KARG database)

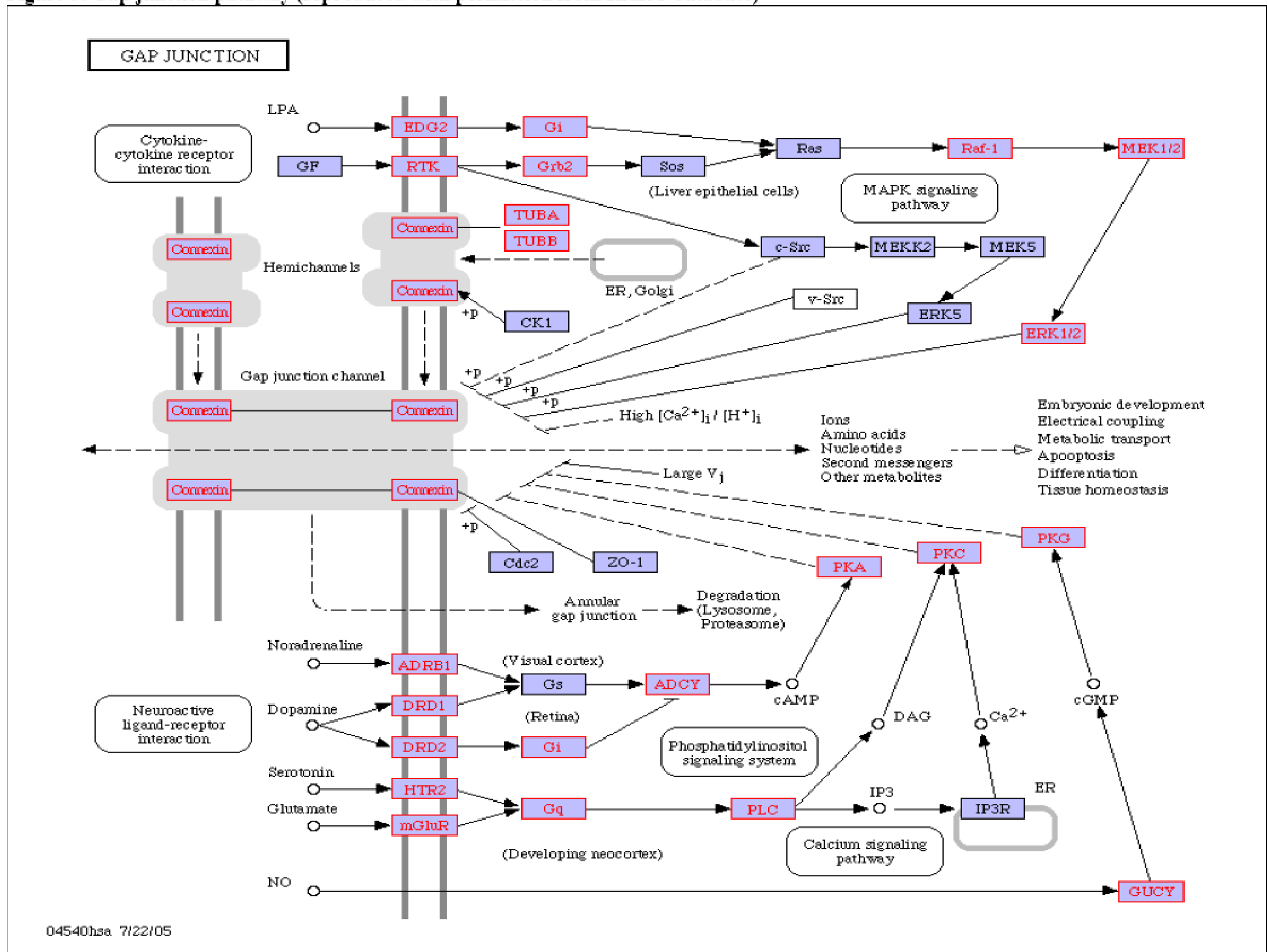
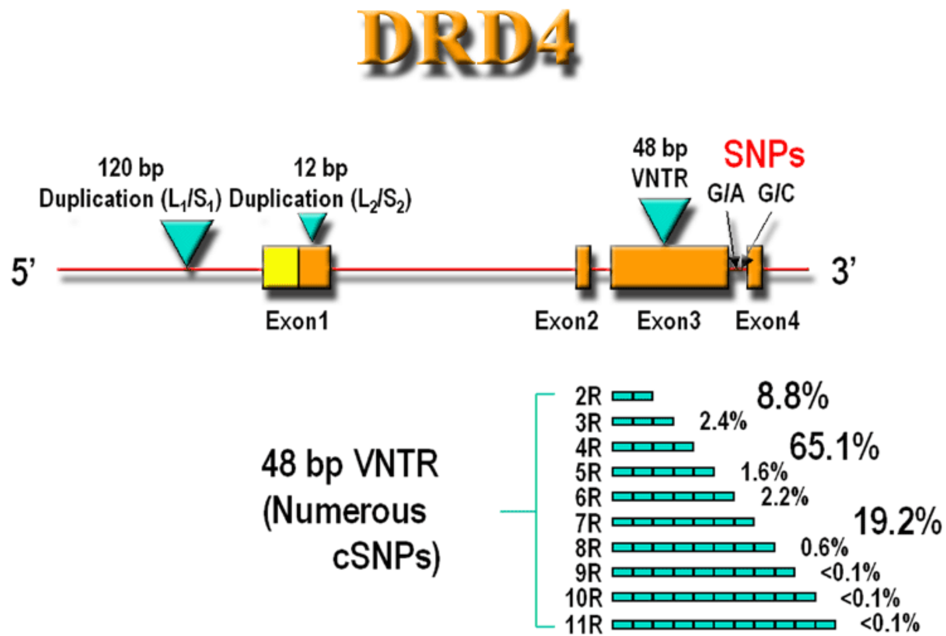
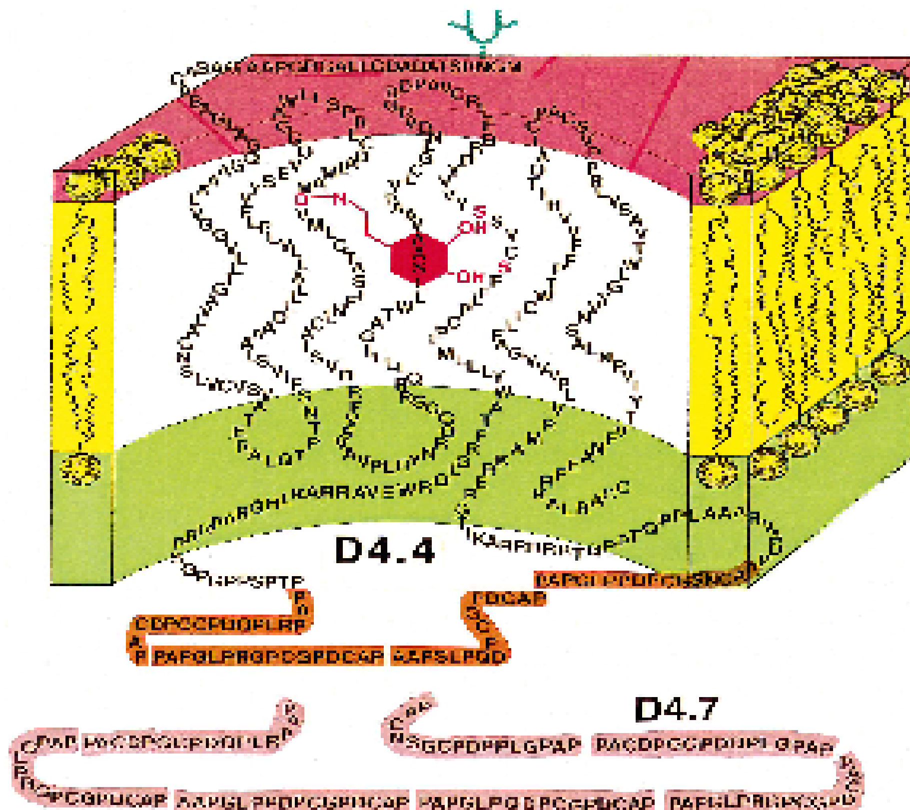


Figure 6: Diagrammatic Representation of the DRD4 gene and DRD4 exon III VNTR.



Representation of the DRD4 gene and the alleles found in the DRD4 exon III VNTR polymorphism. Each allele is named after the number of repetitions of a 48bp sequence present in that allele, i.e. the 4-repeat allele has 4 repetitions of the 48bp sequence. Percentages represent frequency of the alleles in Caucasian population. (Reproduced with permission from: Ding et al., PNAS January 8, 2002 vol. 99 (1): 309-314)

Figure 7: Dopamine D4 receptor graphical representation:



The DRD4 gene synthesizes the D4 receptor. Repeated sequences in the DRD4 gene (more specifically located in the exon III of the gene) are responsible for synthesizing a loop in the receptor (loop synthesized by the 4-repeat allele represented in orange). If the gene has more repeats (e.g. 7-repeat allele), the receptor loop will be longer (loop synthesized by the 7-repeat allele represented in pink).

Figure 8: Screenshot of the sequence alignment (DRD4 exon III)

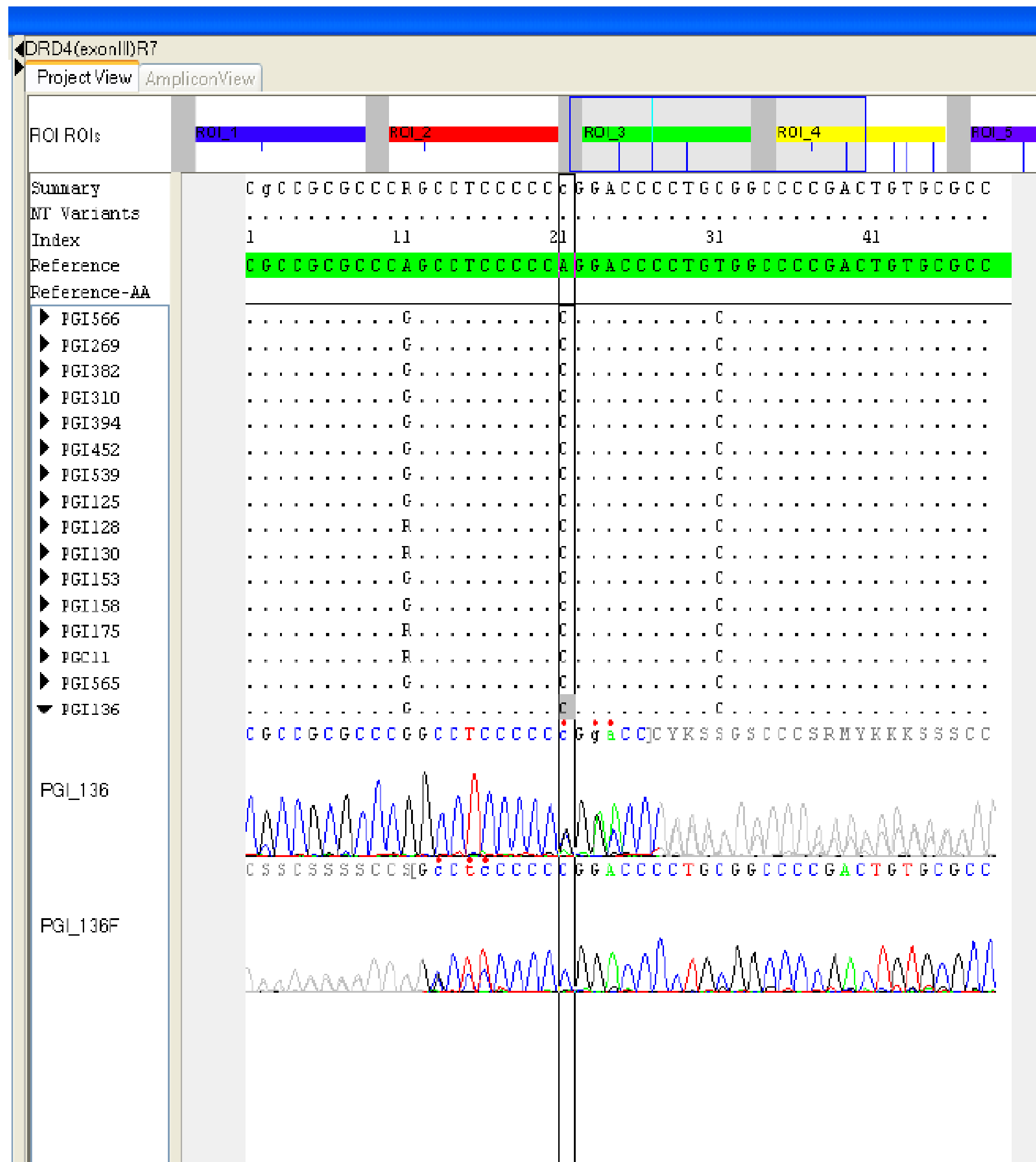


Figure 8: Screenshot of the sequence alignment showing one novel single-nucleotide deletion variant in one PG subject homozygous for the 7-repeat allele. The expected (reference) sequence is highlighted in green near the top of the screenshot. Whereas all other subjects presented the C nucleotide (complimentary to the A nucleotide in the reference sequence), PGI_136 had a deletion in that location, as indicated by a lower peak on the spectrophotometry, compared to the expected peaks for nucleotide C (blue peaks).

Figure 9: Screenshot of the deletion on DRD4 exon III from one subject in our sample

5'3' Frame 2

```
acccgcgccccgcctcccccaggacccctgcggccccgactgtgcgccccccgcgcccggc
  P  A  P  R  L  P  Q  D  P  C  G  P  D  C  A  P  P  A  P  G
cttccccggggtccctgcggccccgactgtgcgcccgccgcgcccagcctcccccggacc
 L  P  R  G  P  C  G  P  D  C  A  P  A  A  P  S  L  P  R  T
cctgtggccccgactgtgcgcccccccgcgcccggcctcccccggacccctgcggctcca
 P  V  A  P  T  V  R  P  P  R  P  A  S  P  R  T  P  A  A  P
actgtgctcccccccgcgcccggcctcccccaggacccctgcggccccgactgtgcgcccg
 T  V  L  P  P  R  P  A  S  P  R  T  P  A  A  P  T  V  R  P
ccgcgcccggcctcccccggacccctgcggccccgactgtgcgcccccccgcgcccggcc
 P  R  P  A  S  P  R  T  P  A  A  P  T  V  R  P  P  R  P  A
ttccccaggacccctgtggccccgactgtgcgccccccgacgccgtcagagccgccgcgc
 F  P  R  T  P  V  A  P  T  V  R  P  P  T  P  S  E  P  P  R
tcccaccccagactccaccgcagacccgcaggaggcggcgtgccaagatcaccggccggg
 S  H  P  R  L  H  R  R  P  A  G  G  G  V  P  R  S  P  A  G
agcgcaaggccatgagggtcctgccggtggtggtcg
 S  A  R  P  -  G  S  C  R  W  W  S
```

Figure 9. A novel variant in the *DRD4* gene. Through our sequencing efforts at this locus we discovered a novel single-nucleotide deletion in two (out of eleven) individuals homozygous for the 7-repeat allele in our PG sample (Figure A). Through in silico translation, we determined that either one of these deletions would result in a shift in the reading frame and premature chain termination at the 377th codon of the DRD4 mRNA, resulting in a truncated predicted amino-acid sequence, i.e. this deletion would cause a defective protein to be translated (produced) by the DRD4 gene.