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Abstract

Candidate Gene Analysis of Panic Disorder

Laura M. Hodges

Panic disorder (PD) is a common anxiety disorder of unknown etiology, with a modest contribution estimated to come from multiple genes. Although pharmacological and behavioral remedies are helpful, PD carries a significant personal and socioecomonic burden in the population. Evidence from basic and pharmaceutical research implicates a number of genes in PD. To date, among many candidate gene studies, only two candidate genes, COMT and ADORA2A, have been established to show significant association and/or linkage to PD in different study populations. Pursuing a convergence of biological, ethological, and pharmaceutical evidence, we investigated genetic variation in previously untested neuropeptide system genes (GRP, GRPR, TACR1, CRHR1), and fine-mapped the PD-linked GABA neurotransmitter receptor genes (GABRB3 and GABRA5), as risk factors for PD in 120 multiplex pedigrees. A second goal was to characterize the heritability of COMT and GABRB3 expression patterns in a control population of nuclear families, in order to identify variants in a ~440-480 kb region around the genes that may contribute to gene function.

We found marginal evidence for association and/or linkage in GRP, TACR1 and CRHR1 that prohibits ruling them out as candidates for PD. Suggestive and significant evidence for linkage and association of PD to

GABRB3 and GABRA5 encouraged mutation screens of each gene in a subset of ninety-six probands. A number of potentially functional variants, including a synonymous SNP, 5' and 3' untranslated region SNPs, exon-flanking SNPs, and two mutations that alter transcription factor binding sites were discovered in PD probands. In lymphoblastoid cell lines from Northern European samples represented in the HapMap, variants associated with the heritable expression of GABRB3 were located in introns and 5' to the gene. Significant evidence for heritable COMT expression occurred for variants in genic as well as distant regions, including a gene-poor region ~340 kb from COMT, which was previously linked and associated to PD. This is the first evidence connecting potential long-range regulation of COMT expression to possible COMT dysregulation in PD. As well, nearer upstream and downstream variants to COMT were associated with COMT gene expression, providing new positional targets for putative COMT dysregulation in PD.

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1.1. Background

1.1.1. Description of PD

Panic disorder (PD) is a common psychiatric disorder, of unknown etiology, characterized by paroxysmal panic attacks and impairment from ensuing anticipatory anxiety. Panic attacks manifest with intense cardiorespiratory, autonomic, and neurobehavioral symptoms, often with the fear of dying and an urge to flee or escape[1,2] as coded in the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition, revised (DSM-IV-R). PD is defined by the presence of at least four of thirteen somatic and/or cognitive symptoms which develop abruptly, listed here with their associated frequency of occurrence: palpitations, pounding heart or tachycardia (87%), dyspnea (difficulty breathing) (77%), fear of dying (70%), dizziness (64%) or faintness (56%), chest pain or discomfort (60%), trembling or shaking (59%), sweating (55%), paresthesias (numbing or tingling sensations) (51%), hot flushes (or chills) (50%), feeling of choking (40%), nausea or abdominal distress (31%), depersonalization (self detachment) (29%) or derealization (feelings of unreality) (27%), and/or fear of going crazy or losing control (27%). These symptoms crescendo within ten minutes and can last for minutes to hours. PD is diagnostically qualified by the recurrence of spontaneous panic attacks, followed by at least a month of one or more of the following behavioral symptoms: anticipatory anxiety about the recurrence of attacks, worry about dying or losing

emotional or behavioral control, or impairment in the form of phobic avoidance. Resulting maladaptive avoidance behavior and persistent apprehension are the cardinal features of PD. This contrasts with individuals who experience panic attacks, which occurs at about five times the rate of PD[3].

1.1.2. Physiological traits and potential endophenotypes of PD

Compared to most other psychiatric disorders, PD presents with discernable physiological symptoms and a predisposition for certain endophenotypes. For example, exposure to various agents, such as caffeine, cocaine, cystokinin, beta-carbolines, marijuana, pentagastrin, sodium lactate infusion, and carbon dioxide (CO_2) inhalation, elicit panic attacks in patients with PD at a significantly higher rate than the general population. Heritable aspects of this vulnerability are suggested by the observation that CO_2 provocation in asymptomatic relatives of PD probands is associated with higher rates of anxiety than other controls[4].

Other characteristics of PD are observed in both the peripheral and central nervous systems. For example, the differential activation of brain regions[5-7] and heightened autonomic tone[8] can be seen as electroencephalogram (EEG) abnormalities. Brain imaging also shows discrete differences in regional brain activation at baseline[9,10] and during spontaneous panic attacks[11]. Deficits in prepulse inhibition (PPI), with estimated 50-70% heritability, are characteristic in PD patients[12], and altered in schizophrenia. Additionally, subjects with PD exhibit differences in skin conductance[13] related to amygdalar-based subjective

fear processing. In fact, PD patients demonstrate less variability in heart rate, skin conductance[14], and blood pressure at baseline, and during spontaneous or CO_2–induced panic attacks[15], reflecting decreased flexibility in the homeostatic control of vagal tone, and physiological vulnerability to stress[16], with a reduced ability to buffer autonomic responses in PD[14]. Despite these characteristic endophenotypes, variability in the individual physiological responses among individuals with PD, such as individual differences in vulnerability to different challenge agents, highlight a source of phenotypic heterogeneity in PD[15]. We do not know if this level of locus heterogeneity contributes to phenotypic heterogeneity, or is merely due to variable penetrance of a singular underlying disorder. In the following chapters, related phenotypes that inform the hypothesis-driven selection of our candidate genes are highlighted in order to propose a larger integrative hypothesis for PD.

1.1.3. Epidemiology of PD

Epidemiological studies estimate the lifetime prevalence of PD between 1.1-3.7%[3,17,18] in the United States, with between 1.4-2.9%[19] among nine of ten world cultures. The lowest prevalence rate for PD is in Taiwan at 0.4%, with unknown causes for being an outlier[19]. Inclusion of individuals with PD with co-occurring agoraphobia, or phobic avoidance of situations or places in which escape might be difficult or embarassing, raises the lifetime prevalence of PD to between 4.8%[3] and 5.1%[20] in the United States. PD with agoraphobia

constitutes between 20-50% of all subjects with PD[20,21], depending upon the population.

Age, gender, and ethnicity influence PD. Individuals with PD rarely fall outside the ages of 20-50 years old[3], which suggests the involvement of some age-related factor. The genetic risk of PD in relatives of probands increases if the age of onset less than 20 years old in probands [22]. Interestingly, PD has a sexually dimorphic prevalence rate, with the female-specific risk between 1.9[23] and 3.7-fold greater[24] than that of males across countries. This disparity becomes more pronounced with age, as prevalence rates in males decline more precipitously than in females[23]. Symptoms can significantly vary by gender and ethnicity as well. For example, females report more chronic and severe agoraphobic avoidance symptoms[25]; males report more respiratory symptoms[26], and African Americans exhibit nearly 8 times greater incidence of isolated sleep paralysis than Caucasians[27]. Some of the gender-specific disparity is attributed to a doubling in treatment relapse in females[102], ostensibly due to known hormonal interactions with the pharmacological targets (e.g. GABAergic system genes)[28,29]. Fluctuations in hormones correlate with PD symptoms, as is recapitulated by the adminstration of exogenous hormones to reduce the provocation of panic attacks in a controlled setting[30]. One hormone-mediated mechanism thought to underlie greater risk of PD in females is the precipitous spike in CO_2 levels and decreased respiratory stimulation with a drop in progesterone, not unlike states of hyperventilation during panic attacks and provocation of attacks with CO_2 inhalation. These sex-specific effects are

estimated to act upon the same genetic factors in males and females[31] according to a twin study that showed no difference in genetic or environmental factors between the sexes. Thus, it is possible that hormonal influences may explain the non-genetic, non-environmental variability in PD risk, prevalence, and symptomology differences due to age and gender.

1.1.4. The etiology of PD

Evidence for genetic determinants for PD is observed in family and twin studies. Family studies suggest an 8 to 20-fold increased relative risk to first-degree relatives of probands compared to controls, depending upon the age of onset[31], while twin data suggests that less than 12% of the variance in anxiety disorders is estimated to be due to shared environment[32]. Estimates of the relative risk to siblings of PD probands at 5 to 10-fold higher than the population risk ($lambda_s$)[33]. PD exhibits moderate heritability of 0.48[34], with locus heterogeneity[35], and with the remaining liability presumably due to environmental factors. Segregation studies[36-38] prescribe no consistent, simple mode of inheritance for PD. Thus the diathesis for PD is thought to result from complex, polygenic origins that are modified by epistatic interactions and environmental factors[39-41].

1.1.5. Comorbidity and syndromic features for PD

A number of psychiatric disorders are comorbid with primary panic disorder, including major depressive disorder (35-39%), agoraphobia (50%),

recurrent nocturnal panic attacks (18-45%), alcohol (25-37%) and/or substance abuse (18-36%), social anxiety disorder (31-70%), specific phobia (34-75%), generalized anxiety disorder (GAD) (15-21%), suicide attempts (20%), bipolar affective disorder (14-30%), posttraumatic stress disorder (22-40%), and obsessive-compulsive disorder (8-19%)[3,42,43]. Combining the prevalence of multiple comorbidities, 83% of individuals with PD are likely to have a comorbid disorder, while all individuals with PD and agoraphobia have comorbidities[3].

Heterogeneity in the diathesis to PD is observed in the variability in symptoms and outcomes, in agreement with putative multigenic origins of the disorder. Many features of PD are nonuniform across patients. PD also exhibits somatic or visceral syndromic features[44], which may reflect pleiotropic effects of a common, underlying origin. Comorbidity between PD and neurological, gastrointestinal, cardiac, and respiratory conditions is well-documented in the clinical literature[45]. Comorbid physical disorders[46,47], such as bladder, kidney, thyroid[48], or gastrointestinal[49-51] problems[52], mitral valve prolapse[35,37], and migraine[53] are notably enriched in PD patients, suggesting a shared etiology. Various studies show that this phenomenon is not due to ascertainment bias. For example, independent analysis of patients with interstitial cystitis showed a correlative increase in PD (odds ratio=4, p=0.02)[52]. Using these syndromic features as a diagnostic subclassification for PD has yielded evidence for significant genetic linkage to multiple regions of a genome scan of sixty multiplex pedigrees[44]. This sample is a subset of the larger set of pedigrees studied here. Hypothetical pleiotropy underlying PD and the syndromic features is supported

by known pleiotripic effects of PD candidate genes (e.g. GABAergic-mediated cerebral blood flow) and overlapping therapeutic interventions. For example, the "brain-gut" disorder, irritable bowel syndrome (IBS), is 46% prevalent among PD patients seeking treatment[54], as compared to the 10-20% prevalence of IBS in the general population. And psychotropic drugs, including $GABR_A$-targeting benzodiazepines, such as Valium (diazepam) and Xanax (alprazolam), are used to treat IBS[51,55]. In addition, the GABRB3 gene is upregulated in animal models of IBS, via vagal sensory relays[56], and corticotropin releasing-hormone is implicated in mediating IBS symptoms[51]. Finally, neonatal maternal separation, which is an environmental modifier of PD risk, is a testing paradigm for stress-induced IBS in animal models[57]. Therefore, genetic, pharmaceutical, and environmental factors overlap in their involvement with PD and related syndromic features, which reinforce the selection of our candidate genes in PD.

1.1.6. The societal and personal relevance of PD

PD exacts a heavy socioeconomic cost[58,59]. The abrupt and often unprovoked physical manifestations of panic attacks commonly lead to overuse of emergency medical care centers[60-63]. Outside of a psychiatric setting misinterpretation of the symptoms incurs high medical testing costs that are inconclusive[64]. Up to 98% of individuals with PD are left undiagnosed by emergency departments[65,66]. Maladaptive avoidance behavior and persistent apprehension, which are hallmark features of PD, also lead to significant personal and societal detriment in the form of work disability[59,66-69]. Nearly one

third of PD patients are financially dependent[68], with an average work disability of 2.7 years[70], and 25% unemployment[71].

Likewise, a high personal cost is incurred in the deterioration of interpersonal relationships[68], risk of alcohol and substance abuse[3,72,73], and depression and suicide[46,74,75], including detrimental generational effects in children of suicidal mothers with PD by showing an eight-fold increased risk of depressive and anxiety disorders compared to other children[76,77].

1.1.7. Genetic studies of PD

A number of genome scans in PD pedigrees demonstrate multiple, non-overlapping regions of genetic linkage to PD[40,44,53,78-81]. One group carried out a targeted genome scan in regions syntenic to mouse quantitative trait loci for anxiety and found evidence of linkage in several of those regions in a single extended pedigree[82]. In addition, basic neuroscience research has discovered a number of potential biological mechanisms that underlie the manifestations of PD. Despite this convergence of genetic and biological evidence, only two hypothesis-based candidate genes on chromosome 22, catechol-*O*-methyltransferase (COMT, 18.3Mb)[83,84] and adenosine 2A receptor (ADORA2A, 23.1Mb)[85,86], are established candidate genes for PD, showing replication in more than one population with ethnic-specific differences in the valence of allelic association[83,87]. Among the genes studied in this thesis, only the chr15q GABRA candidate genes have prior postional support from linkage studies to complement biological data. However, the successful replication results for COMT and

ADORA2A, which did not have prior positional data to support their involvement in PD, illustrates the utility of the hypothesis-driven candidate gene approach in identifying loci for PD. The sensitivity of association testing to alleles with small effects, combined with the relatively high sibling risk for PD (lambda$_s$) that is required for gene-mapping efforts[88] (see section 1.1.4.), make the genetic study of PD a tractable endeavor. There are currently no published genome-wide association studies for PD.

1.1.8. Treatment of PD

Besides cognitive behavioral therapy, which is particularly effective in combination with medication for individuals with PD and agoraphobia, a number of drugs are effective for PD. Current treatment of PD includes anxiolytic and antidepressant medications such as the GABR$_A$-targeting benzodiazepines (BZs), and numerous monoamine-targeting drugs, such as the tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), selective serotonin reuptake inhibitors (SSRIs), inhibitors of alpha-2-adrenergic receptors (NaSSAs), and serotonergic-noradrenergic reuptake inhibitors (SNRIs)[89]. These drugs modulate GABAergic, noradrenergic, dopaminergic, and serotonergic signalling. Some of these drugs also remedy syndromic features, such as migraine, enuresis, and comorbid disorders, such as melancholic depression characterized by hypercortisolemia[90,91]. The variability in pharmacological effects in relation to PD symptomatology may shed light on the underlying biology. For example, imipramine is shown to ameliorate PD patients presenting with more

dyspneic symptoms, while alprazolam appears to better treat PD in individuals without dyspnea[92]. Insight into the effects of PD drugs, vis-à-vis their respective molecular targets and effectiveness in quelling symptoms of PD, as well as the syndromic sequelae in PD, gives insight into the complexity of the underlying mechanisms of the disorder and related pleiotropic effects. Treatment of PD using these drugs, by direct or indirect effects, supports the "monoamine hypothesis" for anxiety and depression.

Despite the utility of the current therapies, more appropriate molecular targets for treatment are needed. A study undertaken at 6 to 10 years post-treatment showed that drug response was incomplete in 40-50%, or without improvement in 20-30% of PD patients[93-95]. Due to their broad mechanism of action, side effects, (i.e. drug dependence, drug withdrawal, sexual dysfunction, sedation, and unwanted somatic symptoms), are responsible for much non-compliance and discontinuation. From a research standpoint, their utility is also limited. Their broad actions are imprecise, and none of these drugs exhibits plasma drug concentrations, or other indices (e.g. circulating GABA levels), that are predictive of their response. Furthermore, the monoaminergic drugs have a delayed pharmacodynamic response on the order of weeks, despite their immediate molecular effects[96]. This lack of correlation in the pharmacokinetic-pharmacodynamic (PK-PD) relationship implies possible alternative effectors for therapeutic response. In summary, poor efficacy[97,98], non-specificity, and lack of PK-PD correlation in our current arsenal of PD drugs warrants the identification of alternative molecular targets. Neuropeptides have been of recent interest in

drug development for their fine-tuned role in mediating neurotransmitter signaling in animal models. This has lead to some success in clinical trials of inhibitors of neuropeptide receptors. Our investigation of genes involved in the neuropeptidergic, GABAergic, and catecholaminergic signaling systems are in part based on the pharmaceutical evidence supporting their influence on PD and anxiety.

1.1.9. Fear network structures in PD

Biological support for the involvement of our chosen candidate genes in PD is based in part on evidence of fear signaling networks in the central and peripheral nervous systems via brain structures in the amygdala, hippocampus, thalamus, midbrain, caudal pons, medulla, cortex and cerebellum[10,11], as well as neuroendocrine cascades via the hypothalamic-pituitary-adrenal (HPA) axis. Somatic, cognitive and behavioral symptoms in PD parallel specific physiological and psychological effects that occur with acute and chronic activation of the stress response system. Panic attacks trigger programmed autonomic nervous system (ANS) responses via the HPA axis. Hallmark anticipatory anxiety that ensues with recurring panic attacks arethe hallmark effect of PD, distinguishes PD from idiosyncratic panic attacks, and is hypothesized to engage higher order fear learning and memory mechanisms in the cortex via limbic and brainstem regions. Chronic stress may reflect a biological alteration in the response threshold for somatosensory and psychogenic stimuli. Healthy stress responses ensure adaptive “fight or flight” or freeze behaviors, by activating the

parasympathetic and sympathetic arms of the ANS in response to a perceived external or internal assault (e.g. physical predation, immune challenge). PD symptoms appear to manifest inappropriately, due to putative dysregulation of the stress response system. Aberrant activation of stress response is hypothesized to underlie a number of psychiatric disorders (depression, posttraumatic stress disorder, anorexia nervosa, and PD) as evinced by molecular changes in the effectors of the stress pathway (neurotransmitters, neuropeptides, hormones, and their cognate receptors), and their corresponding ANS responses (respiratory, hemodynamic, cardiac, gastrointestinal, immunoinflammatory), and behavioral adaptations (avoidance, anxiety, somnolence, libido, addiction, feeding, arousal, escape, emotionality). The molecular actions of neuropeptidergic genes, interacting with neurotransmitter system genes, in controlling many aspects of PD pathology are natural candidates for genetic contribution to the etiology of PD.

1.1.10. Candidate genes for PD

A number of hypothesis-based candidate genes, supported by positional data and/or biological evidence, have been studied in PD (e.g. MAOA, ABCG1, and serotinergic genes)[99-101]. Despite numerous genetic studies, scant reproducible evidence exists for linkage and association of candidate genes to PD. Two exceptions are the catechol-*O*-methyltransferase (COMT) and adenosine 2A receptor (ADORA2A) genes, with evidence for linkage and association to PD in more than one study population. Genes encoding other

neurotransmitter and neuropeptide system genes are of particular interest to us based on their role in anxiety circuitry, their use as molecular targets of anxiety-modulating agents (e.g. anxiolytic benzodiazepines), ethological evidence from gene disruption studies, and overlapping mechanisms in other peripheral and central nervous system processes in the syndromic features and comorbid disorders of PD. In this thesis we detail the results from the investigation of seven neurotransmitter and neuropeptide system genes in affected familes and/or ethnically matched controls to pinpoint genetic determinants that may lead to the diathesis of PD.

1.2. Aims

To identify genetic determinants for PD we used a three-tiered approach in studying candidate genes with differing levels of evidence for their involvement in PD: exploratory, suggestive, and well-established candidates. Genetic linkage data from low-resolution genome scans in PD pedigrees implicates large regions of the genome, on the order of 10 centimorgans, for which there may be a hundred resident genes. Hypothesis-based selection of candidate genes is an approach to prioritize the search. We chose four neuropeptidergic candidate genes (gastrin releasing peptide (GRP), gastrin releasing peptide receptor (GRPR), tachykinin receptor (TACR1), and corticotropin releasing hormone receptor (CRHR1)) on the basis of biological data to test for association and linkage in PD pedigrees. We also investigated two gama-aminobutyric acid (GABA) type A receptor subunit genes (GABRA5, GABRB3), for which there was a convergence of both biological and positional data for PD as suggestive

candidates. Lastly, we investigated the heritable influences of *cis*-acting variants on the expression of a well-established candidate gene, catechol-*O*-methyltransferase (COMT), for which there is more substantial evidence of its involvement in PD in multiple populations. This pursuit is aimed to increase the understanding of genetic risk factors and their biological consequences to improve diagnosis and treatment of PD and related disorders, by providing new targets for therapeutic intervention.

Outline of aims:

1) Test for linkage and allelic association of variants for the candidate genes, GABRB3, GABRA5, GRP, GRPR, TACR1, and CRHR1, in 120 Caucasian, multiplex PD pedigrees.
2) Screen ninety-six PD probands for mutations in GABRB3 and GABRA5, for which there was evidence of linkage and association in aim 1.
3) Characterize COMT and GABRB3 expression in unphenotyped Caucasian trios, ethnically-matched to our PD pedigrees, to determine the association of *cis* variants to expression and heritable expression patterns.

1.3. References

1. Marquez M *et al. (*2001) Is panic disorder with psychosensorial symptoms (depersonalization-derealization) a more severe clinical subtype? *J.Nerv.Ment.Dis.* 189 (5):332-335.

2. Starcevic V *et al. (*1993) The phenomenology of panic attacks in panic disorder with and without agoraphobia. *Compr.Psychiatry* 34 (1):36-41.

3. Kessler RC *et al. (*2006) The epidemiology of panic attacks, panic disorder, and agoraphobia in the National Comorbidity Survey Replication. *Arch.Gen.Psychiatry* 63 (4):415-424.

4. Coryell W *et al. (*2006) Anxiety responses to CO2 inhalation in subjects at high-risk for panic disorder. *J.Affect.Disord.* 92 (1):63-70.

5. Dantendorfer K *et al. (*1996) High frequency of EEG and MRI brain abnormalities in panic disorder. *Psychiatry Res.* 68 (1):41-53.

6. Knott VJ *et al. (*1996) Quantitative EEG correlates of panic disorder. *Psychiatry Res.* 68 (1):31-39.

7. Lepola U *et al. (*1990) Cerebrospinal fluid monoamine metabolites and neuropeptides in patients with panic disorder. *Ann.Med.* 22 (4):237-239.

8. Yavuzkir M *et al. (*2007) P-wave dispersion in panic disorder. *Psychosom.Med.* 69 (4):344-347.

9. Boshuisen ML *et al. (*2002) rCBF differences between panic disorder patients and control subjects during anticipatory anxiety and rest. *Biol.Psychiatry* 52 (2):126-135.

10. Sakai Y *et al. (*2005) Cerebral glucose metabolism associated with a fear network in panic disorder. *Neuroreport* 16 (9):927-931.

11. Pfleiderer B *et al. (*2007) fMRI amygdala activation during a spontaneous panic attack in a patient with panic disorder. *World J.Biol.Psychiatry* 8 (4):269-272.

12. Anokhin AP *et al. (*2003) Genetic influences on prepulse inhibition of startle reflex in humans. *Neurosci.Lett.* 353 (1):45-48.

13. Michael T *et al. (*2007) Fear conditioning in panic disorder: Enhanced resistance to extinction. *J.Abnorm.Psychol.* 116 (3):612-617.

14. Hoehn-Saric R *et al. (*2004) Somatic symptoms and physiologic responses in generalized anxiety disorder and panic disorder: an ambulatory monitor study. *Arch.Gen.Psychiatry* 61 (9):913-921.

15. Bystritsky A *et al. (*2000) Autonomic reactivity of panic patients during a CO2 inhalation procedure. *Depress.Anxiety.* 11 (1):15-26.

16. Porges SW (1992) Vagal tone: a physiologic marker of stress vulnerability. *Pediatrics* 90 (3 Pt 2):498-504.

17. Fyer AJ and Weissman MM (1999) Genetic linkage study of panic: Clinical methodology and description of pedigrees. *American Journal of Medical Genetics* 88 (2):173-181.

18. Roy MA and Kendler KS (1994) The effect of diagnostic hierarchy in genetic epidemiological studies of psychiatric disorders. *Arch.Gen.Psychiatry* 51 (11):926-927.

19. Weissman MM *et al. (*1997) The cross-national epidemiology of panic disorder. *Arch.Gen.Psychiatry* 54 (4):305-309.

20. Grant BF *et al. (*2006) The epidemiology of DSM-IV panic disorder and agoraphobia in the United States: results from the National Epidemiologic Survey on Alcohol and Related Conditions. *J.Clin.Psychiatry* 67 (3):363-374.

21. Reed V and Wittchen HU (1998) DSM-IV panic attacks and panic disorder in a community sample of adolescents and young adults: how specific are panic attacks? *J.Psychiatr.Res.* 32 (6):335-345.

22. Goldstein RB *et al. (*1997) Familial aggregation and phenomenology of 'early'-onset (at or before age 20 years) panic disorder. *Arch.Gen.Psychiatry* 54 (3):271-278.

23. Eaton WW *et al. (*1994) Panic and panic disorder in the United States. *Am.J.Psychiatry* 151 (3):413-420.

24. Joyce PR *et al. (*1989) The epidemiology of panic symptomatology and agoraphobic avoidance. *Compr.Psychiatry* 30 (4):303-312.

25. Yonkers KA *et al. (*1998) Is the course of panic disorder the same in women and men? *Am.J.Psychiatry* 155 (5):596-602.

26. Sheikh JI, Leskin GA, and Klein DF (2002) Gender differences in panic disorder: findings from the National Comorbidity Survey. *Am.J.Psychiatry* 159 (1):55-58.

27. Paradis CM, Friedman S, and Hatch M (1997) Isolated sleep paralysis in African Americans with panic disorder. *Cult.Divers.Ment.Health* 3 (1):69-76.

28. Herbison AE (1994) Immunocytochemical evidence for oestrogen receptors within GABA neurones located in the perinuclear zone of the supraoptic nucleus and GABAA receptor beta 2/beta 3 subunits on supraoptic oxytocin neurones. *J.Neuroendocrinol.* 6 (1):5-11.

29. Pierson RC, Lyons AM, and Greenfield LJ, Jr. (2005) Gonadal steroids regulate GABAA receptor subunit mRNA expression in NT2-N neurons. *Brain Res.Mol.Brain Res.* 138 (2):105-115.

30. Le Mellédo J *et al. (*2001) Effect of medroxyprogesterone pretreatment on pentagastrin-induced panic symptoms in females with panic disorder. *Psychiatry Res.* 101 (3):237-242.

31. Knowles JA, Weissman MM (1995) Panic disorder and agoraphobia. In: Oldham JM, Riba MB (eds) Review of Psychiatry, Volume 14. American Psychiatric Press: Washington, DC, pp 383-404.

32. Hettema JM *et al. (*2005) The structure of genetic and environmental risk factors for anxiety disorders in men and women. *Arch.Gen.Psychiatry* 62 (2):182-189.

33. Smoller JW and Tsuang MT (1998) Panic and phobic anxiety: defining phenotypes for genetic studies. *Am.J.Psychiatry* 155 (9):1152-1162.

34. Hettema JM, Neale MC, and Kendler KS (2001) A review and meta-analysis of the genetic epidemiology of anxiety disorders. *Am.J.Psychiatry* 158 (10):1568-1578.

35. Crowe RR (1985) The genetics of panic disorder and agoraphobia. *Psychiatr.Dev.* 3 (2):171-185.

36. Crowe RR *et al. (*1983) A family study of panic disorder. *Arch.Gen.Psychiatry* 40 (10):1065-1069.

37. Pauls DL *et al. (*1980) A genetic study of panic disorder pedigrees. *Am.J.Hum.Genet.* 32 (5):639-644.

38. Vieland VJ *et al. (*1996) New segregation analysis of panic disorder. *Am.J.Med.Genet.* 67 (2):147-153.

39. Kendler KS, Gardner CO, and Prescott CA (2001) Panic syndromes in a population-based sample of male and female twins. *Psychol.Med.* 31 (6):989-1000.

40. Knowles JA *et al. (*1998) Results of a genome-wide genetic screen for panic disorder. *American Journal of Medical Genetics* 81 (2):139-147.

41. Skre I *et al. (*1993) A twin study of DSM-III-R anxiety disorders. *Acta Psychiatr.Scand.* 88 (2):85-92.

42. Craske MG *et al. (*2002) Does nocturnal panic represent a more severe form of panic disorder? *J.Nerv.Ment.Dis.* 190 (9):611-618.

43. Mellman TA and Uhde TW (1989) Sleep panic attacks: new clinical findings and theoretical implications. *Am.J.Psychiatry* 146 (9):1204-1207.

44. Hamilton SP *et al. (*2003) Further genetic evidence for a panic disorder syndrome mapping to chromosome 13q. *Proc.Natl.Acad.Sci.U.S.A* 100 (5):2550-2555.

45. Zaubler TS and Katon W (1996) Panic disorder and medical comorbidity: a review of the medical and psychiatric literature. *Bull.Menninger Clin.* 60 (2 Suppl A):A12-A38.

46. Sareen J *et al. (*2005) Anxiety disorders and risk for suicidal ideation and suicide attempts: a population-based longitudinal study of adults. *Arch.Gen.Psychiatry* 62 (11):1249-1257.

47. Sareen J *et al. (*2006) Disability and poor quality of life associated with comorbid anxiety disorders and physical conditions. *Arch.Intern.Med.* 166 (19):2109-2116.

48. Rogers MP *et al. (*1994) Prevalence of medical illness in patients with anxiety disorders. *Int.J.Psychiatry Med.* 24 (1):83-96.

49. Endo Y (2006) [Psychological symptoms in IBS]. *Nippon Rinsho* 64 (8):1471-1476.

50. Kumano H *et al. (*2004) Comorbidity of irritable bowel syndrome, panic disorder, and agoraphobia in a Japanese representative sample. *Am.J.Gastroenterol.* 99 (2):370-376.

51. Lydiard RB (2001) Irritable bowel syndrome, anxiety, and depression: what are the links? *J.Clin.Psychiatry* 62 Suppl 8:38-45.

52. Weissman MM *et al. (*2004) Interstitial cystitis and panic disorder: a potential genetic syndrome. *Arch.Gen.Psychiatry* 61 (3):273-279.

53. Weissman MM *et al. (*2000) Potential panic disorder syndrome: clinical and genetic linkage evidence. *Am.J.Med.Genet. (Neuropsychiatr.Genet.)* 96:24-35.

54. Garakani A *et al. (*2003) Comorbidity of irritable bowel syndrome in psychiatric patients: a review. *Am.J.Ther.* 10 (1):61-67.

55. Wald A (2002) Psychotropic agents in irritable bowel syndrome. *J.Clin.Gastroenterol.* 35 (1 Suppl):S53-S57.

56. Aerssens J *et al. (*2007) Alterations in the brain-gut axis underlying visceral chemosensitivity in Nippostrongylus brasiliensis-infected mice. *Gastroenterology* 132 (4):1375-1387.

57. Mayer E and Collins S (2002) Evolving pathophysiologic models of functional gastrointestinal disorders. *Gastroenterology* 122 (7):2032-2048.

58. Katon W (1996) Panic disorder: relationship to high medical utilization, unexplained physical symptoms, and medical costs. *J.Clin.Psychiatry* 57 Suppl 10:11-18.

59. Smit F *et al. (*2006) Costs of nine common mental disorders: implications for curative and preventive psychiatry. *J.Ment.Health Policy Econ.* 9 (4):193-200.

60. Boyd JH (1986) Use of mental health services for the treatment of panic disorder. *Am.J.Psychiatry* 143 (12):1569-1574.

61. Deacon B, Lickel J, and Abramowitz JS (2008) Medical utilization across the anxiety disorders. *J.Anxiety.Disord.* 22 (2):344-350.

62. Korczak DJ, Goldstein BI, and Levitt AJ (2007) Panic disorder, cardiac diagnosis and emergency department utilization in an epidemiologic community sample. *Gen.Hosp.Psychiatry* 29 (4):335-339.

63. Roy-Byrne PP *et al. (*1999) Panic disorder in the primary care setting: comorbidity, disability, service utilization, and treatment. *J.Clin.Psychiatry* 60 (7):492-499.

64. Weissman MM *et al. (*1990) Panic disorder and cardiovascular/cerebrovascular problems: results from a community survey. *Am.J.Psychiatry* 147 (11):1504-1508.

65. Fleet RP *et al. (*1998) Comparing emergency department and psychiatric setting patients with panic disorder. *Psychosomatics* 39 (6):512-518.

66. Zane RD *et al. (*2003) Panic disorder and emergency services utilization. *Acad.Emerg.Med.* 10 (10):1065-1069.

67. Leon AC, Portera L, and Weissman MM (1995) The social costs of anxiety disorders. *Br.J.Psychiatry Suppl* (27):19-22.

68. Markowitz JS *et al. (*1989) Quality of life in panic disorder. *Arch.Gen.Psychiatry* 46 (11):984-992.

69. Roy-Byrne PP *et al. (*2003) Unemployment and emergency room visits predict poor treatment outcome in primary care panic disorder. *J.Clin.Psychiatry* 64 (4):383-389.

70. Edlund MJ (1990) The economics of anxiety. *Psychiatr.Med.* 8 (2):15-26.

71. Ettigi P *et al. (*1997) The quality of life and employment in panic disorder. *J.Nerv.Ment.Dis.* 185 (6):368-372.

72. Cosci F *et al. (*2007) Alcohol use disorders and panic disorder: a review of the evidence of a direct relationship. *J.Clin.Psychiatry* 68 (6):874-880.

73. Klerman GL *et al. (*1991) Panic attacks in the community. Social morbidity and health care utilization. *JAMA* 265 (6):742-746.

74. Diaconu G and Turecki G (2007) Panic disorder and suicidality: is comorbidity with depression the key? *J.Affect.Disord.* 104 (1-3):203-209.

75. Weissman MM *et al. (*1989) Suicidal ideation and suicide attempts in panic disorder and attacks. *N.Engl.J.Med.* 321 (18):1209-1214.

76. Pilowsky DJ *et al. (*2006) Children of currently depressed mothers: a STAR*D ancillary study. *J.Clin.Psychiatry* 67 (1):126-136.

77. Weissman MM *et al. (*2006) Remissions in maternal depression and child psychopathology: a STAR*D-child report. *JAMA* 295 (12):1389-1398.

78. Crowe RR *et al. (*2001) Genome scan of panic disorder: Results of nonparametric linkage analyses. *American Journal of Medical Genetics* 105 (7):578.

79. Fyer AJ *et al. (*2006) A Third-Pass Genome Scan in Panic Disorder: Evidence for Multiple Susceptibility Loci. *Biol.Psychiatry* 60 (4):388-401.

80. Gelernter J *et al. (*2001) Linkage genome scan for loci predisposing to panic disorder or agoraphobia. *American Journal of Medical Genetics* 105 (6):548-557.

81. Thorgeirsson TE *et al. (*2003) Anxiety with Panic Disorder Linked to Chromosome 9q in Iceland. *Am J Hum.Genet.* 72 (5).

82. Smoller JW *et al. (*2001) Targeted genome screen of panic disorder and anxiety disorder proneness using homology to murine QTL regions. *American Journal of Medical Genetics* 105 (2):195-206.

83. Domschke K *et al. (*2007) Meta-analysis of COMT val158met in panic disorder: ethnic heterogeneity and gender specificity. *Am.J.Med.Genet.B Neuropsychiatr.Genet.* 144 (5):667-673.

84. Hamilton SP *et al. (*2002) Evidence for a susceptibility locus for panic disorder near the catechol-O-methyltransferase gene on chromosome 22. *Biol.Psychiatry* 51 (7):591-601.

85. Deckert J *et al. (*1998) Systematic mutation screening and association study of the A1 and A2a adenosine receptor genes in panic disorder suggest a contribution of the A2a gene to the development of disease. *Mol Psychiatry* 3 (1):81-85.

86. Hamilton SP *et al. (*2004) Evidence for genetic linkage between a polymorphism in the adenosine 2A receptor and panic disorder. *Neuropsychopharmacology* 29 (3):558-565.

87. Lam P, Hong CJ, and Tsai SJ (2005) Association study of A2a adenosine receptor genetic polymorphism in panic disorder. *Neurosci.Lett.* 378 (2):98-101.

88. Lander ES and Schork NJ (1994) Genetic dissection of complex traits. *Science* 265 (5181):2037-2048.

89. Saeed SA and Bruce TJ (1998) Panic disorder: effective treatment options. *Am.Fam.Physician* 57 (10):2405-2420.

90. Contarino A *et al. (*1999) Reduced anxiety-like and cognitive performance in mice lacking the corticotropin-releasing factor receptor 1. *Brain Research* 835 (1):1-9.

91. Gold PW, Goodwin FK, and Chrousos GP (1988) Clinical and biochemical manifestations of depression. Relation to the neurobiology of stress (1). *N.Engl.J.Med.* 319 (6):348-353.

92. Klein DF (1998) Panic and phobic anxiety: phenotypes, endophenotypes, and genotypes. *Am.J.Psychiatry* 155 (9):1147-1149.

93. Pollack M, Otto MW, Rosenbaum J (2008) Treatment of panic disorder. Pharmacologic perspectives. In: Challenges in clinical practice: pharmacologic and psychosocial strategies. Guilford Press: New York.

94. Scott EL *et al. (*1999) Clinician Response to Treatment Refractory Panic Disorder: A Survey of Psychiatrists. *Journal of Nervous & Mental Disease* 187 (12):755-757.

95. Roy-Byrne PP and Cowley DS (1994) Course and outcome in panic disorder: a review of recent follow-up studies. *Anxiety*. 1 (4):151-160.

96. Ballenger JC *et al. (*1998) Consensus statement on panic disorder from the International Consensus Group on Depression and Anxiety. *J.Clin.Psychiatry* 59 Suppl 8:47-54.

97. Bakker A *et al. (*1999) Paroxetine, clomipramine, and cognitive therapy in the treatment of panic disorder. *J.Clin.Psychiatry* 60 (12):831-838.

98. Lecrubier Y *et al. (*1997) A comparison of paroxetine, clomipramine and placebo in the treatment of panic disorder. Collaborative Paroxetine Panic Study Investigators. *Acta Psychiatr.Scand.* 95 (2):145-152.

99. Nakamura M *et al. (*1999) Polymorphisms of the human homologue of the Drosophila white gene are associated with mood and panic disorders. *Mol.Psychiat.* 4 (2):155-162.

100. Domschke K *et al. (*2006) Association of the functional -1019C/G 5-HT1A polymorphism with prefrontal cortex and amygdala activation measured with 3 T fMRI in panic disorder. *Int.J.Neuropsychopharmacol.* 9 (3):349-55.

101. Maron E and Shlik J (2006) Serotonin function in panic disorder: Important, but why? *Neuropsychopharmacology* 31:1-11.

102. Yonkers KA *et al. (*2003) Chronicity, relapse, and illness-course of panic disorder, social phobia, and generalized anxiety disorder: findings in men and women from 8 years of follow-up. *Depress.Anxiety.* 17 (3):173-179.

2. Chapter Two. Linkage and Association of Neuropeptide Genes in PD

2.1. Abstract

The polygenic origins of panic disorder (PD) remain elusive. Following the hypothesis of altered neurotransmitter tone in PD putatively underlying COMT and ADORA2A[1] involvement in PD, the neuropeptidergic system genes are natural candidates for PD. Ethological and pharmacological data show neuropeptidergic modulation of anxiety and stress via neurotransmitters. The genes encoding the corticotropin releasing hormone receptor 1 (CRHR1), tachykinin receptor 1 (TACR1), gastrin releasing peptide (GRP), and gastrin releasing peptide receptor (GRPR) were selected as candidates for linkage and association analysis in 120 multiplex, Caucasian PD pedigrees. Modest logarithm of odds (LOD) scores for linkage (TACR1 and CRHR1, LOD ~1) and association (GRP and TACR1, p=0.02) suggest that these genes cannot be ruled out in the genetic susceptibility for PD.

2.2. Background

Dysregulation of the stress response system, including molecular changes in the effectors of the stress pathway (i.e. neurotransmitters, neuropeptides, hormones, and their cognate receptors) are thought to underlie the pathogenesis of PD. The molecular actions of CRHR1, TACR1, GRPR and GRP in controlling many aspects of PD pathology are natural candidates for genetic contribution to the etiology of PD.

2.2.1. Pharmacological mechanisms of PD

Antagonists of CRHR1 and TACR1, such as R121919[3,4] and MK869[5], have been developed for treatment of anxiety and depression, while a GRPR antagonist (RC3095) results in increased anxiety[6] and altered memory processing in animals[6-9], although its development as a therapeutic target for psychiatric disorders, including anxiety, is currently under investigation[10]. These antagonists are potential tools in elucidating the role of neuropeptidergic signaling systems as modulators of anxiety and cognition in ethological studies, and suggest that genetic variation in the genes encoding their molecular targets may influence the susceptibility to PD.

2.2.2. Effectors of the stress response system

GRPR, TACR1, and CRHR1 are G-protein coupled receptors that enhance the release of co-localized neurotransmitters in response to extracellular signals[11-13]. These neuropeptide receptors densely populate central and peripheral neural structures responsible for modulating the stress response system. Central distribution includes the hypothalamus, pituitary and adrenals (HPA axis), hippocampus, amygdala, prefrontal cortex (PFC), locus coeruleus (LC), septum, substantia nigra (SN), nucleus of the solitary tract (NST), raphe nucleus (RN), periaqueductal gray area (PAG), bed nucleus stria terminalis (BNST), and nucleus accumbens (NAcc). Ligands for these receptors (GRP, tachykinin, and CRH) are secreted in response to humoral, somatosensory, and psychogenic stressors. Downstream effects of GRPR, TACR1, and CRHR1 activation include enhanced release of neurotransmitters coexisting within the

cell (gamma-amino butyric acid (GABA), glutamate, dopamine (DA)[11,12], serotonin (5HT), norepinephrine (NE), and acetylcholine (ACh)[11]. Experimental evidence reveals highly integrated interactions between these signaling systems. For example, GRP and CRH are coordinately released with stress[13-15], and stress-induced hypothalamic serotonin (5HT) activity can be blocked by antagonists of both CRH and GRP receptors[16,17]. CRH and tachykinin mediate dorsal raphe nucleus firing and 5HT release with stress[13], while activation of TACR1 in turn inhibits CRH synthesis and the hypothalamic-pituitary-adrenal (HPA) axis response[18].

Stress responses are mediated via the HPA axis and higher order central processes (e.g., at the level of the hippocampus and amygdala). Stress signals received by the hypothalamus evoke CRH secretion, targeting CRHR1 in the anterior pituitary, where Gs-coupled signal transduction induces adrenocorticotropin (ACTH) and beta-endorphin release. The latter two peptides are both cleaved from the proopiomelanocortin (POMC) precursor protein. ACTH secretion into the blood stream targets the adrenal gland to elicit epinephrine secretion from the adrenal medulla, and glucocorticoid (GC) secretion from the adrenal cortex (cortisol in humans, corticosterone in rodents). ACTH upregulates the expression of TACR1, preprotachykinin-A (tachykinin precursor), and initiates tachykinin/TACR1 signaling[23]. GCs simultaneously elicit SNS responses while initiating feedback inhibition via glucocorticoid and mineralocorticoid receptors (GR, MR) that reside along all various relay points in the stress response regulatory regions, including the hippocampus,

hypothalamus, and pituitary. GRP/GRPR and tachykinin/TACR1 signaling also evoke the HPA axis response to transmit effects of the sympathetic and parasympathetic nervous systems. Outside of the HPA axis, all of the candidate neuropeptidergic signaling systems have multiple site-specific roles in modulating the classic neurotransmitters to manifest specific central effects. For example, GRPR mediates memory formation in the hippocampus and amygdala via excitation of inhibitory GABAergic, GRPR-positive interneurons to inhibit CA1 principal cells[19].

In the periphery, cellular responses include Gq-coupled, TACR1 and GRPR-mediated cardiovascular and other smooth muscle contractility[20] to mount the sympathetic nervous system "fight or flight" response by increasing heart rate, which is a primary somatic symptom of panic attacks in PD. Gs-coupled, CRHR1 and TACR1[21] signaling alternatively evokes vasodilatory response in arterioles, and bronchial/tracheal constriction, reminiscent of PD symptoms of dizziness and dyspnea, and characteristic of the parasympathetic nervous system "fright, freeze or faint" response. The Gq and Gs-coupled second messenger cascades reflect opposing mechanisms of autonomic nervous system response delineated by the PNS and SNS, respectively. TACR1 signaling may evoke PNS or SNS responses depending upon ligand concentration. TACR1 coupling to Gs (high ligand affinity) or Gq (low ligand affinity) occurs in a biphasic manner, with distinct cellular outcomes[22]. Therefore, manifold stress-response effects of these neuropeptide signal systems are substrate concentration-

dependent[22], tissue-specific[24], and context-dependent in integrating adaptive responses.

These neuropeptidergic GCPR systems respond to signals sensed by humoral (blood gases, osmolality, hormones), somatosensory (pain, infection, trauma, ingestion), and psychogenic (predation, threat) inputs that in turn modulate classical synaptic transmission and effect paracrine and endocrine responses in the autonomic nervous system as adaptive compensation. Among their duties in organismal homeostasis, the neuropeptidic signaling systems controlled by GRP/GRPR, CRHR1, and TACR1 include the adaptive responses of: immunoinflammatory, salt-water balance, blood acid-base balance, blood gas balance, blood pressure, tissue growth and repair, reproductive efficiency, digestion, energy use or storage, thermoregulation, muscle contractility, cardiovascular, and nociception, all to shunt molecular resources to the task at hand. Outward indices of these changes motivate behaviors that benefit the adaptive internal molecular changes, such as: escape/exploration, sleep/arousal, social/sexual, temperature regulating, feeding, water-seeking, and behaviors. PD and depression, which are both stress-related disorders, are characterized by chronic alterations in a number of the abovementioned molecular and behavioral indices.

2.2.3. Pleiotropic effects of neuropeptidergic signaling

These neuropeptide modulators are implicated in PD for their direct involvement in activating numerous somatic and cognitive responses observed

with acute panic attacks and with ensuing anticipatory anxiety in PD. Important to resolving the complex genetic etiology of PD, non-psychological symptoms implicated with these genes may represent PD endophenotypes as potential markers of underlying biological mechanisms for PD. Given the likelihood of dysregulation of the stress-response system in PD, our neuropeptidergic candidate genes appear to be associated with somatic and psychological effects that parallel many PD symptoms. Examples include peripheral autonomic reflexes in the skin and bladder, regulated by peptidergic signaling systems. Observed differences in skin conductance is biological marker for PD[25-27]. Threshold set points for the "fight or flight" sympathetic tone and homeostasis, marked by vasoconstriction of skin blood capillaries may be dysregulated in PD. Dense peripheral innervation of the skin by TACR1[28] and GRPR (43% of the epidermal cells)[29] is responsible for many important vascular[30], immunological, and inflammatory[31] effects. In the amygdala, where all of our candidate genes probably play important anxiety-mediating functions, surgical resection for refractory epilepsy results in changes in skin conductance associated with reduced anxiety[32]. Thus, skin conductance reflects centrally mediated autonomic tone. Similarly, bladder function is regulated by neuropeptidergic regulation of ANS roles for smooth muscle control, salt-water balance, and inflammation. Interstitial cystitis (IC), a chronic and painful disorder, is characterized by increased urinary histamine[33], accumulation of lymphocytes and mast cells[34,35] and a breach in the bladder permeability barrier to urine[36], and is attributed to HPA axis dysregulation. IC is 2 to 11 times more common in females than males

in general[37,38], and enriched in our PD cohort. Reciprocal enrichment of PD exists in patients with urinary interstitial cystitis[39,40]. Patients with IC exhibit pronounced TACR1 mRNA expression by bladder biopsy[138], and significantly increased catecholamine-synthesizing tyrosine hydroxylase in the LC[41] and bladder[42]. Animal models of the tachykinin/TACR1 system (TACR1 gene deletion) reveal similar symptoms of cystitis, with four fold greater mast cells in the bladder[43], and impaired inflammatory response to antigen challenge (including AP-1 responsive fos and TNF-beta genes)[44]. Treatment of IC includes the anxiolytic TCA, amitriptyline; and relevant effects of TACR1 antagonists have been found to increase bladder capacity without changing micturition pressure[45]. On the basis of common psychological (anxiety, fear) and somatic (skin conductance, bladder function) phenotypes in PD, these neuropeptidergic genes are good candidate genes.

2.2.4. Gastrin releasing-peptide signaling

GRP is secreted in response to restraint stress[15], and administration of GRP or GRPR antagonists are shown to alter memory of aversive events[6-9,46] in a region-specific and dose-dependent manner. GRPR knockout mice exhibit enhanced learned fear memory[12] due to increased long-term potentiation in the cortex and amygdala. GRPR is distributed in both excitatory, glutamatergic neurons, and inhibitory, GABAergic neurons, leading to memory enhancement[12] or inhibition[6-9], respectively. Thus, manifold effects of GRP/GRPR signaling should depend upon specific neurocircuitry. Together these studies implicate

GRP and GRPR in a number of autonomic nervous system and behavioral responses, some of which may reflect PD symptomology.

2.2.5. Corticotropin releasing-hormone signaling

Exogenous administration[48-51] or overexpression[52] of CRH induces anxiety that can be blocked by the administration of the anxiolytic, $GABR_A$-targeting benzodiazepine (BZ), chlorodiazepoxide[50]. Heightened CRH levels are suggested to be an endophenotype of anxiety, as indexed by greater freezing behavior and adrenal-pituitary activity in non-human primates[53]. Patients with PD exhibit altered ACTH and cortisol responses[54,55], which normalizes with alprazolam treatment[55].

Knockdown of CRHR1 by antisense mRNA reduces anxiety that is provoked by stress testing paradigms, or CRH administration[56-61]. Stress-mediated surges in ACTH and GC are blunted in CRHR1 knockouts[62], as with administration of CRHR1 antagonist[63]. CRHR1 knockout animals exhibit reduced anxiety, along with impaired cognition, and low basal plasma levels of glucocorticoid stress hormones from adrenal agenesis and atrophy[62,64-65]. Thus, CRHR1 is critical to proper adrenal development[64], not only for mounting a robust stress response, but also for maintaining homeostasis with endocrine fluctuations. Hyperactive CRH effectively causes functional adrenal hyperplasia, with possible down-regulation of pituitary CRHR1, and lessened feedback inhibition at hippocampal glucocorticoid receptors, typical of hypercortisolemic subjects with depression or PD[66]. Similar to CRHR1 gene deficiency,

antagonists of CRHR1 cause diminution of learned fear[69], anxiety[67-74], and depression[75,76] in animal behavior models. The CRHR1 antagonist R121919, has shown efficacy, tolerability, and safety in trials for clinical treatment of anxiety and depression[77].

2.2.6. Tachykinin signaling

TACR1 is the receptor for tachykinin (also called substance P), which is secreted in response to stress[11,78]. Treatment with antidepressants[79] and anxiolytics[80] reduce tachykinin levels in the rat brain, while administration of tachykinin induces anxiety in animals[11,81-84]. Tachykinin acts to enhance inhibitory avoidance learning[85] and conditioned place preference[86,87].

TACR1 knockout animals exhibit reduced nociception and psychogenic anxiety provoked by TACR1 agonist or maternal separation[88], but not anxiety from exploration[89]. Other TACR1 deletion phenotypes include reductions in aggression[89], reward behavior[90], and stress-induced analgesia[91], as well as altered pain perception[91].

Similarly, TACR1 antagonists lead to reductions in anxiety-like behaviors in a number of paradigms[5,83, 84,92-95], reduction in aggression[89], aversive memory conditioning[89], defensive rage[96], anxiety in maternal-separation[83], anxiety in social interaction tests[93], and anxiety in some[84], but not all exploration-hiding conflict tests[93].

Both disruption of TACR1 gene[88,97,98] and pharmacological blockade by TACR1 antagonists[99,100] result in increased hippocampal 5HT neurotransmission,

greater frequency of spontaneous 5HT-firing in the DRN[88,101] and subsequent inactivation of $5HT_{1A}$ autoreceptors that mimics the actions of antidepressants, such as SSRIs, MAOIs, $5HT_{1A}$ antagonists, and alpha 2-adrenoreceptor antagonists (e.g. mirtazapine, bupropion). TACR1 antagonists, however, exhibit less motor impairment and sedative side effects than traditional antidepressants, as measured by rotorod test for performance in locomotion and balance[89]. TACR1-mediated effects are complex due to differential cleavage of the tachykinin precursor[102], and variability in tachykinin ligand concentration[103]. This reflects the biphasic Gs and Gq-coupled actions of TACR1-mediated signal transduction inherent to substrate concentration[22]. Lastly, there are region-specific differences in colocalized neurotransmitters within TACR1-positive cells, such as ACh in ventral pallidum[104], glutamate in raphe and PAG[13], and NE in locus coeruleus.

In humans, TACR1-mediated signaling is implicated in the clinical observation of sustained increases in plasma tachykinin levels with increased anxiety[105], and decreased cortical TACR1 in depression[106]. The TACR1 antagonist, MK869, has shown efficacy with less side effects than the selective serotonin reuptake inhibitor (SSRI), paroxetine, in clinical trials for treatment of anxiety and depression[5], although this drug has yet to be pursued in large-scale trials. Another TACR1 antagonist, GR205171, has proved comparable to the SSRI, citalopram, in treating patients with social phobia[107], which is up to 70% comorbid in subjects with PD[3].

Given the compelling biological support involving these four genes with anxiety, we investigated the hypothesis that genetic variations at these loci are involved in the susceptibility to PD. No previous studies have been carried out in PD with the candidate genes studied here.

2.3. Methods

2.3.1. Subject recruitment and sample collection

A total of 1,591 individuals (for which we collected 992 DNAs for genotyping) in 120 PD pedigrees of primarily Caucasian Americans of Western European descent were recruited from anxiety clinics, therapists, and support groups from the Northeastern United States. Subjects were independently assessed per the *Diagnostic and Statistical Manual of Mental Disorders*, Third Edition, Revised (DSM III-R) criteria for PD by psychiatrists blinded to family relationships[108]. Diagnostic categories were defined as definite, probable, possible, or any panic, and unaffected, or unknown, as described elsewhere[108]. These were used to establish three phenotypic designations for statistical analysis: narrow (definite or probable PD), intermediate (adding possible PD), and broad (adding any panic) PD phenotypes[109]. These diagnostic categories allow for the inclusion of individuals who did not meet the strict DSM III-R criteria for PD ± agoraphobia, but who might otherwise contribute to the genetic susceptibility of PD. For example, the “any” panic category allows for inclusion of individuals with some sign or symptom of PD, such as a single, severe paroxysmal panic attack without sequelae, or medically unexplained bouts of “air

hunger" [108]. The individuals for whom we had no DNA were intermediate relatives who were necessary to establish familial relationships among affecteds.

According to a disproportionate co-occurrence of PD with syndromic features, such as mitral valve prolapse, migraines or serious headaches, thyroid conditions, bladder or kidney problems (see section 1.1.5.), two additional diagnostic categories were used, as described elsewhere[139,140]. Briefly, affected status was broadened to include family members with syndromic or bladder conditions. For example, the bladder and renal conditions included family members with a history of enuresis, mild renal insufficiency with secondary hypertension, or cystitis. Mechanistic and genetic studies show evidence for a link between PD with the syndromic features enriched in PD pedigrees. These mechanistic overlaps reflect potential pleiotropic effects of the candidate genes for PD (sections 2.2.3., 3.2.5., 4.2.5., and 4.2.6.).

Genomic DNA was previously isolated from blood or lymphoblastoid cell lines[110]. All subjects provided informed consent, and the experimental protocol was approved by the institutional review boards of the University of California, San Francisco and New York State Psychiatric Institute.

2.3.2. Genotyping methods

Five to six single nucleotide polymorphisms (SNPs) per gene, with at least 10% minor allele frequencies (MAF), were selected for genotyping from the Celera and National Center for Biotechnology Information (NCBI) databases.

Methods for genotyping DNA included fluorescence polarization template-directed dye-terminator incorporation (FP-TDI)[111] and 5'-nuclease (TaqMan)[112].

2.3.3. FP-TDI genotyping

Polymerase chain reaction (PCR) was performed in 5μL reactions using 2ng/μL DNA, 200nM primers, 50-200μM deoxyribonucleotide triphosphate (dNTPs), 1.0 or 1.5M anhydrous betaine (Acros Organics, Geel, Belgium), 50mM KCl, 20mM Tris-HCl (pH 8.4), 1.0 to 2.5mM $MgCl_2$, and 0.05U/μL Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Oligonucleotide primers and probes were designed using Primer3 software for synthesis (Invitrogen). Conditions for PCR using ABI GeneAmp PCR System (Applied Biosystems (ABI), Foster City, CA) thermal cyclers were as follows: 94°C for 3min, followed by 7 three-step touchdown cycles (94°C for 30s, decreasing 1C per cycle 65-59°C for 30s, 72°C for 30s), followed by 38 three-step cycles (90°C for 30s, 58°C for 30s, 72°C for 30s), and 72°C for 10min. Excess primers and dNTPs were enzymatically digested with the addition of a 2μL PCR Clean-Up Reagent diluted in PCR Clean-Up Dilution Buffer (USB Corporation, Cleveland, OH) and incubation at 37°C for 90min, followed by enzyme deactivation at 95°C for 15min. Incorporation of acycloNTP terminators followed with the addition of 13μL mixture containing 250nM TDI probe, 0.4U Acyclopol DNA polymerase (Perkin Elmer Life Sciences), 50mM Tris-HCl, 50mM KCl, 50mM $MgCl_2$, 5mM NaCl, 8% glycerol, and 125nM AcycloTerminator mix. Cycling conditions for terminator incorporation were: 95°C for 2min, 10-45 two-step cycles (94°C for 15s, 55°C for

30s). SNP detection was performed using a fluorescence plate reader (Victor^2 Multilabel Counter, PerkinElmer, Boston, MA) at 480nm/535nm and 544nm/595nm excitation/emission wavelengths for R110 and TAMRA-labeled acycloNTP terminators, respectively. Genotypes were manually determined by clustering on a 2-by-2 biallelic scatter plot[113] using internally developed software. See table 2.1. for marker information.

2.3.4. TaqMan genotyping

TaqMan reactions were performed according to manufacturer's specifications for Assays-by-Design and Assays-on-Demand (Applied Biosystems). PCR reactions contained 4ng/μL DNA with 1x concentration of TaqMan Universal PCR master mix containing AmpliTaq Gold DNA polymerase and 0.5x or 0.66x concentration of SNP genotyping assay mix in 5μL reactions. Thermocycling conditions included 95°C for 10min, followed by 50 two-step cycles (92°C for 15s, and 60°C for 60s). Thermocyling, data acquisition, and analyses were performed using ABI Prism 7900HT Sequence Detection System. PCR was performed in 10μL reactions using 3ng/μL DNA, 1M anhydrous betaine, 2.5mM $MgCl_2$, 50nM dNTPs, 0.025U/μL Platinum *Taq* polymerase, 300nM primers, 75mM KCl, and 30mM Tris-HCl (pH 8.4). PCR conditions for STR genotyping using ABI GeneAmp PCR System 9700 thermal cyclers were as follows: 94°C for 3min, followed by 7 three-step touchdown cycles (94°C for 30s, decreasing 1.14C per cycle 65-58°C for 30s, 72°C for 30s), followed by 38 three-step cycles (90°C for 30s, 58°C for 30s, 72°C for 30s), and 72°C for 10min.

Quality control procedures for genotyping included monitoring concordant genotypes for duplicate samples, as well as routine mendelization error checks. Inconsistent genotypes were re-typed, and unresolved incongruent genotypes were dropped.

2.3.5. Linkage and association analysis

Parametric linkage analysis was performed assuming 1% and 20% disease allele frequencies in probands using dominant and recessive genetic models, respectively[114,115]. Maximum logarithm of odds (LOD) scores for genetic linkage were reported as maximized over 0 to 0.5 recombination fraction, assuming locus homogeneity or heterogeneity disease partition models, 1% phenocopy rate, and 50% reduced penetrance. Parametric assumptions in the models were based upon previous segregation analyses[116]. Based upon sexually dimorphic prevalence rates in PD, unknown or unaffected phenotypes were assigned 66% and 33% penetrance, and 14% and 7% phenocopy rate in females and males, respectively. Parametric linkage scores were generated using FASTLINK[117,118] given locus homogeneity, and using HOMOG under the assumption of locus heterogeneity. Identity-by-descent nonparametric linkage (NPL) LOD scores were also determined for allele sharing among family members using GENEHUNTER[119]. Allelic association analysis in nuclear trios and extended pedigrees was determined using the family-based association test (FBAT)[120,121]. Multimarker haplotypes that were inferred by the maximum-

likelihood method imbedded in GENEHUNTER, were tested for association using FBAT[120]. We report uncorrected *p*-values for association analyses.

2.4. Results

In a previous genome-wide scan of the 120 multiplex PD pedigrees, using 371 markers at a low-resolution spacing of 9 cM on average, we did not find significant positional support for the neuropeptidergic candidate genes tested[122]. Using a more focused candidate gene approach, we used twenty-one SNPs at the candidate loci in 120 PD pedigrees with 992 available DNAs to test for linkage and association to PD.

2.4.1. Allelic association

Three SNPs in two genes (GRP and TACR1) showed nominally significant *p*-values of between $p=0.02$ and $p=0.05$ for allelic association under the phenotypic designations of broad, intermediate or narrow PD (table 2.2.). Observed allelic association was robust to at least two of the three phenotypic designations for analysis. For example, two markers in GRP each showed nominally significant association to PD for the narrow and intermediate diagnostic categories, but not for the broad phenotype. Due to its being an X-linked locus, GRPR was analyzed with a family-based association test within ANALYZE[123], and the results were not significant.

2.4.2. Multimarker haplotypic association

Global *p*-values for haplotypic association were calculated using the family-based association test. Given the multiple tests generated in the haplotype association testing, the threshold for significance in association was not met by any of the two-, three-, or four-marker haplotypes.

*2.*4.3. Two point parametric genetic linkage

Modest linkage signals were observed for TACR1 at rs3755460 (SNP 3, LOD>1.1) and rs1477157 (SNP 6, LOD>1.2) given the broad diagnostic category, dominant genetic model, and assuming locus heterogeneity (figure 2.1.). No other neuropeptidergic genes showed linkage of LOD>1.

2.4.4. Multipoint genetic linkage and non-parametric linkage scores

Using multipoint analysis all SNPs in TACR1 and CRHR1 (figures 2.1. and 2.2.) gave LOD scores >1.0. Locus heterogeneity multipoint LODs between 1.1 and 1.5 were found for all of the TACR1 SNPs given the broad diagnostic category, dominant mode of inheritance. The maximum multipoint score for CRHR1 was 1.35 for the narrow diagnostic category, assuming locus heterogeneity, in 11% of the families. Non-parametric linkage (NPL) scores were determined and converted to LOD scores for ease of comparison. LOD-transformed NPL scores by multipoint analysis reached a maximum of 1.2 (p=0.014) for two TACR1 SNPs, and 1.1 (p<0.018, or p<0.015) for the three SNPs, using both dominant and recessive genetic models. The linkage and association results presented in this report are without correction for multiple

testing as it is yet unclear how to interpret data generated from candidate genes with a prior probability of hypothetical correlation to PD, given the biological and pharmacological support for their involvement in anxiety. Given the negative single marker LOD results for each of the GRPR SNPs, GRPR was not analyzed for multipoint linkage.

2.5. Discussion

Modest linkage and/or association results for GRP, CRHR1, and TACR1 suggest that they cannot be ruled out as genetic contributors to PD. To our knowledge, our cohort constitutes the largest PD pedigree collection for study in linkage analysis. It may be that linkage analysis simply cannot detect the small effects that may underlie complex disorders like PD[124]. These candidate genes may constitute major susceptibility loci for PD, but contribute only a minor portion of the variance observed in our cohort.

Genetic background plays an important role in the presentation of traits, vis-à-vis our neuropeptidergic candidate genes, suggesting that regional *cis* variants may modify the effects of linked and/or associated SNPs. For example, differences in spontaneous locomotor activity in the GRPR knockout are dependent upon the background strain[125,126], and breed-specific differences in CRHR1 and tachykinin levels have been associated with increased anxiety in five behavioral testing paradigms, and blunted reduction in tachykinin with stress[127]. A three-marker CRHR1 haplotype (rs1876828-rs242939-rs242941) was associated with reduction in anxiety in response to antidepressant treatment for a subset of depressed patients who had high trait anxiety[128]. Similarly, comparison

of animal strains showed that anxiolytic effects of CRHR1 antagonist, R121919, correlated with strains having high innate emotionality[129]. Expression profiles suggest that this may be related to different mRNA patterns observed between high and low anxiety strains[130].

One can speculate as to the potential *cis* variants that could modify CRHR1 effects. For example, an intronic CRHR1 variant, rs1876831, lying in an SP1 transcription factor binding site (TFBS), is associated with alcohol abuse[131], which is also highly comorbid with PD. Characterization of linkage disequilibrium patterns may show that this SP1 TFBS SNP segregates with the CRHR1 SNP linked to PD. Interestingly, this SP1 transcription factor is significantly downregulated in females with PD[132], so there is at least a rationale for further characterization of this locus, with potential influences from gender-specific traits, and/or specific gene-gene interactions (e.g. CRHR1 and SP1).

Heritable gender effects occur for GRPR as well. GRPR, which lies on chromosome Xp22, is inherited at a double dose in females, due to escape from X-inactivation[133]. This is seen in baseline GRPR mRNA expression in lung fibroblasts[134], which makes females more susceptible to hyperactive, mitogenic GRP signaling. Hormonal regulation is also known to modify the effects of tachykinin in the brain. The concept of genetic epistasis, defined by the masking or enhancing of one gene by another[135], is characterized by non-mendelian segregation ratios that are typical of complex genetic traits, such as PD. Genetic programming of the fetus *in utero*, for example, results in heritable hypertension (ANS dysregulation)[136] and reduced learning memory. In the progeny of mothers

with excess glucocorticoids, this was associated with baseline elevation in plasma glucocorticoid levels, region-specific alterations in GR mRNA, and increased hypothalamic CRH mRNA levels[137].

In summary, heritable alteration of gene regulation in clinical and animal data suggest a mechanism for global and gender-specific genetic background differences that modify genes involved in PD, perhaps explaining phenotypic heterogeneity and sexual-dimorphic prevalence rates in PD. Our work is limited by the few markers used per gene to describe the LD pattern of these genes. To understand potential genetic background influences, further mapping and characterization of linkage disequilibrium patterns in these candidate genes may help to pinpoint or exclude the involvement of these neuropeptidergic candidate genes in PD. In summary, genetic background, hormone status, and gender-specific effects may be important factors influencing the actions of these candidate genes.

2.6. References

1. Tebano MT *et al.* (2004) Adenosine A2A receptor blockade differentially influences excitotoxic mechanisms at pre- and postsynaptic sites in the rat striatum. *J.Neurosci.Res.* 77 (1):100-107.

2. Hodges LM *et al. (*2008) Association and Linkage Analysis of Candidate Genes GRP, GRPR, CRHR1 and TACR1 in Panic Disorder. *Am.J.Med.Genet. (Neuropsychiatric Genet.)* In press.

3. Kunzel HE *et al. (*2003) Treatment of depression with the CRH-1-receptor antagonist R121919: endocrine changes and side effects. *Journal of Psychiatric Research* 37 (6):525-533.

4. Zobel AW *et al. (*2000) Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated. *J.Psychiatr.Res.* 34 (3):171-181.

5. Kramer MS *et al. (*1998) Distinct Mechanism for Antidepressant Activity by Blockade of Central Substance P Receptors. *Science* 281 (5383):1640-1645.

6. Martins MR *et al. (*2005) Non-associative learning and anxiety in rats treated with a single systemic administration of the gastrin-releasing peptide receptor antagonist RC-3095. *Peptides* 26 (12):2525-2529.

7. Roesler R *et al. (*2004) RC-3095, a bombesin/gastrin-releasing peptide receptor antagonist, impairs aversive but not recognition memory in rats. *European Journal of Pharmacology* 486 (1):35-41.

8. Roesler R *et al. (*2004) Bombesin/gastrin-releasing peptide receptors in the basolateral amygdala regulate memory consolidation. *European Journal of Neuroscience* 19 (4):1041-1045.

9. Roesler R *et al. (*2003) Intrahippocampal infusion of the bombesin/gastrin-releasing peptide antagonist RC-3095 impairs inhibitory avoidance retention. *Peptides* 24 (7):1069-1074.

10. Roesler R, Henriques JA, and Schwartsmann G (2006) Gastrin-releasing peptide receptor as a molecular target for psychiatric and neurological disorders. *CNS.Neurol.Disord.Drug Targets.* 5 (2):197-204.

11. Hasenohrl RU *et al. (*2000) Substance P and its role in neural mechanisms governing learning, anxiety and functional recovery. *Neuropeptides* 34 (5):272-280.

12. Shumyatsky GP *et al. (*2002) Identification of a signaling network in lateral nucleus of amygdala important for inhibiting memory specifically related to learned fear. *Cell* 111 (6):905-918.

13. Valentino RJ and Commons KG (2005) Peptides that fine-tune the serotonin system. *Neuropeptides* 39 (1):1-8.

14. Garrido MM, Manzanares J, and Fuentes JA (1999) Hypothalamus, anterior pituitary and adrenal gland involvement in the activation of adrenocorticotropin and corticosterone secretion by gastrin-releasing peptide. *Brain Res.* 828 (1-2):20-26.

15. Merali Z *et al. (*1998) Aversive and Appetitive Events Evoke the Release of Corticotropin-Releasing Hormone and Bombesin-Like Peptides at the Central Nucleus of the Amygdala. *Journal of Neuroscience* 18 (12):4758-4766.

16. Garrido MM *et al. (*1998) Role of corticotropin-releasing hormone in gastrin-releasing peptide-mediated regulation of corticotropin and corticosterone secretion in male rats. *Neuroendocrinology* 68 (2):116-122.

17. Garrido MM, Fuentes JA, and Manzanares J (2002) Gastrin-releasing peptide mediated regulation of 5-HT neuronal activity in the hypothalamic paraventricular nucleus under basal and restraint stress conditions. *Life Sci.* 70 (25):2953-2966.

18. Nussdorfer GG and Malendowicz LK (1998) Role of tachykinins in the regulation of the hypothalamo-pituitary-adrenal axis. *Peptides* 19 (5):949-968.

19. Lee K *et al. (*1999) Bombesin-like peptides depolarize rat hippocampal interneurones through interaction with subtype 2 bombesin receptors. *J.Physiol* 518 (Pt 3):791-802.

20. Lingappa VR FK (2000) Physiological Medicine A Clinical Approach to Basic Medical Physiology. McGraw-Hill Companies, Inc., New York.

21. Kwatra MM *et al. (*1993) The substance P receptor, which couples to Gq/11, is a substrate of beta-adrenergic receptor kinase 1 and 2. *J.Biol.Chem.* 268 (13):9161-9164.

22. Holst B *et al. (*2001) Two active molecular phenotypes of the tachykinin NK1 receptor revealed by G-protein fusions and mutagenesis. *J.Biol.Chem.* 276 (23):19793-19799.

23. Maloof PB *et al. (*2001) Induction of preprotachykinin-I and neurokinin-1 by adrenocorticotropin and prolactin. Implication for neuroendocrine-immune-hematopoietic axis. *J.Neuroimmunol.* 112 (1-2):188-196.

24. Garcia LJ *et al. (*1997) The gastrin-releasing peptide receptor is differentially coupled to adenylate cyclase and phospholipase C in different tissues. *Biochim.Biophys.Acta* 1356 (3):343-354.

25. Argyle N (1991) Skin conductance levels in panic disorder and depression. *J.Nerv.Ment.Dis.* 179 (9):563-566.

26. Roth WT, Wilhelm FH, and Trabert W (1998) Autonomic instability during relaxation in panic disorder. *Psychiatry Res.* 80 (2):155-164.

27. Wilhelm FH, Gerlach AL, and Roth WT (2001) Slow recovery from voluntary hyperventilation in panic disorder. *Psychosom.Med.* 63 (4):638-649.

28. Theoharides TC *et al. (*1998) Corticotropin-releasing hormone induces skin mast cell degranulation and increased vascular permeability, a possible explanation for its proinflammatory effects. *Endocrinology* 139 (1):403-413.

29. Staniek V *et al. (*1996) Expression of gastrin-releasing peptide receptor in human skin. *Acta Derm.Venereol.* 76 (4):282-286.

30. Ruocco I *et al. (*2001) Light and electron microscopic study of the distribution of substance P-immunoreactive fibers and neurokinin-1 receptors in the skin of the rat lower lip. *J.Comp Neurol.* 432 (4):466-480.

31. Orsal AS *et al. (*2006) The progesterone derivative dydrogesterone down-regulates neurokinin 1 receptor expression on lymphocytes, induces a Th2 skew and exerts hypoalgesic effects in mice. *J.Mol.Med.* 84 (2):159-167.

32. Masaoka Y *et al. (*2003) Effects of left amygdala lesions on respiration, skin conductance, heart rate, anxiety, and activity of the right amygdala during anticipation of negative stimulus. *Behav.Modif.* 27 (5):607-619.

33. Okragly AJ *et al. (*1999) Elevated tryptase, nerve growth factor, neurotrophin-3 and glial cell line-derived neurotrophic factor levels in the urine of interstitial cystitis and bladder cancer patients. *J.Urol.* 161 (2):438-441.

34. Elbadawi A (1997) Interstitial cystitis: a critique of current concepts with a new proposal for pathologic diagnosis and pathogenesis. *Urology* 49 (5A Suppl):14-40.

35. Sant GR and Theoharides TC (1994) The role of the mast cell in interstitial cystitis. *Urol.Clin.North Am.* 21 (1):41-53.

36. Lavelle JP *et al. (*1998) Disruption of guinea pig urinary bladder permeability barrier in noninfectious cystitis. *Am.J.Physiol* 274 (1 Pt 2):F205-F214.

37. Curhan GC *et al. (*1999) Epidemiology of interstitial cystitis: a population based study. *J.Urol.* 161 (2):549-552.

38. Simon LJ *et al. (*1997) The Interstitial Cystitis Data Base Study: concepts and preliminary baseline descriptive statistics. *Urology* 49 (5A Suppl):64-75.

39. Talati A *et al. (*2008) Panic disorder, social anxiety disorder, and a possible medical syndrome previously linked to chromosome 13. *Biol Psychiatry.* 63 (6):594-601.

40. Weissman MM *et al. (*2004) Interstitial cystitis and panic disorder: a potential genetic syndrome. *Arch.Gen.Psychiatry* 61 (3):273-279.

41. Reche JA and Buffington CA (1998) Increased tyrosine hydroxylase immunoreactivity in the locus coeruleus of cats with interstitial cystitis. J.Urol. 159 (3):1045-1048.

42. Peeker R *et al. (*2000) Increased tyrosine hydroxylase immunoreactivity in bladder tissue from patients with classic and nonulcer interstitial cystitis. *J.Urol.* 163 (4):1112-1115.

43. Saban R *et al. (*2000) Neurokinin-1 (NK-1) receptor is required in antigen-induced cystitis. *Am.J.Pathol.* 156 (3):775-780.

44. Dozmorov I *et al. (*2003) Neurokinin 1 receptors and neprilysin modulation of mouse bladder gene regulation. *Physiol Genomics* 12 (3):239-250.

45. Lecci A *et al. (*1993) Evidence for a role of tachykinins as sensory transmitters in the activation of micturition reflex. *Neuroscience* 54 (3):827-837.

46. Flood JF and Morley JE (1988) Effects of bombesin and gastrin-releasing peptide on memory processing. *Brain Research* 460 (2):314-322.

47. Santo-Yamada Y, Yamada K, and Wada K (2001) Posttraining administration of gastrin-releasing peptide improves memory loss in scopolamine- and hypoxia-induced amnesic mice. *Physiol Behav.* 74 (1-2):139-143.

48. Butler PD *et al. (*1990) Corticotropin-releasing factor produces fear-enhancing and behavioral activating effects following infusion into the locus coeruleus. *J.Neurosci.* 10 (1):176-183.

49. Sahuque LL *et al. (*2006) Anxiogenic and aversive effects of corticotropin-releasing factor (CRF) in the bed nucleus of the stria terminalis in the rat: role of CRF receptor subtypes. *Psychopharmacology (Berl)* 186 (1):122-132.

50. Swerdlow NR *et al. (*1986) Corticotropin-releasing factor potentiates acoustic startle in rats: blockade by chlordiazepoxide. *Psychopharmacology (Berl)* 88 (2):147-152.

51. Yang M *et al. (*2006) Central infusion of ovine CRF (oCRF) potentiates defensive behaviors in CD-1 mice in the Mouse Defense Test Battery (MDTB). *Behav.Brain Res.* 171 (1):1-8.

52. Stenzel-Poore MP *et al. (*1994) Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. *J.Neurosci.* 14 (5 Pt 1):2579-2584.

53. Kalin NH, Shelton SE, and Davidson RJ (2004) The role of the central nucleus of the amygdala in mediating fear and anxiety in the primate. *J.Neurosci.* 24 (24):5506-5515.

54. Brambilla F *et al. (*1992) Psychoimmunoendocrine aspects of panic disorder. *Neuropsychobiology* 26 (1-2):12-22.

55. Curtis GC, Abelson JL, and Gold PW (1997) Adrenocorticotropic hormone and cortisol responses to corticotropin-releasing hormone: changes in panic disorder and effects of alprazolam treatment. *Biol.Psychiatry* 41 (1):76-85.

56. Heinrichs SC *et al. (*1997) Corticotropin-releasing factor CRF1, but not CRF2, receptors mediate anxiogenic-like behavior. *Regul.Pept.* 71 (1):15-21.

57. Liebsch G *et al. (*1999) Differential behavioural effects of chronic infusion of CRH 1 and CRH 2 receptor antisense oligonucleotides into the rat brain. *J.Psychiatr.Res.* 33 (2):153-163.

58. Liebsch G *et al. (*1995) Chronic infusion of a CRH1 receptor antisense oligodeoxynucleotide into the central nucleus of the amygdala reduced anxiety-related behavior in socially defeated rats. *Regul.Pept.* 59 (2):229-239.

59. Skutella T *et al. (*1994) Corticotropin-releasing hormone (CRH) antisense oligodeoxynucleotide induces anxiolytic effects in rat. *Neuroreport* 5 (16):2181-2185.

60. Skutella T *et al. (*1994) Corticotropin-releasing hormone (CRH) antisense oligodeoxynucleotide treatment attenuates social defeat-induced anxiety in rats. *Cell Mol.Neurobiol.* 14 (5):579-588.

61. Skutella T *et al. (*1998) Corticotropin-releasing hormone receptor (type I) antisense targeting reduces anxiety. *Neuroscience* 85 (3):795-805.

62. Smith GW *et al. (*1998) Corticotropin Releasing Factor Receptor 1-Deficient Mice Display Decreased Anxiety, Impaired Stress Response, and Aberrant Neuroendocrine Development. *Neuron* 20 (6):1093-1102.

63. Gulyas J *et al. (*1995) Potent, structurally constrained agonists and competitive antagonists of corticotropin-releasing factor. *Proc.Natl.Acad.Sci.U.S.A* 92 (23):10575-10579.

64. Contarino A *et al. (*1999) Reduced anxiety-like and cognitive performance in mice lacking the corticotropin-releasing factor receptor 1. *Brain Research* 835 (1):1-9.

65. Timpl P *et al. (*1998) Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. *Nat.Genet.* 19 (2):162-166.

66. von BU and Holsboer F (1988) Human corticotropin releasing hormone: clinical studies in patients with affective disorders, alcoholism, panic disorder and in normal controls. *Prog.Neuropsychopharmacol.Biol.Psychiatry* 12 Suppl:S165-S187.

67. Baldwin HA *et al. (*1991) CRF antagonist reverses the "anxiogenic" response to ethanol withdrawal in the rat. *Psychopharmacology (Berl)* 103 (2):227-232.

68. Heinrichs SC *et al. (*1992) Corticotropin-releasing factor antagonist reduces emotionality in socially defeated rats via direct neurotropic action. *Brain Res.* 581 (2):190-197.

69. Hikichi T *et al. (*2000) Suppression of conditioned fear by administration of CRF receptor antagonist CP-154,526. *Pharmacopsychiatry* 33 (5):189-193.

70. Koob GF (1996) Drug addiction: the yin and yang of hedonic homeostasis. *Neuron* 16 (5):893-896.

71. Lelas S *et al. (*2004) Anxiolytic-like effects of the corticotropin-releasing factor1 (CRF1) antagonist DMP904 [4-(3-pentylamino)-2,7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo-[1,5 -a]-pyrimidine] administered acutely or chronically at doses occupying central CRF1 receptors in rats. *J.Pharmacol.Exp.Ther.* 309 (1):293-302.

72. Lundkvist J *et al. (*1996) A non peptidic corticotropin releasing factor receptor antagonist attenuates fever and exhibits anxiolytic-like activity. *Eur.J.Pharmacol.* 309 (2):195-200.

73. Millan MJ *et al. (*2001) Anxiolytic properties of the selective, non-peptidergic CRF(1) antagonists, CP154,526 and DMP695: a comparison to other classes of anxiolytic agent. *Neuropsychopharmacology* 25 (4):585-600.

74. Radulovic J *et al. (*1999) Modulation of learning and anxiety by corticotropin-releasing factor (CRF) and stress: differential roles of CRF receptors 1 and 2. *J.Neurosci.* 19 (12):5016-5025.

75. Griebel G *et al. (*2002) 4-(2-Chloro-4-methoxy-5-methylphenyl)-N-[(1S)-2-cyclopropyl-1-(3-fluoro-4- methylphenyl)ethyl]5-methyl-N-(2-propynyl)-1, 3-thiazol-2-amine hydrochloride (SSR125543A), a potent and selective corticotrophin-releasing factor(1) receptor antagonist. II. Characterization in rodent models of stress-related disorders. *J.Pharmacol.Exp.Ther.* 301 (1):333-345.

76. Mansbach RS, Brooks EN, and Chen YL (1997) Antidepressant-like effects of CP-154,526, a selective CRF1 receptor antagonist. *Eur.J.Pharmacol.* 323 (1):21-26.

77. Ising M and Holsboer F (2007) CRH-sub-1 receptor antagonists for the treatment of depression and anxiety. *Exp.Clin.Psychopharmacol.* 15 (6):519-28.

78. Rosen A *et al. (*1992) Short-term restraint stress and s.c. saline injection alter the tissue levels of substance P and cholecystokinin in the peri-aqueductal grey and limbic regions of rat brain. *Acta Physiol Scand.* 146 (3):341-348.

79. Shirayama Y *et al. (*1996) Reduction of substance P after chronic antidepressants treatment in the striatum, substantia nigra and amygdala of the rat. *Brain Res.* 739 (1-2):70-78.

80. Brodin E *et al. (*1994) Effects of sequential removal of rats from a group cage, and of individual housing of rats, on substance P, cholecystokinin and somatostatin levels in the periaqueductal grey and limbic regions. *Neuropeptides* 26 (4):253-260.

81. Aguiar MS and Brandao ML (1996) Effects of microinjections of the neuropeptide substance P in the dorsal periaqueductal gray on the behaviour of rats in the plus-maze test. *Physiol Behav.* 60 (4):1183-1186.

82. Commons KG and Valentino RJ (2002) Cellular basis for the effects of substance P in the periaqueductal gray and dorsal raphe nucleus. *J.Comp Neurol.* 447 (1):82-97.

83. Rupniak NMJ *et al. (*2000) Pharmacological blockade or genetic deletion of substance P (NK1) receptors attenuates neonatal vocalisation in guinea-pigs and mice. *Neuropharmacology* 39 (8):1413-1421.

84. Teixeira RM *et al. (*1996) Effects of central administration of tachykinin receptor agonists and antagonists on plus-maze behavior in mice. *European Journal of Pharmacology* 311 (1):7-14.

85. Hasenohrl RU, Gerhardt P, and Huston JP (1990) Substance P enhancement of inhibitory avoidance learning: mediation by the N-terminal sequence. *Peptides* 11 (1):163-167.

86. Aguiar MS and Brandao ML (1994) Conditioned place aversion produced by microinjections of substance P into the periaqueductal gray of rats. *Behav.Pharmacol.* 5 (3):369-373.

87. Holzhauer-Oitzl MS, Hasenohrl R, and Huston JP (1988) Reinforcing properties of substance P in the region of the nucleus basalis magnocellularis in rats. *Neuropharmacology* 27 (7):749-756.

88. Santarelli L *et al. (*2001) Genetic and pharmacological disruption of neurokinin 1 receptor function decreases anxiety-related behaviors and increases serotonergic function. *Proc.Natl.Acad.Sci.U.S.A* 98 (4):1912-1917.

89. Rupniak NM *et al. (*2001) Comparison of the phenotype of NK1R-/- mice with pharmacological blockade of the substance P (NK1) receptor in assays for antidepressant and anxiolytic drugs. *Behav.Pharmacol.* 12 (6-7):497-508.

90. Ripley TL *et al. (*2002) Lack of self-administration and behavioural sensitisation to morphine, but not cocaine, in mice lacking NK1 receptors. Neuropharmacology 43 (8):1258-1268.

91. De Felipe C *et al. (*1998) Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. *Nature* 392 (6674):394-397.

92. Boyce S *et al. (*2001) Intra-amygdala injection of the substance P [NK(1) receptor] antagonist L-760735 inhibits neonatal vocalisations in guinea-pigs. *Neuropharmacology* 41 (1):130-137.

93. Cheeta S *et al. (*2001) Anxiolytic actions of the substance P (NK1) receptor antagonist L-760735 and the 5-HT1A agonist 8-OH-DPAT in the social interaction test in gerbils. *Brain Research* 915 (2):170-175.

94. File SE (1997) Anxiolytic action of a neurokinin1 receptor antagonist in the social interaction test. *Pharmacol.Biochem.Behav.* 58 (3):747-752.

95. Varty GB *et al. (*2002) The gerbil elevated plus-maze II: anxiolytic-like effects of selective neurokinin NK1 receptor antagonists. *Neuropsychopharmacology* 27 (3):371-379.

96. Shaikh MB, Steinberg A, and Siegel A (1993) Evidence that substance P is utilized in medial amygdaloid facilitation of defensive rage behavior in the cat. *Brain Res.* 625 (2):283-294.

97. Froger N *et al. (*2001) 5-hydroxytryptamine (5-HT)1A autoreceptor adaptive changes in substance P (neurokinin 1) receptor knock-out mice mimic antidepressant-induced desensitization. *J.Neurosci.* 21 (20):8188-8197.

98. Santarelli L *et al. (*2002) Behavioral and physiologic effects of genetic or pharmacologic inactivation of the substance P receptor (NK1). *J.Clin.Psychiatry* 63 Suppl 11:11-17.

99. Conley RK *et al. (*2002) Substance P (neurokinin 1) receptor antagonists enhance dorsal raphe neuronal activity. *J.Neurosci.* 22 (17):7730-7736.

100. Haddjeri N and Blier P (2001) Sustained blockade of neurokinin-1 receptors enhances serotonin neurotransmission. *Biol.Psychiatry* 50 (3):191-199.

101. Blier P *et al. (*2004) Impact of substance P receptor antagonism on the serotonin and norepinephrine systems: relevance to the antidepressant/anxiolytic response. *J.Psychiatry Neurosci.* 29 (3):208-218.

102. Blumberg S *et al. (*1980) Cleavage of substance P to an N-terminal tetrapeptide and a C-terminal heptapeptide by a post-proline Cleaving enzyme from bovine brain. *Brain Res.* 192 (2):477-486.

103. Hasenohrl RU *et al. (*1998) Anxiolytic-like action of neurokinin substance P administered systemically or into the nucleus basalis magnocellularis region. *Eur.J.Pharmacol.* 354 (2-3):123-133.

104. Mitrovic I and Napier TC (1996) Interactions between the mu opioid agonist DAMGO and substance P in regulation of the ventral pallidum. *Synapse* 23 (3):142-151.

105. Fehder WP *et al. (*1997) Substance P as an immune modulator of anxiety. *Neuroimmunomodulation.* 4 (1):42-48.

106. Stockmeier CA *et al. (*2002) Neurokinin-1 receptors are decreased in major depressive disorder. *Neuroreport* 13 (9):1223-1227.

107. Furmark T *et al. (*2005) Cerebral blood flow changes after treatment of social phobia with the neurokinin-1 antagonist GR205171, citalopram, or placebo. *Biol.Psychiatry* 58 (2):132-142.

108. Fyer AJ and Weissman MM (1999) Genetic linkage study of panic: clinical methodology and description of pedigrees. *Am.J.Med.Genet.* 88 (2):173-181.

109. Hamilton SP *et al. (*1999) Lack of Genetic Linkage or Association Between a Functional Serotonin Transporter Polymorphism and Panic Disorder. *Psychiatric Genetics* 9 (1):1-6.

110. Knowles JA *et al. (*1998) Results of a genome-wide genetic screen for panic disorder. *American Journal of Medical Genetics* 81 (2):139-147.

111. Chen X, Levine L, and Kwok PY (1999) Fluorescence polarization in homogeneous nucleic acid analysis. *Genome Res.* 9 (5):492-498.

112. Livak KJ (1999) Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genet Anal.* 14 (5-6):143-149.

113. Xiao M, Latif SM, and Kwok PY (2003) Kinetic FP-TDI assay for SNP allele frequency determination. *Biotechniques* 34 (1):190-197.

114. Greenberg DA, Abreu P, and Hodge SE (1998) The power to detect linkage in complex disease by means of simple LOD-score analyses. *Am.J.Hum.Genet.* 63 (3):870-879.

115. Hodge SE, Abreu PC, and Greenberg DA (1997) Magnitude of type I error when single-locus linkage analysis is maximized over models: a simulation study. *Am.J.Hum.Genet.* 60 (1):217-227.

116. Vieland VJ *et al. (*1996) New segregation analysis of panic disorder. *Am.J.Med.Genet.* 67 (2):147-153.

117. Cottingham RW, Jr., Idury RM, and Schaffer AA (1993) Faster sequential genetic linkage computations. *Am.J.Hum.Genet.* 53 (1):252-263.

118. Schaffer AA *et al. (*1994) Avoiding recomputation in linkage analysis. *Hum.Hered.* 44 (4):225-237.

119. Kruglyak L *et al. (*1996) Parametric and nonparametric linkage analysis: a unified multipoint approach. *Am.J.Hum.Genet.* 58 (6):1347-1363.

120. Horvath S, Xu X, and Laird NM (2001) The family based association test method: strategies for studying general genotype--phenotype associations. *Eur.J Hum Genet* 9 (4):301-306.

121. Laird NM, Horvath S, and Xu X (2000) Implementing a unified approach to family-based tests of association. *Genet.Epidemiol.* 19 Suppl 1:S36-S42.

122. Fyer AJ *et al. (*2006) A Third-Pass Genome Scan in Panic Disorder: Evidence for Multiple Susceptibility Loci. *Biol.Psychiatry* 60 (4):388-401.

123. Terwilliger JD and Ott J (1992) A haplotype-based 'haplotype relative risk' approach to detecting allelic associations. *Hum.Hered.* 42:337-346.

124. Risch N and Merikangas K (1996) The future of genetic studies of complex human diseases. *Science* 273 (5281):1516-1517.

125. Wada E *et al. (*1991) cDNA cloning, characterization, and brain region-specific expression of a neuromedin-B-preferring bombesin receptor. Neuron 6 (3):421-430.

126. Wada E *et al. (*1997) Generation and characterization of mice lacking gastrin-releasing peptide receptor. Biochem.Biophys.Res.Commun. 239 (1):28-33.

127. Sudakov SK *et al. (*2001) Differences in genetic predisposition to high anxiety in two inbred rat strains: role of substance P, diazepam binding inhibitor fragment and neuropeptide Y. *Psychopharmacology (Berl)* 154 (4):327-335.

128. Licinio J *et al. (*2004) Association of a corticotropin-releasing hormone receptor 1 haplotype and antidepressant treatment response in Mexican-Americans. *Mol.Psychiatry* 9 (12):1075-1082.

129. Keck ME *et al. (*2001) The anxiolytic effect of the CRH(1) receptor antagonist R121919 depends on innate emotionality in rats. *Eur.J.Neurosci.* 13 (2):373-380.

130. Wigger A *et al. (*2004) Alterations in central neuropeptide expression, release, and receptor binding in rats bred for high anxiety: critical role of vasopressin. *Neuropsychopharmacology*. 29 (1):1-14.

131. Treutlein J *et al. (*2006) Genetic association of the human corticotropin releasing hormone receptor 1 (CRHR1) with binge drinking and alcohol intake patterns in two independent samples. *Mol.Psychiatry* 11 (6):594-602.

132. Philibert RA *et al. (*2007) Transcriptional profiling of lymphoblast lines from subjects with panic disorder. *Am.J.Med.Genet.B Neuropsychiatr.Genet.* 144 (5):674-682.

133. Ishikawa-Brush Y *et al. (*1997) Autism and multiple exostoses associated with an X;8 translocation occurring within the GRPR gene and 3' to the SDC2 gene. *Hum.Mol.Genet.* 6 (8):1241-1250.

134. Shriver SP *et al. (*2000) Sex-specific expression of gastrin-releasing peptide receptor: relationship to smoking history and risk of lung cancer. *J.Natl.Cancer Inst.* 92 (1):24-33.

135. Moore JH (2005) A global view of epistasis. *Nat.Genet.* 37 (1):13-14.

136. Seckl JR *et al. (*1995) Placental 11 beta-hydroxysteroid dehydrogenase and the programming of hypertension. *J.Steroid Biochem.Mol.Biol.* 55 (5-6):447-455.

137. Welberg LA, Seckl JR, and Holmes MC (2000) Inhibition of 11beta-hydroxysteroid dehydrogenase, the foeto-placental barrier to maternal glucocorticoids, permanently programs amygdala GR mRNA expression and anxiety-like behaviour in the offspring. *Eur.J.Neurosci.* 12 (3):1047-1054.

138. Marchand JE, Sant GR, and Kream RM (1998) Increased expression of substance P receptor-encoding mRNA in bladder biopsies from patients with interstitial cystitis. *Br.J.Urol.* 81 (2):224-228.

139. Weissman, M. M. *et al.* Potential panic disorder syndrome: clinical and genetic linkage evidence. *Am. J. Med. Genet.* 96, 24-35 (2000).

140. Hamilton SP *et al. (*2003) Further genetic evidence for a panic disorder syndrome mapping to chromosome 13q. *Proc.Natl.Acad.Sci.U.S.A* 100 (5):2550-2555.

2.7. Figures and tables

Figure 2.1. Two point and multipoint linkage results for TACR1 in 120 PD pedigrees.

Phenotypic diagnostic categories for panic disorder (PD) are broad, intermediate, or narrow, for dominant or recessive mode of inheritance, given locus heterogeneity. LOD = logarithm of odds, SNPs =single nucleotide polymorphisms, Mb = megabase (May 2004, build 35, hg 17). Locus homogeneity scores were negative.

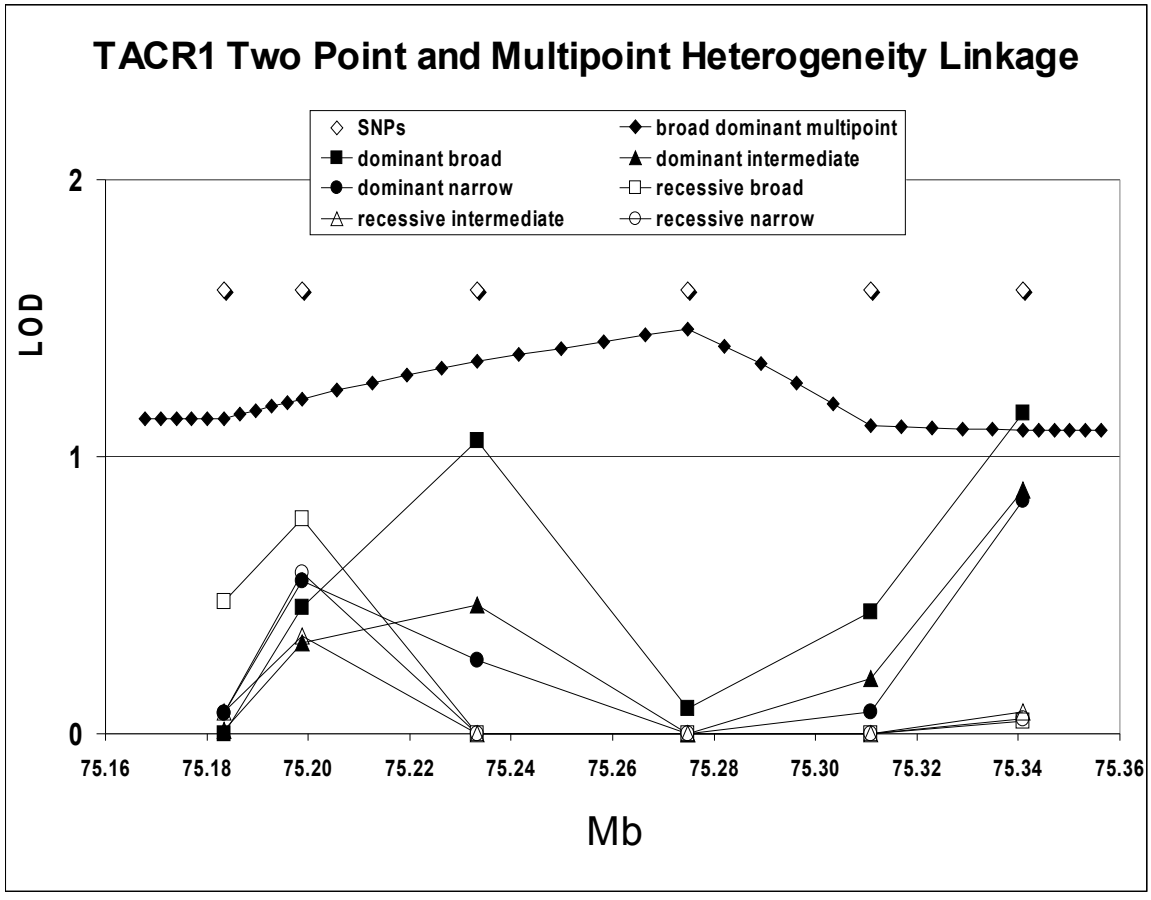

Figure 2.2. Multipoint heterogeneity and nonparametric linkage results for CRHR1 in 120 PD pedigrees.

Parametric logarithm of odds (LOD) scores are given for broad, intermediate, or narrow phenotypic diagnostic categories for panic disorder, given a dominant or recessive mode of inheritance, under the assumption of locus heterogeneity (Het). NPL = nonparametric linkage, SNPs =single nucleotide polymorphisms, Mb = megabase (May 2004, build 35, hg 17). Locus homogeneity scores were negative.

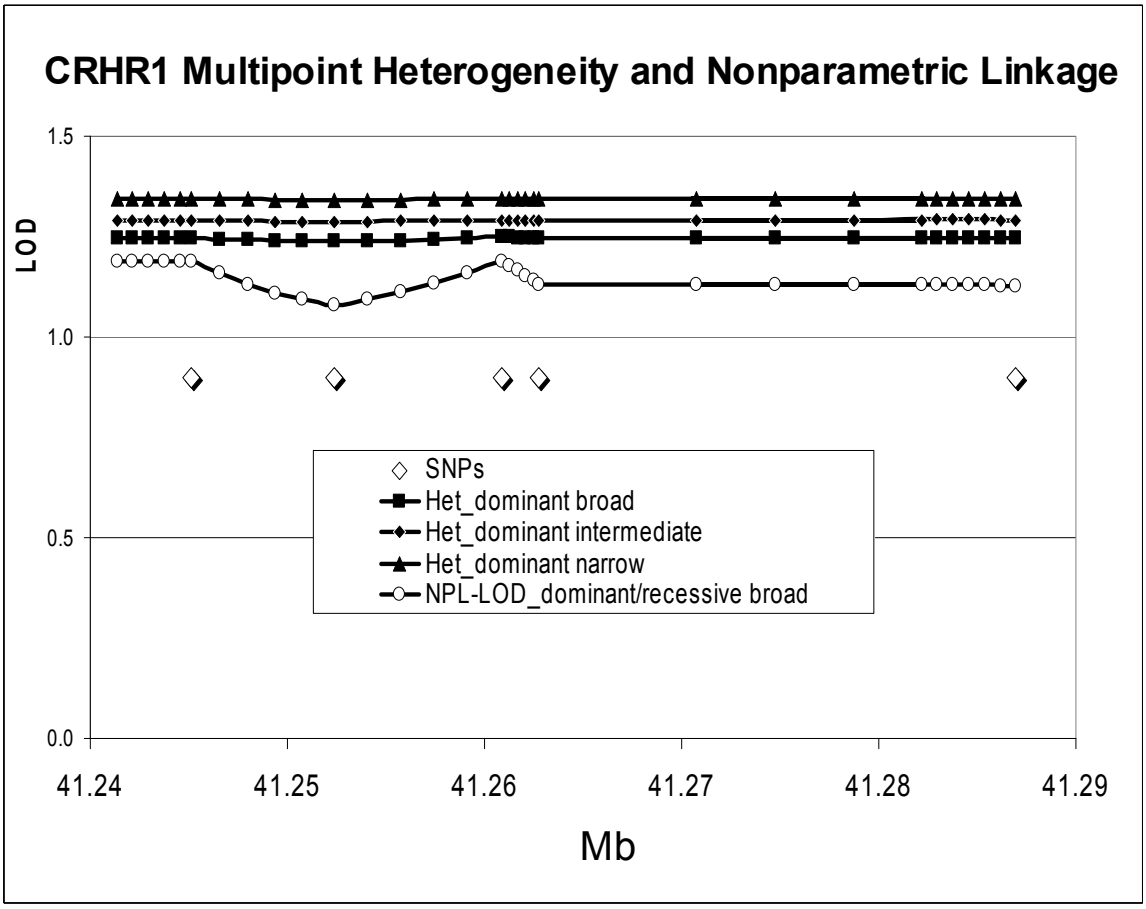

Table 2.1. Neuropeptide variant information

The minor allele frequency (MAF) for each single nucleotide polymorphism is given according to HapMap (H), Celera (C), and NCBI (N) databases. ND = no data, S>R = serine to arginine, I>I = isoleucine synonymous change, R>H = arginine to histidine, IMP5 = intramembrane protease 5 gene, TFBS = transcription factor binding site, UTR = untranslated region.

Marker ID	Variant	May 2004 NCBI position	Location	Alleles	MAF
GRP-1	rs755719	Chr18:55028469	5' of gene	A>G	0.45^{H}; 0.40^{N}
GRP-2	rs1517036	Chr18:55029342	5' of gene	C>T	0.13^{H}
GRP-3	rs1517035	Chr18:55029464	5' of gene	C>T	ND
GRP-4	rs4108697	Chr18:55034501	5' of gene	C>T	ND
GRP-5	rs1062557	Chr18:55038487	Exonic S>R	A>C	0.20^{H}; 0.10^{N}
GRP-6	rs936431	Chr18:55039736	Intronic	A>C	0.17^{H}; 0.17^{C}
GRPR-1	rs4084832	ChrX:15895480	5' of gene	C>T	ND
GRPR-2	rs2353577	ChrX:15926919	Intronic	C>T	0.23^{H}
GRPR-3	rs4986945	ChrX:15928124	Exonic S>S	C>T	0.23^{H}
GRPR-4	rs4986946	ChrX:15928334	Exonic I>I	C>T	0.37^{H}
GRPR-5	rs3747411	ChrX:15930734	3'UTR; TFBS	A>T	0.18^{H}
GRPR-6	rs2353576	ChrX:15931617	3' of gene	G>T	0.46^{H}
CRHR1-1	rs242924	Chr17:41241147	Intronic	G>T	0.45^{H}; 0.38^{C}
CRHR1-2	rs242940	Chr17:41248380	Intronic	A>G	0.43^{C}
CRHR1-3	rs173365	Chr17:41256855	Intronic	A>G	0.40^{H}; 0.49^{C}
CRHR1-4	rs1396862	Chr17:41258778	Intronic	A>G	0.20^{H}; 0.28^{C}
CRHR1-5	rs242944	Chr17:41278960	3' of CRHR1; Exonic R>H in gene IMP5	A>G	0.40^{H}; 0.48^{C}
TACR1-1	rs754978	Chr2:75183451	3' of gene	A>C	0.43^{H}; 0.44^{C}
TACR1-2	rs4439987	Chr2:75198761	Intronic	A>G	0.49^{H}; 0.45^{C}
TACR1-3	rs3755460	Chr2:75233290	Intronic	A>G	0.39^{C}
TACR1-4	rs741418	Chr2:75274841	Intronic	A>G	0.41^{H}; 0.40^{C}
TACR1-5	rs2058860	Chr2:75310956	Intronic	C>T	0.45^{C}
TACR1-6	rs1477157	Chr2:75340883	5' of gene	A>G	0.45^{H}; 0.45^{C}

Table 2.2. Allelic association ($p \leq 0.05$) of neuropeptidergic variants

Nominally significant allelic associations are given for narrow, intermediate and broad diagnostic categories for panic disorder using the family-based association test. NS = non-significant.

Marker ID	SNP	*p*-values		
		Narrow	Intermediate	Broad
GRP-3	rs1517035	0.05	0.02	NS
GRP-4	rs4108697	0.02	0.03	NS
TACR1-2	rs4439987	0.05	0.02	0.04

3. Chapter Three. The contribution of the chr15q12 $GABR_A$ genes to PD*

3.1. Introduction

Pharmacological, ethological, and clinical evidence implicates the gamma-aminobutyric acid (GABA) type A receptor ($GABR_A$) in the pathogenesis of PD. Dysregulation of the GABAergic system is hypothesized to underlie PD pathology, according to observed phenotypes resulting from $GABR_A$-targeting compounds and $GABR_A$ gene mutation, as well as trait differences in GABAergic tone in subjects with PD. Supporting the biological rationale for the $GABR_A$ genes as candidates for PD, we previously revealed positional evidence for linkage to $GABR_A$ genes on chromosome 15q12 in a gene-agnostic scan of the genome in 120 Caucasian, multiplex PD pedigrees[1], which was sustained in finemapping efforts using eight microsatellite markers in the chr15q0-13.1 region of interest (0-19.3 cM). A mutation screen in a subset of probands revealed a number of potentially functionally relevant variants in the beta 3 (GABRB3) and alpha 5 (GABRA5) subunit genes. Additionally, we characterized the heritable expression of ribonucleic acid (RNA) as a biomarker of GABAergic regulation in thirty-five Caucasian controls. Our combined results reveal evidence for linkage and association, as well as novel identified variants in PD, and heritable expression patterns in GABRA5 and/or GABRB3.

* The material in this chapter is in preparation for publication (Hodges LM *et al*.).

3.2. Background

Prior to the aims detailed in this thesis, previous work to this thesis showed positional evidence for linkage in Caucasian, multiplex PD pedigrees to a cluster of $GABR_A$ genes at chr15q12[1-3], which led to the investigation the beta 3 (GABRB3) and alpha 5 (GABRA5) subunits as candidate genes for PD. Two other genome scans by other investigators[4,5] plus a scan of PD comorbid with agoraphobia[6], in which chromosome 15 was interrrogated, failed to show genome-wide level of suggestive linkage to PD[1]. However, previously a genome scan using twenty-three Caucasian, multiplex PD pedigrees showed nominal linkage to chr15q11-14 (LOD>1.7)[2]. In sixty PD pedigrees overlapping with the earlier study a separate scan for syndromic features revealed a LOD-transformed, multipoint NPL score of 3.0[3]. And augmenting the sample to 120 PD pedigrees revealed suggestive linkage (LOD=1.9-2.0) to marker D15S822 in chr15q12 (12cM) in a convergence of analytical approaches[1], including multipoint non-parametric analysis (LOD=2.56)[7]. Thus, there is sufficient positional evidence in support of the involvement of the GABRB3 and GABA5 genes in PD.

3.2.1. $GABR_A$ function, distribution, and gene structure

Membrane-bound $GABR_A$s are the principal molecular mediators of neuronal excitability in the brain[8-11], able to integrate signals from the central and peripheral nervous systems[12] (CNS, PNS), where they transduce somato-behavioral “fight or flight” stress responses[13], psychogenic anxiety, vigilance, seizures, muscle tension[14], and nociception[15]. $GABR_A$s are distributed

throughout the CNS, neuroendocrine thyroid[16,17], and ganglia relays, including the thalamocortical[18], hypothalamic-pituitary adrenal (HPA)[48], somatic and central sensory structures[19-21], such as the olfactory bulb, bladder[22], skin[23], viscera, intestine[16,17,21], caridorespiratory[24], and other autonomic axes, interconnected with limbic amygdala, hippocampus[25], hypothalamus, thalamus and prefrontal cortex regions.

$GABR_A$-potentiating ligands, such as GABA, neuroactive steroids, benzodiazepines, alcohol, and barbiturates exert anxiolytic, sedative, analgesic, anticonvulsant, and muscle-relaxing effects, with a reduction of autonomic system outflow[26]. Alternatively, decreased $GABR_A$ function, achieved by $GABR_A$ antagonists[27] or physiological signals for metabolic or respiratory demand[28,29], evoke autonomic excitation[27], arousal, convulsions, pain hypersensitivity[31], and panic attacks.

$GABR_A$s come from a superfamily of ligand-gated amino acid channels, including the glutamate and nicotinic receptors, and are characterized by structural and sequence homology. All superfamily members form multimeric complexes composed of five receptor subunits, forming a ligand-gated pore at synaptic and extrasynaptic membranes. Binding of $GABR_A$ ligands translate into membrane potentials by alosterically permitting monovalent anions (chloride, bicarbonate[30,32-34], thiocyanate[35,36]) to influx or efflux, depending upon the electrochemical gradient established by other passive and active transport systems[37]. $GABR_A$s convey both fast and slow kinetics for different effects[38]. Transient, multiphasic currents recruit pathways for acute neurobehavioral and

somatic responses, while tonic[39-46] signalling allows for neuroplasticity[47], for example, that are important in long-term learning and memory. $GABR_A$ dysregulation can lead to paroxsymal symptoms and/or changes in homeostasis that are pathological, which correspond to the paroxysmal panic attacks and chronic anticipatory anxiety characterizing PD.

A family of $GABR_A$ genes encode eighteen known human receptor subunits[50], divided into eight classes (α1–6, β1–3, γ1–3, δ, ρ1-2[51], π, ε and θ)[52] based on sequence homology[49]. The extant structure of $GABR_A$ gene isofoms reflects evolutionary duplication, translocation and expansion events[53,54] that dispersed the genes among six chromosomes (X, 5, 15, 4, 6, 1), typically in gene clusters that aid in the putative coordinate expression of multiple subunits for efficient coassambly into the pentameric channel complex. In part, due to abundant stretches of repeat nucleotides and high GC content of the chr15q12 $GABR_A$ genes, *de novo* microdeletions, as well as duplications, are relatively common in the population, as seen in the neurodevelopmental disorders, Prader-Willi syndrome (PWS) and Angelman syndrome (AS)[55,56].

$GABR_A$ genes each contain extracellular amino and carboxy terminals, four transmembrane domains, and two intracellular loops. The sequence for the second and largest intracellular loop (IC2) contains the greatest nucleotide diversity among the subunit isoforms, as well as a highly conserved consensus sequence for intracellular modulation of $GABR_A$ activity[57].

3.2.2. Low $GABR_A$ mutability

Important parts of the $GABR_A$ genes exhibit low mutability and nucleotide diversity due to critical structural and functional constraints, for extracellular ligand binding[8,58-63], permeant ion selectivity (an exclusive property of GABRB3)[64], subunit-subunit coassembly[65,66], and subcellular trafficking[47,67]. For example, nucleotides unique to beta subunits[68] are responsible for insertion of the oligomeric receptor complex into the membrane through interaction with intracellular proteins[67]. Four amino-terminal signal sequence residues in GABRB3 have been found to promote cell surface function[69]. A single residue in GABRA5 causes decreased expression with chronic diazepam administration, and is responsible for the development of tolerance to its sedative effects[70]. Residues at the interface of alpha and beta subunits, and alpha and gamma subunits allow for GABA[37] and BZ[71] binding, respectively. Critical cytoplasmic residues are relevant for long-lasting $GABR_A$ effects mediated by intracellular enzymes. Intracellular kinases and phosphatases[72-75] interact with a consensus sites in IC2 for neuroplasticity[76-78]. Phosphorylation of conserved tyrosine and serine residues in GABRB3 causes upregulation of activity[47] by enhancing the efficacy of inhibitory post-synaptic currents (IPSCs) and receptor density at the membrane[79]. Kinase activity also enhances the actions of BZs, barbiturates, and neurosteroids, and has been shown to specifically modulate GABRA5-directed tonic inhibition[47].

Given the multiple functional constraints of $GABR_A$s, few mutations resulting in a residue change have been found to be associated with neurological phenotypes[80]. However, a number of non-coding SNPs and microsatellite

markers for GABRB3 and GABRA5 have been linked or associated with neuropsychatric disorders, such as epilepsy[81], bipolar disorder[82,83], depression[84], schizophrenia[85], and autism[86-90]. One such example is a promoter variant associated with childhood absence epilepsy that alters GABRB3 expression by disrupting a transcription factor binding site[81]. Our mutation screen of GABRB3 and GABRA5 in ninety-six PD probands identified only one low frequency synonymous SNP in GABRA5. However, given the relatively low nucleotide diversity observed among the $GABR_A$ isoforms, there may be regulatory significance to the seven novel SNPs that we found in untranslated regions (UTRs) for the two genes.

3.2.3. Evidence for anxiolysis via $GABR_A$ remodeling

The molecular actions of various PD drugs inform the role of the GABAergic system in PD. Treatment of PD has evolved with improved drug development in order to acheive greater molecular target selectivity, optimized drug response, and minimized side effects. Prior to the development of BZs, PD treatment included the tricyclic antidepressant (TCA), imipramine, with broad effects on the serotonergic, noradrenergic, dopaminergic and opiate systems. Librium in 1960, followed by the prototypical 1,4-BZ, diazepam (Valium) in 1963, replaced TCAs as treatment for PD. With further drug development, BZs became a second-line drug, chiefly due to the development of tolerance and potential for dependence/abuse with chronic use, although other 1,4-BZ class drugs, alprazolam (Xanax), lorazepam (Ativan) and clonazepam, are still used as

treatment adjuncts and in refractory cases. Current first-line treatments include the selective serotonin reuptake inhibitors (SSRIs), monoamine oxidase inhibitors (MAOI: phenelzine), and other mixed target drugs (e.g. serotonin-noradrenergic reuptake inhibitor, venlafaxine (Effexor)[91]), which all enhance monamine levels. In contrast to the BZs, however, the newer generation therapeutics show a much delayed drug response on the order of weeks. Consequently, concommitant BZ administration is often prescribed to control acute symptoms while the pharmacodynamic response accrues for the non-$GABR_A$-targeting drugs. Related to this pharmacological difference, GABAergic mechanisms are thought to underlie the anxiolytic effects of many PD drugs, including those that do not directly target $GABR_A$s. For example, transcriptional profiles of MAOI and TCA drugs show an upregulation of $GABR_A$ genes in a time frame commensurate with the delayed onset of their therapeutic effects. Meanwhile, acute SSRI and MAOI administration normalizes levels of $GABR_A$ ligands, such as GABA[92-94] and neurosteroids[95]; and pretreatment with TCA can block $GABR_A$ antagonist-induced anxiogenic responses[96]. These data suggest that $GABR_A$ remodeling is a key mechanism for the long-term efficacy of TCA, MAOI, and SSRI anxiolytics[97], accompanied by rapid changes in GABAergic signaling.

3.2.4. Low GABAergic tone in PD recapitulated in genetic and pharmacological manipulation

Low GABAergic tone promotes anxiety and serves as a putative endophenotype for PD. Pharmacological manipulation of upstream GABAergic

pathway genes is consistent with downstream alterations in $GABR_A$-mediated signalling. Inhibition of the proteins responsible for GABA degradation (GABA transaminase; GABA-T) and reuptake (neuronal and glial GABA transporter; GATs), have anxiolytic effects by enhancing GABAergic tone. For example, the anti-ischemic tiagabine[98] (GAT inhibitor), and valproate[99,100] or vigabatrin[101,102] (GABA-T inhibitors) are among drugs that treat PD, anxiety, and block the experimental provocation of panic attacks. Likewise, low GABAergic tone, achieved by mutation of GABRB3 or GABRA5, shows an anxiogenic response. Targetted deletion of GABRB3 in mice and low GABAergic tone reveal increased cardiorespiratory stress responses[103], spontaneous fear behavior[103-107], avoidance behavior[108], risk assessment behavior[107], hypersensitivity to sensory stimuli[15,106,108], and altered fear conditioning[108], among other developmental (cleft palate[106,109]; noradrenergic neurogenesis[107,110]) behavioral, and phenotypic effects, including hyperactivity, EEG abnormalities[111] and seizures[106,108]. Disruption of GABRA5 results in less dramatic neurological deficits[112]. However, GABRA5 deficiency by gene deletion or point mutation is associated with altered BZ pharmacodynamics[70], hippocampal disinhibition[113,114], and selective learning deficits, such as altered conditioned aversive learning[58,115], and reduced inhibition of intrusive or irrelevant stimuli (prepulse inhibition, PPI)[116].

PD subjects exhibit less habituation to startle, and significantly reduced PPI[117]. Mechanistically, this may be explained by the actions of bicarbonate, which is a permeant ion of $GABR_A$ channels, causing *excitatory*, $GABR_A$–mediated long-term potentiation of habituation responses and neuroplasticity[33].

Alprazolam shows significant amelioration of auditory startle, as measured by decreases in anxiety and skin conductance (an index of autonomic output) in subjects with PD[118].

In the special case of the $GABR_A$ genes, whose function is dependent upon the proper expression and co-assembly with other subunits genes, aberrations in one subunit requisitely affect the function of the pentameric complex. Their interdependence, for example, has been demonstrated with *in vitro* recombinant gene studies, as well as in developmental studies. Switches in subunit expression[19] can lead to stoichiometric shifts in the combinatorial subunit composition of $GABR_A$s, which is reflected in changes in membrane expression[67], ligand sensitivity[8], and related neurobehavioral effects. Thus, changes in either or both the GABRB3 or the GABRA5 subunits should have highly integrated effects in GABAergic signalling, especially in regions of their coexpression. Thus, gene-gene interactions are the rule, rather than the exception in this case.

3.2.5. GABAergic drugs highlight $GABR_A$-mediated syndromes and comorbidities in PD

GABAergic dysfunction, which is implicated in PD pathogenesis, may also manifest in syndromic features and comorbidities seen in the same PD proband. GABAergic drugs accordingly treat alcohol and substance abuse, migraine, thyroid, bladder, and intestinal problems, obsessive-compulsive disorder (OCD), and bipolar disorder (BP), in addition to PD. For example, BZs, such as

diazepam, have treat alcohol withdrawal syndrome and opiate abuse; and other $GABR_A$-potentiating drugs (valproate, gabapentin, vigabatrin) aid in alcohol[125] and substance abuse[244]. Diazepam (with high affinity for GABRA5) and alprazolam also treat irritable bowel syndrome (IBS), interstitial cystitis (IC), and downregulate thyroid hormone signalling[119]. Migraine is treated with gabapentin (GABA analogue), valproate[120], and prophylatically with $GABR_A$ agonists[121]. Gabapentin (off-label) and valproate are indicated for BP[122]. Gabapentin is also used to treat IC[123,124] and OCD[102]. Thus, GABAergic pharmacology elucidates pleiotropic effects that parallel pervasive syndromic features and comorbidities in PD.

The $GABR_A$ genes have important developmental functions. Congenital $GABR_A$ deficiencies in animals and in humans can be recapitulated by GABAergic drug exposure during development. For example, vigabatrin causes tetratogenic cleft palate. Cleft palate is a trait of the murine GABRB3 knockout[106,109], and patients with Angelman syndrome (AS) and Prader Willi syndrome (PWS) who carry microdeletion of chr15q11-13[56]. Other $GABR_A$–mediated disturbances seen in AS and PWS, are also seen in PD. Similarities to PD include abnormal electroencephalography (EEG), tremulousness, hyperactivity, and behavioral paroxysms seen in AS; and hypotonia, hypothyroidism[126], obsessive behavior, learning deficits, and anxiety observed in PWS. Thus, low GABAergic tone causes common effects in AS, PWS, and PD.

3.2.6. $GABR_A$ physiology reflects PD symptoms, comorbidity, and syndromic features

Paroxysmal dyspnea (shortness of breath) and tachycardia (pounding heart, or increased heart rate) are common somatic symptoms of a panic attack. Homeostatic signals for hypoxia, hyperventilation, and increased metabolic demand are detected by changes in endogenous levels of carbon dioxide (CO_2), lactate, and its metabolite, bicarbonate, which can trigger reflex tachycardia. In controlled settings, the administration of exogenous "challenge agents", like CO_2 inhalation or sodium lactate infusion, provokes panic attacks in PD subjects at a significantly higher rate than in healthy controls. This characteristic hypersensitivity is also observed in the first degree relatives of probands[127,128], although not in all studies[129]. Mechanistically, subjects with PD exhibit exaggerated metabolic response to alkalosis, and delayed recovery to normal arterial CO_2 pressure (normocapnia) after hyperventilation, exercise, and lactate infusion[130]. Klein[131] proposed the "false suffocation alarm" hypothesis for PD to explain the observed hyperexcitation of autonomic and neuroendocrine cascades in response to these physiological cues.

$GABR_A$-mediated mechanisms are implicated in studies demonstrating precipitation of somatic and behavioral stress symptoms by $GABR_A$ inverse agonists[132]. Wildtype animals are also rendered sensitive to lactate-induced anxiogenic and sympathoexcitatory responses by administration of $GABR_A$ antagonists in the amygdala[133] and hypothalamus[104,134]. As well, cardiorespiratory and anxiogenic responses (e.g. increased heart rate, blood pressure, respiration) to psychogenic stressors, sympathetic excitation, and provocation agents, can be abolished or attenuated by anxiolytic $GABR_A$

agonists, such as diazepam[135], alprazolam[136], clonazepam[96], muscimol[134], and the beta carboline abecarnil[136]. BZs are generally prescribed for patients with hyperventilation syndrome[137]. The 1,4-BZ agonist, midazolam (Versed), which has high affinity for GABRA5, is used for rapid anxiolysis with concommitant depression of carotid body chemoreceptor activity to counteract the depolarizing effects of hypoxia during respiratory distress[138]. In summary, pharmacological $GABR_A$ blockade[27,29,136] induces psychogenic and hypoxic stress, which is reversed by $GABR_A$ agonists.

Migraine is a frequent syndromic feature of PD[139]; and the same $GABR_A$-targetting drugs are used to treat PD and migraine, as discussed earlier. A possible mechanistic explanation for the apparent pleiotropic $GABR_A$ effects underlying PD and migraine may result from disturbances in $GABR_A$'s role in maintaining metabolic homeostasis. $GABR_A$-mediated inhibitory effects are sensitive to metabolic demand, and appear to be dependent on GABR expression, as illustrated by comparison of different species. $GABR_A$ inhibitory activity is overridden by metabolic demand. However, an interesting finding in freshwater turtles demonstrates a species-specific mechanism for underwater survival in response to prolonged anoxia, by sustaining an upregulation of $GABR_A$ (and inhibitory GABAergic actions) despite high metabolic demand. In this way the turtle can maintain a hypometabolic state at least for twenty-four hours[245]. The rat also initially upregulates GABAergic tone in response to hypoxia, but subsequently loses GABAergic inhibition, which leads to a compensatory increase in arterial cerebral blood flow to restore the normoxic

state. Likewise, in humans, the cardiorespiratory response to dyspnea in panic attacks, as well as to $GABR_A$ antagonism, causes hypertension[27] and increased blood flow. This undoing of the $GABR_A$ inhibitory activity has shared mechanistic and genetic overlap with migraine. Paroxysmal migraines can occur with an oxidative metabolic trigger that results in cortical spreading depression (a wave of cellular ischemia and depolarization with vasodilation, followed by vasoconstriction). $GABR_A$-potentiating drugs counter these effects. The BZ, lorazepam, treats especially severe migraines and acutely reduces cortical blood flow[140]. Other antimigraine drugs include GABAergic-potentiating valproate and gabapentin, as well as $GABR_A$ agonists[121] for prophylatic use.

Multiple symptoms are observed during both migraine attacks and panic attacks alike, including disruption of metabolic homeostasis, EEG abnormalities, decreased vagal tone, and abnormal levels of GABA and lactate, as measured in cerebral spinal fluid[141]. Similar to PD, migraine has also been genetically linked to GABRB3 (max LOD=5.6; multipoint LOD=6.5)[142], although yet unreplicated[143,144]. PD and migraine also share similar gender-specific prevalences[145] with hormonal implications[146]. For example, protective neuroactive steroids, such as progesterone and its derivatives, are potent allosteric modulators of $GABR_A$s as well as transcriptional regulators of $GABR_A$ gene expression[147,242]. Their effects extend to dampening symptoms of panic attack (see section 1.1.3.) and countering the neurotoxic effects of ischemia or chronic hyperventilation[148].

Putative pleiotropic effects of underlying $GABR_A$ dysregulation in thyroid

problems are another example to reinforce the possible biological underpinnings of PD. Thyroid dysfunction, which is a frequent syndromic feature of PD[139,149] (usually hypothyroidism), and observed in PWS subjects with chr15q11-13 microdeletions, is mechanistically linked to the multiple effects of $GABR_A$ dysregulation and psychiatric disorders[150]. Hyperthyroidism enduces HPA axis activity, hyperactivity, and increases oxygen consumption; while hypothyroidism shows contradicting excitatory and depressing effects during development versus adulthood, respectively[150]. Thiocyanate is a permeant ion of $GABR_A$ channels, with powerful antithyroid activity, particularly in subclinical and overt hypothyroidism. It inhibits thyroid hormone[151], which in turn inhibits $GABR_A$ actions[152], and is also shown to alter binding affinities of $GABA_A$ antagonists and agonists[153]. Thiocyanate can be found in dietary and toxic xenobiotics, and is a detoxification product of cyanide, which is contained in cigarettes[154]. Relatedly, smoking is thought to be a risk factor for PD[155]; while nicotine plus the BZ, midazolam, synergistically enhances GABRA5-mediated IPSCs[156]. Thus, $GABA_A$ mechanisms impinge on psychiatric and somatic effects of thyroid hormone dysregulation and related mechanisms. Experimentally, acute administration of the BZ, diazepam, is shown to reduce thyroid activity and a reduction in thyroid receptor density in the CNS and liver[119]. Thus, there is a potential mechanistic overlap in $GABA_A$–mediated thyroid and psychiatric health. Furthermore, thyroid homeostasis, which can be altered in at least by environmental factors, provides potential non-genetic, epigenetic and/or genetic contributions to PD via $GABR_A$ actions.

3.2.7. Subunit-specific kinetics and phenotypes of GABRB3 and GABRA5

Complex $GABR_A$ kinetics yield a range of effects, depending upon subunit-specific, neuroanatomical, and subcellular expression. GABRB3 is more promiscuously distributed than GABRA5 throughout the brain[157] in both extrasynaptic[158] and synaptic sites[159]. However, GABRB3 colocalizes with GABRA5 in granule cells[19] and the multiple pyramidal hippocampal cells[160] they innervate[161]. Their respective kinetic effects can be elucidated by subunit-specific GABAergic ligands. For example, GABRA5 subunit exerts *tonic* inhibitory effects, primarily (although not exclusively[162,163]) at extrasynaptic sites[58] (soma, axons, base and spines of dendrites) in the hippocampus[37,162,164], where low micromolar or submicromolar[40] ambient concentrations of GABA exist. Increasing extracellular GABA with GAT antagonists enhances tonic inhibition at high affinity extrasynaptic $GABR_A$s[46], which do not become desensitized to ligands. This change in ambient GABA "spillover" does not influence phasic neurotransmission conveyed by fast IPSCs[219]. Likewise, the 1,4-BZ agonist, midazolam, which is highly selective for GABRA5, increases *tonic* current in a functionally and pharmacologically distinct way from synaptic BZ effects[165]. In contrast, at the synaptic cleft, where high concentrations of vesicular-released GABA exerts quantally discrete pulses of phasic inhibition by IPSCs, the GABRA5-insensitive[243] BZ agonist, zolpidem (Ambien), causes a 66% increased *phasic* inhibition by prolonging the decay of IPSCs. However, by using alternative $GABR_A$ antagonists, both the tonic and phasic inhibitory currents can be blocked[47,166]. Thus, the selectivity of ligand affinity for specific $GABR_A$s, and

their cellular location manifest different kinetic profiles with distinct neurbehavioral outcomes. These differences may be important in elucidating the relative genetic and biological contribution of the chr15q12 genes to PD, since their differential effects can lead to distinct mechanistic and neurobehavioral outcomes.

3.2.8. Trait GABAergic differences in subjects with PD and animal models of anxiety

Inherent trait-like GABAergic differences are observed in subjects with PD. Probands show reduced cortical GABA levels[167], and BZ binding[168-174] at baseline as well as during spontaneous[171], or provoked panic attacks[171,175,177]. Compared to controls, BZ administration is associated with attenuated GABAergic response in PD patients[178], and blunted behavioral sensitivity[176] in animal models of anxiety. These findings further support the idea of an overall decrease in GABAergic flux in PD. Further evidence extends to differences in endogenous neuroactive steroids derived from progesterone that are potent nanomolar ligands of $GABR_A$s, binding at two specific sites on the receptor[60]. Subjects with PD exhibit a significant disequilibrium toward the $GABR_A$-potentiating steroids at baseline, compared to controls and patients with depression, which is thought to reflect a compensatory mechanism for low GABAergic tone[181]. Provocation of panic attacks by challenge agents in PD patients causes a dramatic shift in the composition of the 3-alpha-reduced pregnane steroids. During induced panic attacks, there is a 5-fold increase in

$GABR_A$-antagonizing isopregnanolone (3β,5α-THP), with a concomitant decrease in the $GABR_A$-potentiating metabolites, allopregnanolone (3α,5α-THP) and pregnanolone (3α,5β-THP)[177]. In contrast, a shift toward $GABR_A$-potentiaing steroids usually occurs during stress[180], pregnancy[183], and acute ethanol consumption[184] in humans, and in response to CO_2 challenge[180] or stress testing paradigms[179] in wildtype animals. Thus, neurosteroidal modulation of $GABR_A$ genes may factor into the complex etiology of PD.

Lastly, PD subjects exhibit trait brain activity differences in "fear network"[103] regions associated with altered $GABR_A$ BZ binding. Provocation agents and states of anticipatory anxiety in PD patients show region-specific activity differences (f.g. amygdala, insula, hypothalamus), compared to controls[185]. Subjects with PD exhibit baseline increases in incidental and specific rythmic wave activity using EEG, MRI, and computerized tomography, which measures the summation of post-synaptic conductances across regions of the brain[186-188]. $GABR_A$-mediated EEG abnormalities are a putative endophenotype of PD. As well, imaging studies have delineated differences between autonomic fear processes and cortical ones using skin conductance as an index of autonomic excitation. Functional magnetic resonance imaging (fMRI) demonstrates that visceral, subjective fear processing, activates an amygdala-medial frontal network with increased skin conductance, while contextual fear engages a hippocampus-lateral frontal network alone[189]. Measurements in subjects with PD shows a prolonged skin conductance in fear conditioning during extinction[190], and an overall smaller variability in skin conductance with

spontaneous[191] or CO_2–induced panic attacks[192]. Similarly, PD subjects exhibit decreased variability in heart rate and vagal tone, which reflects a physiological vulnerability to stress[193]. Together these data suggest a reduced ability to buffer autonomic responses in PD[191], all implicated in $GABR_A$ dysfunction.

3.2.9. Gender-specific phenotypes, $GABR_A$ mechanisms, and linkage to PD

Gender differences in PD symptoms, biometric indices, and $GABR_A$-mediated phenotypes[115,174] may contribute to the gender-specific prevalence rates of PD. For example, males with PD exhibit higher hematological indicators of respiratory distress, similar to that of patients with chronic obstructive pulmonary disease and inhabitants of high altitudes[194], which simultates the effect of chronic hypoxia. However, in CO_2 provocation studies, females with PD exhibit greater increases in respiratory rate[195] relative to males, who exhibit more somatic symptoms[196]. Studies in rats, comparing the influence of gender-specific hormones, reveal a female-specific diminution of hormones responsible for $GABR_A$-mediated tolerance to chronic diazepam administration, due to pregnane steroid-mediated reduction in cortical BZ binding[197]. A large body of work supports the hypothesis of a gender-specific hormonal contribution to anxiety; and GABAergic neurotransmission is tightly regulated by the menstrual cycle[198]. Progesterone metabolite synthesis is sensitive to fluctuations in estrogen[199], and the allopregnanolone (3α,5α-THP) isoform is shown to upregulate $GABR_A$ expression in the dentate gyrus of the hippocampus to enhance tonic inhibition[200].

During the mid-luteal menstrual phase, the GABAergic system is upregulated, when progesterone and estrogen levels are high, and downregulated in the late-luteal and early follicular phases with autonomic upregulation[201,236]. Females with PD exhibit greater skin conductance response to anxiogenic stimuli during the premenstrual phase[201]. Females with PD with agoraphobia exhibit greater severity of symptoms[202,203], and a 3-fold greater rate of relapse[204] in PD compared to males. Females have 1.9 to 3.7-fold greater prevalence of PD than males, depending upon age (see gender section 3.2.8.)[205,206]. Thus, hormonal status plays an important role in PD. The linkage data from our initial genome scan revealed a gender differences for the microsatellite marker, D15S822 at 12cM, which gave an HLOD (locus heterogeneity logrithm of odds) of 2.0 (given broad PD phenotype and recessive genetic model) for the combined sample. When parsed for gender, female probands gave HLOD scores of 1.3, 1.1, and 0.9 for narrow, intermediate, and broad phenotypic definitions for PD, respectively, given a recessive genetic model, versus HLODs of 0.0, 0.1, and 0.4 for corresponding male probands.

3.2.10. Environmental and psychological factors in $GABR_A$ dysfunction

$GABR_A$ activity is also influenced by exogenous ligands such as insecticide, anthelmintic (avermectin, ivermectin) and antibiotic (Ciprofloxacin[207]) antagonists, as well as agonists like dietary flavonoids[208], barbiturates, alcohol[209], and smoking. Sustained xenobiotic exposure is shown to alter $GABR_A$ subunit distribution and activity in animal studies. For example, chronic ethanol exposure

selectively upregulates GABRA5 expression in the hippocampus, and downregulates it in the corticocerebrum[209]. Exogenous $GABR_A$ substrates can also cause transgenerational, epigenetic effects that are transmitted from parent to child. For example, chronic exposure to stress or ethanol acts as a neurobehavioral teratogen in $GABR_A$ remodelling[210,212].

Remodelling of the GABAergic system also results from psychological experiences. For example, chronic exposure to the stress hormone corticosterone (cortisol in humans) is shown to increase GABRB3 expression in the hippocampus[212], which is responsible for the development of contextual fear conditioning by long-term potentiation (LTP)[213]. In animal modelss of anxiety, chronic stress exposure decreases BZ binding in the frontal cortex and hippocampus[212]. Studies in animals show that early adverse experience[214] and separation anxiety is associated with long-term changes, including a loss of $GABR_A$-mediated inhibition of the hypothalamic-pituitary-adrenal axis stress response[215], and trait hypoxic ventilatory responses, both reflecting life-long alterations in GABAergic homeostasis[216]. Fluoxetine BZ treatment significantly abated the usual reductions in DG cell density in pups due to maternal separation stress[217]. A similar connection is observed between PD and early life stressors in humans (disturbed childhood environment, early separation, childhood separation disorder), and is proposed to be predictive of susceptibility to PD[103,218]. And behavioral, electrophysiological, and immunohistochemical studies revealed a $GABR_A$-mediated learned fear circuitry in the amygdala and lateral nucleus involving long-term potentiation for the formation of fear

memory[220] (see section 2.2.4.). Thus, both interoceptive and exteroceptive signals impinge on $GABR_A$s to mediate a multitude of physiological and neurobehavioral outcomes. This complexity may explain the interplay of genetic and environmental cues in PD pathogenesis.

3.3. Aims

In this study, using the same Caucasian sample of 120 multiplex PD pedigrees previously used in the latest genome scan, we fine-mapped the chromosome 15q region of interest (0-19.3 cM) using eight microsatellite markers, and subsequently with ten single nucleotide polymorphisms (SNPs) at finer-resolution to the candidate genes GABRB3 and GABRA5, to pinpoint the linkage signal and test for allelic association to PD. In a subset of ninety-six PD probands we performed a mutation screen of both genes to identify novel variants potentially linked to PD, and/or enrichment of known variants in PD probands. Lastly, to understand possible endophenotypes informing putative GABAergic dysregulation in PD, we sought to characterize the expression of our $GABR_A$ genes in an otherwise normal (unphenotyped) population of Caucasians. We performed a test of genetic association with ribonucleic acid expression levels as a quantitative trait to identify heritable determinants of expression.

3.4. Methods

3.4.1. Subject recruitment and sample collection

Subject recruitment and sample collection was performed as described in Chapter Two.

3.4.2 Genotyping by TaqMan

SNP genotyping was also performed using primers (table 3.1.) designed for the 5' nuclease (TaqMan) assay according to manufacturer's specifications for Assays-on-Demand (ABI). PCR reactions contained 4 ng/μL DNA with 1x concentration of TaqMan Universal PCR master mix containing AmpliTaq Gold DNA polymerase and 0.5x or 0.66x concentration of SNP genotyping assay mix in 5 μL reactions (ABI). Thermocycling conditions included 95°C for 10 min, followed by 50 two-step cycles (92 °C for 15 sec, and 60 °C for 60 sec). Thermocyling, data acquisition, and analyses were performed using ABI Prism 7900HT Sequence Detection System, and allele calls were determined using loaded ABI SDS software. Quality control procedures for all genotyping included monitoring concordant genotypes in duplicate samples, as well as routine Mendelization error checks. Inconsistent duplicate genotypes were re-typed, and unresolved incongruent genotypes were dropped.

3.4.3. Resequencing analysis of GABRB3 and GABRA5

Oligonucleotide primers for PCR and sequencing were designed using Primer 3 software (table 3.2.) for synthesis by Invitrogen to target exonic sequence, exon-flanking non-coding sequence, non-coding regions with high cross-species nucleotide conservation, and regions enriched for clusters of

transcription factor binding sites according to the National Center for Biotechnology Information (NCBI) website, Build 35. PCR conditions varied depending on the amplicon (table 3.3.). Amplification was performed in a 5-10 uL total reaction volume using a final concentration of 2-5 ng/μL genomic DNA, 150-500 nM primers, and 50-200 μM deoxyribonucleotide triphosphate (dNTPs). Possible adjuncts included 1.0-1.5 M anhydrous betaine (Acros Organics, Geel, Belgium), 5% dimethylsulfoxide (DMSO), and 1.25 M BD GC-Melt (Clontech). The final concentration of DNA polymerase was 0.05-0.075 U/uL Platinum Taq (InVitrogen) for most amplicons, or 1x concentration BD Advantage polymerase mix (Clontech), 0.04 U/uL JumpStart AccuTaq LA polymerase (Sigma-Aldrich), or 0.03 U/uL AmpliTaq GOLD polymerase (ABI). Salt content was typically maintained using 1x concentration manufacturer's PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), and 1.5-2.5 mM $MgCl_2$ for most reactions. Some amplicons required the use of salt buffers specified by special polymerase kits, such as 2.75 mM $Mg(OAc)_2$ in BD mix (Clonetech), 1x concentration of LA buffer (Sigma-Aldrich), or 2.5 mM GeneAmp GOLD buffer (ABI). Most reactions followed the usual touchdown PCR protocol using Applied Biosystems Incorporated (ABI) GeneAmp PCR System (Foster City, CA) thermal cyclers as follows (unless otherwise specified in table 3.3.): 94°C for 3 min, followed by 7 three-step touchdown cycles (94 °C for 30 sec, decreasing 1 °C per cycle 65-59 °C for 30 sec, 72 °C for 30 sec), followed by 38 three-step cycles (90 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec), and 72 °C for 10 min. In preparation for sequencing, excess primers and dNTPs were enzymatically digested with the

addition of a 2.5-2.8 µL mix containing a final concentration of 0.7 U/uL *Escherichia coli* exonuclease I (USB, Cleveland, OH), and 0.07 U/uL shrimp alkaline phosphatase (Roche, Indianapolis, IN) in water, and incubated at 37 °C for 2 hr, followed by 80 °C for 15 min, and 95 °C for 1 min. Sequencing reactions were performed using BigDye v3.1 (ABI) scaled to 1/16th in a 5 uL total volume of 1 uL PCR-amplified product, 2.5 pmol PCR primer (used also for sequencing), 0.75 uL ABI buffer and 0.5 uL BigDye v3.1 in water. Sequencing reactions were cycled at 96 °C for 3 min, followed by 25 cycles at 96 °C for 10 sec, 50 °C for 5 sec, and 60 °C for 4 min in 9700 GeneAmp PCR System (ABI). Reactions were purified with Montage MultiScreen-SEQ (Millipore, Bedford, MA) plates and the Hamilton Microlab 4200 (Hamilton, Reno, NV) 96-probe liquid robotic system. Samples were analyzed on a Prism 3730xl DNA Analyzer (ABI), and analysis of mutations was performed SEQUENCHER DNA sequence analysis software (http://sequencher.bio.indiana.edu/).

3.4.4. Linkage and association analysis

Data analysis for parametric and non-parametric linkage and allelic association were performed as previously described in Chapter Two (section 2.8.5.). We report *p*-values that are uncorrected for multiple tests, although we used permutation analysis to increase signal to noise ratio in our exploratory results.

3.4.5. Cell culture

We investigated the expression levels of ribonucleic acid (RNA) in Epstein-Barr virus-transformed lymphoblastoid cell lines from ninety Caucasian individuals from Utah purchased from the Coriell sample repository. These samples consisted of thirty mother-father-child trios, with publicly available genotypes provided by the International HapMap Project. We expanded and maintained the immortalized cell lines in suspension using uniform culture conditions and a target cell density range, according to Coriell recommendations. Briefly, cells were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium (Biowhittaker, Walkersville, MD) with a final concentration of 15% heat-inactivated fetal bovine serum (Sigma), 2 mM L-glutamine (Fisher), and 100 U/ml penicillin, 100 ug/ml streptomycin (HyClone, Fisher), in a humidified incubator at 37 ºC and 5% CO_2. Cell cultures were regularly split to maintain a target range of 0.2-1.0 million cells/mL, as determined by counting cell density with a hemocytometer under a microscope. Cells were harvested by centrifugal pelleting, and washed twice with Dulbecco's phosphate buffered saline (Cambrex Bio Science, Walkersville, MD) before storing at –70 ºC.

3.4.6. RNA preparation

Cell RNA was isolated from up to ten million cells using the Qiagen Rneasy Mini prep kit (Qiagen, Valencia, CA) with beta-2-mercaptoethanol (Fisher, Pittsburg, PA) according to manufacturer's protocol. Total RNA quality and yield was assessed by optical density measurement using the NanoDrop UV-Vis spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and

the RNA 6000 Nano Kit for the 2100 Bioanalyzer (Agilent, Palo Alto, CA) electropheresis instrument according to manufacturer's suggestions. Synthesis and amplification of complementary RNA (cRNA) from 275 ng of RNA was performed using the Illumina RNA Amplification kit (Ambion, Foster City, CA) according to manufacturer's suggestions. Briefly, first strand cDNA synthesis by reverse transcription with T7 promoter-bearing oligo(dT) primers and reverse transcriptase for 2 h at 42 ºC, followed by second strand cDNA synthesis using DNA polymerase for 2 h at 16 ºC to create double-stranded T7 promoter sequence-bearing template for *in vitro* transcription. After subsequent solid phase purification of the cDNA, biotin-16-UTP (Roche, Mannheim, Germany) labeled antisense RNA (aRNA) was synthesized, purified, and amplified for 14-16 h at 37 ºC to generate biotinylated cRNA from the double-stranded cDNA for subsequent visualization via streptavidin-Cy3 (Ambion) binding. Then, 275 ng RNA was then hybridized to complementary 50mer target transcript oligonucleotides embedded on Sentrix BeadChips for quantification by Illumina BeadArray Reader scanner. Detection of RNA levels was determined using the Illumina BeadStation Gene Expression system with BeadStudio data analysis software (Illumina, San Diego, CA).

3.4.7. Heritable expression analysis

We measured RNA levels in ninety cell lines to test for association with *cis* variants for heritable expression patterns. Expression of GABRA5 was below the detection limit in the peripheral lymphoblastoid cell lines, however, GABRB3

expression was reliably detected in thirty-five of the ninety individuals. These thirty-five cell lines formed one nuclear trio, six parent-child dyads, and twenty-six unrelated individuals (20 additional unrelateds) to assess heritable transmission of GABRB3 expression. Preliminary analyses showed very low variation in expression levels for the same cell line sample analyzed on replicate BeadChips (see Chapter Four). However, other sources of biological and technical intrasample variation, including day-to-day variation in culturing and RNA isolation, were addressed by using internal controls for relative gene expression. In a pilot study of cell lines that were cultured, harvested and processed for RNA in replicate, and on different days, we assessed the intra-sample variation of target gene expression. Among the 6,355 transcripts detected by the Illumina BeadChip assay, six reference genes within a five-fold signal intensity of the mean GABRB3 expression (LRRFIP2, TBL1X, FKBP4, VPS13A, C3F, and IL15), were empirically selected for their ability to significantly reduce intra-sample variability, and applied to the larger sample set for normalization.

Using UNPHASED statistical software[237], the relative ratio of GABRB3 to the average of the six reference genes was assessed in a quantitative transmission disequilibrium test (QTDT). Genotypes, available from the International HapMap Project website (Release 20/ phase II Jan 2006 on NCBI B35 assembly, dbSNP b125), for 198 non-redundant SNPs (r^2<1.0) with at least 5% minor allele frequency, were tested for correlation to parent to child transmission of GABRB3 expression levels and genotypic association with expression levels.

3.5. Results

Results from serial genome scans performed in twenty-three, sixty, and 120 PD pedigrees, as additional subjects were recruited and became available for analysis, showed sustained evidence of suggestive linkage to the proximal arm of chr15q at D15S822, near the GABRB3 and GABRA5 candidate genes. Follow-up analysis of the implicated region in chr15q11-13 with eight microsatellites showed further linkage in the same sample (Bautista and Hamilton, unpublished data). Four of the eight microsatellite markers showed suggestive linkage (LOD≥2) using multipoint and two-point analyses given the assumption of locus homogeneity and/or heterogeneity, which prompted finer-resolution mapping using ten SNPs at the candidate genes GABRB3 and GABRA5, based upon ethological, mechanistic and clinical support for their involvement in PD. We observed linkage to GABRB3 (rs11631421, LOD=4.6) (figure 3.1.) and GABRA5 (rs2075716, LOD=2.2) (figure 3.2.) in two-point parametric linkage analysis, and GABRB3 (rs3212337, LOD=1.6) (figure 3.3.) and GABRA5 (rs35399885, rs140682, rs140685 LOD≥2.8) (figure 3.4.) in multipoint analysis under the assumption of locus heterogeneity. Allelic association was found in GABRB3 (rs8024564, p=0.005-0.03; rs8025575, p=0.02) and GABRA5 (rs35399885, p=0.05) (table 3.4.). Further analysis revealed nominally significant p-values (p≤0.05) for two-marker haplotypes that were protective against PD. Three two-marker haplotypes exhibited smaller p-values (p=0.003-0.01) in allele-specific analyses (table 3.5.). Other work in the laboratory suggests that SNPs in the GABRB3 and GABRA5 region are

associated with PD in an independent PD sample of Sardinian trios (Terrill and Hamilton, unpublished data).

A mutation screen of GABRB3 and GABRA5 in a subset of ninety-six PD probands revealed thirteen novel GABRB3 variants (table 3.6.), and seventeen novel GABRA5 variants (table 3.7.) that were predominantly uncommon minor allele frequency (MAF<0.05) and in introns. Two novel GABRB3 variants in the 3' untranslated region (UTR) of the gene are predicted to alter the transcription factor binding sites (TFBSs) for the transcription factors, GATA[221] and NCX (or TLX2, HOX11L1)[222], which are distributed in the brain[223,224], and relevant for neurogenesis[224-226]. Three additional novel GABRB3 variants were detected at high frequency (MAF=0.35-0.50). In GABRA5, novel variants were observed in 3'UTR, 5'UTR, and coding regions (MAF=0.001-0.05), including a synonymous SNP at a wobble codon position (alanine GCc> alanine GCt). Two novel and common (MAF=0.13-0.49) GABRA5 variants were also detected, both within 200bp of a GABRA5 exon, which have not been characterized in control individuals. Among the previously known variants that were observed in our probands, none showed significant deviation in allele frequency from the publicly available databases for Caucasians.

Using the Illumina 6-BeadChip Gene Expression system to detect RNA levels in ninety control lymphoblastoid cell lines from thirty CEU HapMap trios, 6,255 transcripts were reliably detected for all ninety samples. GABRA5 expression was undetected in our samples using this platform; however, GABRB3 was detected in thirty-five of the ninety samples, which provided one

nuclear trio, six parent-child dyads, and twenty-six unrelated individuals (20 additional unrelateds) in which to examine heritable gene expression patterns and genotypic association with GABRB3 transcript levels. Among 198 non-redundant (r^2<1.0), tagging SNPs around GABRB3 genotyped in these samples as part of the HapMap, four variants were significantly associated ($p \leq 0.01$) of GABRB3 expression after 10,000 permutations (table 3.8.). The most significantly associated SNP, rs2315904, gave a *p*-value of 0.00021, which also met Bonferroni correction at $p<0.05$ for 198 tests ($p<0.00025$), prior to permutation analysis. LD relationships between the associated SNPs and untested SNPs (MAF>0.05, r^2=1.0) broaden the implicated region 5' of the gene to ~1100bp around rs6576613.

3.6. Discussion and future directions

GABRB3 and GABRA5 were investigated as candidate genes for the susceptibility to PD based upon prior genetic linkage in serial genome scans in multiplex PD pedigrees, and a convergence of pharmacological, ethological and mechanistic evidence for their role in the pathogenesis of PD. We report association and linkage to markers at GABRB3 and GABRA5 in PD pedigrees, as well as several novel variants in a mutation screen in probands. As well, in an uncharacterized Caucasian population, heritable expression analysis revealed significant association ($p \leq 0.01$, given 10,000 permutations) to three intronic GABRB3 SNPs and one SNP lying 5' to GABRB3 in the intergenic region between the 5' ends of GABRB3 and GABRA5. Future studies include replication of the association and linkage results, and/or finer mapping with

denser marker genotyping in the region. The novel variants found by mutation screening could be characterized in an ethnically-matched control population to look for differences potentially associated with PD. Heritable expression profiles in affecteds would be needed to show potential differences in gene regulation with PD, as compared to the control population characterized here.

A previous study using a simple sequence repeat polymorphism in each of eight $GABR_A$ candidate genes, including GABRB3 and GABRA5, found no evidence for linkage to PD (negative LOD scores) in twenty-six PD pedigrees from the U.S. and Iceland[227]. Here we observed linkage and allelic association of GABRB3 and GABRA5 in PD pedigrees using ten SNPs in the candidate gene regions. Our study used 4.8 times more PD pedigrees and 5 times more markers for testing, which suggests that the previous studies may have been relatively underpowered to detect positive linkage.

Our mutation screen revealed several novel variants of potential functional significance in both GABRB3 and GABRA5. Two low frequency GABRB3 variants were discovered in TFBSs for GATA and NCX transcription factors, which may play an important role in long-term GABAergic tone, due to their relevance during neurogenesis. Three low frequency, genic SNPs were also discovered in the coding region, and 3' and 5' UTRs of GABRA5. Each of these SNPs may have a functional role in regulating gene expression, by potentially altering transcriptional processes. For example, downregulation of GABRB3 in patients with autism[228], Rett syndrome[229], and childhood absence epilepsy (CAE)[81], is associated with GABRB3 variants in regulatory elements or in

mutations of transcription factors themselves. Comparison of the potential expression differences for these variants in PD probands and controls could pinpoint potential regulatory differences between the groups.

A synonymous GABRA5 SNP, which results in a cytosine to thymine transition in the third codon position, found in the mutation screen, is of particular interest. The potential relevance of this SNP, given the relative low mutability of this gene, is suggested in the literature. For example, housekeeping genes, which have low mutability and low rates of protein evolution, exhibit a disproportionate enrichment of cytosines in the wobble codon position[241]. Since housekeeping genes have relatively high mRNA stability[230], it is suggested that this constitutes a role for codon usage bias in humans. For example, tRNA abundance is associated with codon usage in human globin genes[231], and cytosine-enriched codon ends are significantly more common in constitutive versus alternative exons of the human genome[233]. Computational work to derive the thermodynamic changes in mRNA stability *in silico* for over 19,000 human and mouse mRNA sequences shows a significantly higher abundance of C and G nucleotides at the wobble position, suggesting selective pressure for its non-random maintenance[234]. And mechanistic insight comes from studies in *drosophila* showing that silent mutations in alcohol dehydrogenase genes alters gene expression by altering pre-mRNA and mRNA secondary structure. In Chapter Four (section 4.2.11.), a study by Nackley *et al*. decribes a dramatic structural change in the mRNA of COMT, based on the presence of a synonymous SNP, correlated with significant alteration of mRNA stability, mRNA

levels, protein levels, and activity differences[246]. Determination of allele frequency differences for this variant in a control population, compared to PD probands, would ascribe its potential relevance to PD. Studies to determine the functional impact of this SNP could include tests of differential mRNA stability, protein and RNA expression, or ligand binding, for example.

As for the novel mutations found in TFBSs (i.e. NCX and GATA), there is good evidence for their role in psychiatric disease. For example, decreased GABRB3 expression,which is seen in patients with Rett syndrome (RS), autism, and Angleman syndrome, is also observed in mouse strains with deficiency in the transcription factor, methyl-CpG-binding protein 2 (MECP2)[229]. RS is marked by symptoms which overlap with PD, such as hyperventilation, EEG abnormalities, and urinary pH differences[235]. By different mechanisms, decreased GABRB3 expression is also common in patients with autism, which is linked and associated with the chr15q GABR genes[86]. Persons with autism can exhibit EEG abnormalities, altered BZ binding[238], and altered GABAergic tone[239,240], similar to subjects with PD. Thus, future studies to pinpoint epigenetic or other effects that alter gene expression in subjects with PD, will help inform the potential functional causes of the heritablity of PD. Future tests could include characterization of allele frequency differences in cases and controls, followed by functional assays, including differential transcription factor binding (e.g. electrophoretic gel mobility shift assay).

Our data suggests that a proportion of the genetic liability for PD could be explained by mutations in GABRB3 and GABRA5. As the heritability of PD

(0.48) suggests that there are environmental and potential epigenetic effects at play in the susceptibility for PD, we may not be sufficiently powered to show significant association and linkage to these candidate genes to the level of strict multitesting correction, given our sample size. However, we view these findings as exploratory results that can be used for further investigation. Given the overlapping evidence by multiple analytical approaches, including gene-agnostic markers in the genome scans, we are encouraged that GABRB3 and GABRA5 are good candidates to explain at least a portion of the biological and genetic factors in PD path.

3.7. References

1. Fyer, A. J. *et al.* A third-pass genome scan in panic disorder: evidence for multiple susceptibility loci. *Biol. Psychiatry* 60, 388-401 (2006).

2. Knowles, J. A. *et al.* Results of a genome-wide genetic screen for panic disorder. *Am. J. Med. Genet.* 81, 139-147 (1998).

3. Hamilton, S. P. *et al.* Further genetic evidence for a panic disorder syndrome mapping to chromosome 13q. *Proc. Natl. Acad. Sci. U. S. A* 100, 2550-2555 (2003).

4. Crowe, R. R. *et al.* Genomewide survey of panic disorder. *American Journal of Medical Genetics* 105, 105-109 (2001).

5. Thorgeirsson, T. E. *et al.* Anxiety with panic disorder linked to chromosome 9q in Iceland. *American Journal of Human Genetics* 72, 1221-1230 (2003).

6. Gelernter, J. *et al.* Linkage genome scan for loci predisposing to panic disorder or agoraphobia. *American Journal of Medical Genetics* 105, 548-557 (2001).

7. Nyholt, D. R. All LODs are not created equal. *Am. J. Hum. Genet.* 67, 282-288 (2000).

8. Sieghart, W. Structure and pharmacology of gamma-aminobutyric acid A receptor subtypes. *Pharmacol. Rev.* 47, 181-234 (1995).

9. Kulik, A., Nishimaru, H. & Ballanyi, K. Role of bicarbonate and chloride in. *J. Neurosci.* 20, 7905-7913 (2000).

10. Obrietan, K. & van den Pol, A. N. GABA neurotransmission in the hypothalamus: developmental reversal from Ca2+ elevating to depressing. *J. Neurosci.* 15, 5065-5077 (1995).

11. Kirkness, E. F. & Fraser, C. M. A strong promoter element is located between alternative exons of a gene encoding the human gamma-aminobutyric acid-type A receptor beta 3 subunit (GABRB3). *J. Biol. Chem.* 268, 4420-4428 (1993).

12. De Koninck, Y. & Henry, J. L. Prolonged GABAA-mediated inhibition following single hair afferent input to single spinal dorsal horn neurones in cats. *J. Physiol* 476, 89-100 (1994).

13. Martin, D. S., Segura, T. & Haywood, J. R. Cardiovascular responses to bicuculline in the paraventricular nucleus of the rat. *Hypertension* 18, 48-55 (1991).

14. Rudolph, U. *et al.* Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. *Nature* 401, 796-800 (1999).

15. Ugarte, S. D., Homanics, G. E., Firestone, L. L. & Hammond, D. L. Sensory thresholds and the antinociceptive effects of GABA receptor agonists in mice lacking the beta3 subunit of the GABA(A) receptor. *Neuroscience* 95, 795-806 (2000).

16. Kataoka, Y., Niwa, M., Yamashita, K. & Taniyama, K. GABA receptor function in the parasympathetic ganglia. *Jpn. J. Physiol* 44 Suppl 2, S125-S129 (1994).

17. Shirakawa, J., Nakanishi, T., Taniyama, K., Kamidono, S. & Tanaka, C. Regulation of the substance P-induced contraction via the release of acetylcholine and gamma-aminobutyric acid in the guinea-pig urinary bladder. *Br. J. Pharmacol.* 98, 437-444 (1989).

18. Bright, D. P., Aller, M. I. & Brickley, S. G. Synaptic release generates a tonic GABA(A) receptor-mediated conductance that modulates burst precision in thalamic relay neurons. *J. Neurosci.* 27, 2560-2569 (2007).

19. Laurie, D. J., Wisden, W. & Seeburg, P. H. The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J. Neurosci.* 12, 4151-4172 (1992).

20. MacLennan, A. J. *et al.* Independent cellular and ontogenetic expression of mRNAs encoding three alpha polypeptides of the rat GABAA receptor. *Neuroscience* 43, 369-380 (1991).

21. Hara, K., Saito, Y., Kirihara, Y. & Sakura, S. The interaction between gamma-aminobutyric acid agonists and diltiazem in visceral antinociception in rats. *Anesth. Analg.* 98, 1380-4, table (2004).

22. Sillen, U., Persson, B. & Rubenson, A. Involvement of central GABA receptors in the regulation of the urinary bladder function of anaesthetized rats. *Naunyn Schmiedebergs Arch. Pharmacol.* 314, 195-200 (1980).

23. Reith, C. A. & Sillar, K. T. Development and role of GABA(A) receptor-mediated synaptic potentials during swimming in postembryonic Xenopus laevis tadpoles. *J. Neurophysiol.* 82, 3175-3187 (1999).

24. Bouairi, E., Kamendi, H., Wang, X., Gorini, C. & Mendelowitz, D. Multiple types of GABAA receptors mediate inhibition in brain stem

parasympathetic cardiac neurons in the nucleus ambiguus. *J. Neurophysiol.* 96, 3266-3272 (2006).

25. Semyanov, A. & Kullmann, D. M. Relative picrotoxin insensitivity distinguishes ionotropic GABA receptor-mediated IPSCs in hippocampal interneurons. *Neuropharmacology* 43, 726-736 (2002).

26. Song, D. K. *et al.* Central GABAA and GABAB receptor modulation of basal and stress-induced plasma interleukin-6 levels in mice. *J. Pharmacol. Exp. Ther.* 287, 144-149 (1998).

27. Miyawaki, T., Goodchild, A. K. & Pilowsky, P. M. Evidence for a tonic GABA-ergic inhibition of excitatory respiratory-related afferents to presympathetic neurons in the rostral ventrolateral medulla. *Brain Res.* 924, 56-62 (2002).

28. Chung, S., Ivy, G. O. & Reid, S. G. GABA-mediated neurotransmission in the nucleus of the solitary tract alters resting ventilation following exposure to chronic hypoxia in conscious rats. *Am. J. Physiol Regul. Integr. Comp Physiol* 291, R1449-R1456 (2006).

29. Gourine, A. V. & Spyer, K. M. Chemosensitivity of medullary inspiratory neurones: a role for GABA(A) receptors? *Neuroreport* 12, 3395-3400 (2001).

30. Staley, K. J. & Proctor, W. R. Modulation of mammalian dendritic GABA(A) receptor function by the kinetics of Cl- and HCO3- transport. *J. Physiol* 519 Pt 3, 693-712 (1999).

31. Buesa, I. *et al.* Disinhibition of spinal responses to primary afferent input by antagonism at GABA receptors in urethane-anaesthetised rats is dependent on NMDA and metabotropic glutamate receptors. *Neuropharmacology* 50, 585-594 (2006).

32. Kaila, K. & Voipio, J. Postsynaptic fall in intracellular pH induced by GABA-activated bicarbonate conductance. *Nature* 330, 163-165 (1987).

33. White, A. M. & Platt, B. Ionic mechanisms of GABA-induced long-term potentiation in the rat superior colliculus. *Exp. Brain Res.* 140, 486-494 (2001).

34. Chen, J. C. & Chesler, M. Extracellular alkalinization evoked by GABA and its relationship to activity-dependent pH shifts in turtle cerebellum. *J. Physiol* 442, 431-446 (1991).

35. Maksay, G., Fodor, L., Bielik, N. & Tarnawa, I. Bicarbonate and thiocyanate ions affect the gating of gamma-aminobutyric acid(A) receptors in cultured rat cortical cells. *Neurosci. Lett.* 311, 169-172 (2001).

36. Wermuth, C. G. *et al.* The sensitivity of gamma-aminobutyric acid antagonists to thiocyanate is related to the absence of a functional anionic group in their structure. *Eur. J. Pharmacol.* 144, 375-378 (1987).

37. Michels, G. & Moss, S. J. GABAA receptors: properties and trafficking. *Crit Rev. Biochem. Mol. Biol.* 42, 3-14 (2007).

38. Belelli, D. *et al.* Neuroactive steroids and inhibitory neurotransmission: mechanisms of action and physiological relevance. *Neuroscience* 138, 821-829 (2006).

39. Brickley, S. G., Cull-Candy, S. G. & Farrant, M. Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of GABAA receptors. *J. Physiol* 497 (Pt 3), 753-759 (1996).

40. Attwell, D., Barbour, B. & Szatkowski, M. Nonvesicular release of neurotransmitter. *Neuron* 11, 401-407 (1993).

41. Cope, D. W., Hughes, S. W. & Crunelli, V. GABAA receptor-mediated tonic inhibition in thalamic neurons. *J. Neurosci.* 25, 11553-11563 (2005).

42. Hamann, M., Rossi, D. J. & Attwell, D. Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex. *Neuron* 33, 625-633 (2002).

43. McCartney, M. R., Deeb, T. Z., Henderson, T. N. & Hales, T. G. Tonically active GABAA receptors in hippocampal pyramidal neurons exhibit constitutive GABA-independent gating. *Mol. Pharmacol.* 71, 539-548 (2007).

44. Mortensen, M. & Smart, T. G. Extrasynaptic alphabeta subunit GABAA receptors on rat hippocampal pyramidal neurons. *J. Physiol* 577, 841-856 (2006).

45. Park, J. B., Skalska, S. & Stern, J. E. Characterization of a novel tonic gamma-aminobutyric acidA receptor-mediated inhibition in magnocellular neurosecretory neurons and its modulation by glia. *Endocrinology* 147, 3746-3760 (2006).

46. Rossi, D. J., Hamann, M. & Attwell, D. Multiple modes of GABAergic inhibition of rat cerebellar granule cells. *J. Physiol* 548, 97-110 (2003).

47. Mody, I. Aspects of the homeostaic plasticity of GABAA receptor-mediated inhibition. *J. Physiol* 562, 37-46 (2005).

48. Mouginot D., Feltz P., and Schlichter R. Modulation of GABA-gated chloride currents by intracellular Ca2+ in cultured porcine melanotrophs. J.Physiol 437:109-132 (1991).

49. Tyndale R. F., Olsen R. W. and Tobin A. J. GABAA. In: North R. A., Editor, Handbook of Receptors and Channels: Ligand and Voltage-Gated Ion Channels, CRC Press, Boca Raton. 261–286 (1994).

50. Simon, J., Wakimoto, H., Fujita, N., Lalande, M. & Barnard, E. A. Analysis of the set of GABA(A) receptor genes in the human genome. *J. Biol. Chem.* 279, 41422-41435 (2004).

51. Ogurusu, T., Eguchi, G. & Shingai, R. Localization of gamma-aminobutyric acid (GABA) receptor rho 3 subunit in rat retina. *Neuroreport* 8, 925-927 (1997).

52. Barnard, E. A. *et al.* International Union of Pharmacology. XV. Subtypes of gamma-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. *Pharmacol. Rev.* 50, 291-313 (1998).

53. Tsang, S. Y., Ng, S. K., Xu, Z. & Xue, H. The evolution of GABAA receptor-like genes. *Mol. Biol. Evol.* 24, 599-610 (2007).

54. Russek, S. J. Evolution of GABA(A) receptor diversity in the human genome. *Gene* 227, 213-222 (1999).

55. Boer, H. *et al.* Psychotic illness in people with Prader Willi syndrome due to chromosome 15 maternal uniparental disomy. *Lancet* 359, 135-136 (2002).

56. Gole, L., Crolla, J. A., Thomas, S. N., Jacobs, P. A. & Dennis, N. R. Characterization of breakpoints in the GABRG3 and TSPY genes in a family with a t(Y;15)(p11.2;q12). *Am. J. Med. Genet. A* 125, 177-180 (2004).

57. Sweetnam, P. M. *et al.* Phosphorylation of the GABAa/benzodiazepine receptor alpha subunit by a receptor-associated protein kinase. *J. Neurochem.* 51, 1274-1284 (1988).

58. Crestani, F. *et al.* Trace fear conditioning involves hippocampal alpha5 GABA(A) receptors. *Proc. Natl. Acad. Sci. U. S. A* 99, 8980-8985 (2002).

59. Greenfield, L. J., Jr. *et al.* Mutation of the GABAA receptor M1 transmembrane proline increases GABA affinity and reduces barbiturate enhancement. *Neuropharmacology* 42, 502-521 (2002).

60. Hosie, A. M., Wilkins, M. E., da Silva, H. M. & Smart, T. G. Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites. *Nature* 444, 486-489 (2006).

61. Baur, R. & Sigel, E. Replacement of histidine in position 105 in the alpha(5) subunit by cysteine stimulates zolpidem sensitivity of alpha(5)beta(2)gamma(2) GABA(A) receptors. *J. Neurochem.* (2007).

62. Ratra, G. S., Kamita, S. G. & Casida, J. E. Role of human GABA(A) receptor beta3 subunit in insecticide toxicity. *Toxicol. Appl. Pharmacol.* 172, 233-240 (2001).

63. Hosie, A. M., Dunne, E. L., Harvey, R. J. & Smart, T. G. Zinc-mediated inhibition of GABA(A) receptors: discrete binding sites underlie subtype specificity. *Nat. Neurosci.* 6, 362-369 (2003).

64. Jensen, M. L. *et al.* The beta subunit determines the ion selectivity of the GABAA receptor. *J. Biol. Chem.* 277, 41438-41447 (2002).

65. Ehya, N., Sarto, I., Wabnegger, L. & Sieghart, W. Identification of an amino acid sequence within GABA(A) receptor beta3 subunits that is important for receptor assembly. *J. Neurochem.* 84, 127-135 (2003).

66. Sarto, I., Wabnegger, L., Dogl, E. & Sieghart, W. Homologous sites of GABA(A) receptor alpha(1), beta(3) and gamma(2) subunits are important for assembly. *Neuropharmacology* 43, 482-491 (2002).

67. Charych, E. I. *et al.* The brefeldin A-inhibited GDP/GTP exchange factor 2, a protein involved in vesicular trafficking, interacts with the beta subunits of the GABA receptors. *J. Neurochem.* 90, 173-189 (2004).

68. Perez-Velazquez, J. L. & Angelides, K. J. Assembly of GABAA receptor subunits determines sorting and localization in polarized cells. *Nature* 361, 457-460 (1993).

69. Taylor, P. M. *et al.* Identification of amino acid residues within GABA(A) receptor beta subunits that mediate both homomeric and heteromeric receptor expression. *J. Neurosci.* 19, 6360-6371 (1999).

70. van Rijnsoever, C. *et al.* Requirement of alpha5-GABAA receptors for the development of tolerance to the sedative action of diazepam in mice. *J. Neurosci.* 24, 6785-6790 (2004).

71. Chang, C. C., Luntz-Leybman, V., Evans, J. E., Rotter, A. & Frostholm, A. Developmental changes in the expression of gamma-aminobutyric acidA/benzodiazepine receptor subunit mRNAs in the murine inferior olivary complex. *J. Comp Neurol.* 356, 615-628 (1995).

72. McDonald, B. J. & Moss, S. J. Conserved phosphorylation of the intracellular domains of GABA(A) receptor beta2 and beta3 subunits by cAMP-dependent protein kinase, cGMP-dependent protein kinase protein kinase C and Ca2+/calmodulin type II-dependent protein kinase. *Neuropharmacology* 36, 1377-1385 (1997).

73. Raymond, L. A., Blackstone, C. D. & Huganir, R. L. Phosphorylation of amino acid neurotransmitter receptors in synaptic plasticity. *Trends Neurosci.* 16, 147-153 (1993).

74. Chen, Q. X., Stelzer, A., Kay, A. R. & Wong, R. K. GABAA receptor function is regulated by phosphorylation in acutely dissociated guinea-pig hippocampal neurones. *J. Physiol* 420, 207-221 (1990).

75. Leidenheimer, N. J., Browning, M. D. & Harris, R. A. GABAA receptor phosphorylation: multiple sites, actions and artifacts. *Trends Pharmacol. Sci.* 12, 84-87 (1991).

76. Swope, S. L., Moss, S. J., Blackstone, C. D. & Huganir, R. L. Phosphorylation of ligand-gated ion channels: a possible mode of synaptic plasticity. *FASEB J.* 6, 2514-2523 (1992).

77. Kano, M., Rexhausen, U., Dreessen, J. & Konnerth, A. Synaptic excitation produces a long-lasting rebound potentiation of inhibitory synaptic signals in cerebellar Purkinje cells. *Nature* 356, 601-604 (1992).

78. Kano, M. Calcium-induced long-lasting potentiation of GABAergic currents in cerebellar Purkinje cells. *Jpn. J. Physiol* 44 Suppl 2, S131-S136 (1994).

79. Kittler, J. T. *et al.* Phospho-dependent binding of the clathrin AP2 adaptor complex to GABAA receptors regulates the efficacy of inhibitory synaptic transmission. *Proc. Natl. Acad. Sci. U. S. A* 102, 14871-14876 (2005).

80. Buhr, A. *et al.* Functional characterization of the new human GABA(A) receptor mutation beta3(R192H). *Hum. Genet.* 111, 154-160 (2002).

81. Urak, L., Feucht, M., Fathi, N., Hornik, K. & Fuchs, K. A GABRB3 promoter haplotype associated with childhood absence epilepsy impairs transcriptional activity. *Hum. Mol. Genet.* 15, 2533-2541 (2006).

82. Otani, K. *et al.* The GABA type A receptor alpha5 subunit gene is associated with bipolar I disorder. *Neurosci. Lett.* 381, 108-113 (2005).

83. Papadimitriou, G. N. *et al.* Association between the GABA(A) receptor alpha5 subunit gene locus (GABRA5) and bipolar affective disorder. *Am. J. Med. Genet.* 81, 73-80 (1998).

84. Oruc, L. *et al.* Positive association between the GABRA5 gene and unipolar recurrent major depression. *Neuropsychobiology* 36, 62-64 (1997).

85. Papadimitriou, G. *et al.* Association between GABA-A receptor alpha 5 subunit gene locus and schizophrenia of a later age of onset. *Neuropsychobiology* 43, 141-144 (2001).

86. McCauley, J. L. *et al.* A linkage disequilibrium map of the 1-Mb 15q12 GABA(A) receptor subunit cluster and association to autism. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 131, 51-59 (2004).

87. Buxbaum, J. D. *et al.* Association between a GABRB3 polymorphism and autism. *Mol. Psychiatry* 7, 311-316 (2002).

88. Cook, E. H., Jr. *et al.* Linkage-disequilibrium mapping of autistic disorder, with 15q11-13 markers. *Am. J. Hum. Genet.* 62, 1077-1083 (1998).

89. Menold, M. M. *et al.* Association analysis of chromosome 15 gabaa receptor subunit genes in autistic disorder. *J. Neurogenet.* 15, 245-259 (2001).

90. Ashley-Koch, A. E. *et al.* An analysis paradigm for investigating multi-locus effects in complex disease: examination of three GABA receptor subunit genes on 15q11-q13 as risk factors for autistic disorder. *Ann. Hum. Genet.* 70, 281-292 (2006).

91. Ozyalcin, S. N. *et al.* The efficacy and safety of venlafaxine in the prophylaxis of migraine. *Headache* 45, 144-152 (2005).

92. Parent, M. B., Master, S., Kashlub, S. & Baker, G. B. Effects of the antidepressant/antipanic drug phenelzine and its putative metabolite phenylethylidenehydrazine on extracellular gamma-aminobutyric acid levels in the striatum. *Biochem. Pharmacol.* 63, 57-64 (2002).

93. Bhagwagar, Z. *et al.* Increased brain GABA concentrations following acute administration of a selective serotonin reuptake inhibitor. *Am. J. Psychiatry* 161, 368-370 (2004).

94. Sanacora, G., Mason, G. F., Rothman, D. L. & Krystal, J. H. Increased occipital cortex GABA concentrations in depressed patients after therapy with selective serotonin reuptake inhibitors. *Am. J. Psychiatry* 159, 663-665 (2002).

95. Uzunova, V. *et al.* Increase in the cerebrospinal fluid content of neurosteroids in patients with unipolar major depression who are receiving fluoxetine or fluvoxamine. *Proc. Natl. Acad. Sci. U. S. A* 95, 3239-3244 (1998).

96. Shekhar, A. Effects of treatment with imipramine and clonazepam on an animal model of panic disorder. *Biol. Psychiatry* 36, 748-758 (1994).

97. Tanay, V. M., Greenshaw, A. J., Baker, G. B. & Bateson, A. N. Common effects of chronically administered antipanic drugs on brainstem GABA(A) receptor subunit gene expression. *Mol. Psychiatry* 6, 404-412 (2001).

98. Lydiard, R. B. The role of GABA in anxiety disorders. *J. Clin. Psychiatry* 64 Suppl 3, 21-27 (2003).

99. Woodman, C. L. & Noyes, R., Jr. Panic disorder: treatment with valproate. *J. Clin. Psychiatry* 55, 134-136 (1994).

100. Keck, P. E., Jr., Taylor, V. E., Tugrul, K. C., McElroy, S. L. & Bennett, J. A. Valproate treatment of panic disorder and lactate-induced panic attacks. *Biol. Psychiatry* 33, 542-546 (1993).

101. Zwanzger, P. *et al.* Vigabatrin decreases cholecystokinin-tetrapeptide (CCK-4) induced panic in healthy volunteers. *Neuropsychopharmacology* 25, 699-703 (2001).

102. Nemeroff, C. B. The role of GABA in the pathophysiology and treatment of anxiety disorders. *Psychopharmacol. Bull.* 37, 133-146 (2003).

103. Gorman, J. M., Kent, J. M., Sullivan, G. M. & Coplan, J. D. Neuroanatomical hypothesis of panic disorder, revised. *Am. J. Psychiatry* 157, 493-505 (2000).

104. Shekhar, A., Keim, S. R., Simon, J. R. & McBride, W. J. Dorsomedial hypothalamic GABA dysfunction produces physiological arousal following sodium lactate infusions. *Pharmacol. Biochem. Behav.* 55, 249-256 (1996).

105. Stork, O. *et al.* Postnatal development of a GABA deficit and disturbance of neural functions in mice lacking GAD65. *Brain Res.* 865, 45-58 (2000).

106. Homanics, G. E. *et al.* Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and hypersensitive behavior. *Proc. Natl. Acad. Sci. U. S. A* 94, 4143-4148 (1997).

107. Hashemi, E., Sahbaie, P., Davies, M. F., Clark, J. D. & DeLorey, T. M. Gabrb3 gene deficient mice exhibit increased risk assessment behavior, hypotonia and expansion of the plexus of locus coeruleus dendrites. *Brain Res.* 1129, 191-199 (2007).

108. DeLorey, T. M. *et al.* Mice lacking the beta3 subunit of the GABAA receptor have the epilepsy phenotype and many of the behavioral characteristics of Angelman syndrome. *J. Neurosci.* 18, 8505-8514 (1998).

109. Culiat, C. T. *et al.* Deficiency of the beta 3 subunit of the type A gamma-aminobutyric acid receptor causes cleft palate in mice. *Nat. Genet.* 11, 344-346 (1995).

110. Ugarte, S. D., Homanics, G. E. & Hammond, D. L. Effect of embryonic knock-down of GABAA receptors on the levels of monoamines and their metabolites in the CNS of the mouse. *Brain Res.* 904, 290-297 (2001).

111. Liljelund, P., Handforth, A., Homanics, G. E. & Olsen, R. W. GABAA receptor beta3 subunit gene-deficient heterozygous mice show parent-of-origin and gender-related differences in beta3 subunit levels, EEG, and behavior. *Brain Res. Dev. Brain Res.* 157, 150-161 (2005).

112. Culiat, C. T., Stubbs, L. J., Montgomery, C. S., Russell, L. B. & Rinchik, E. M. Phenotypic consequences of deletion of the gamma 3, alpha 5, or beta 3 subunit of the type A gamma-aminobutyric acid receptor in mice. *Proc. Natl. Acad. Sci. U. S. A* 91, 2815-2818 (1994).

113. Glykys, J. & Mody, I. Hippocampal network hyperactivity after selective reduction of tonic inhibition in GABA A receptor alpha5 subunit-deficient mice. *J. Neurophysiol.* 95, 2796-2807 (2006).

114. Collinson, N. *et al.* Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the alpha 5 subunit of the GABAA receptor. *J. Neurosci.* 22, 5572-5580 (2002).

115. Gerdjikov, T. V. *et al.* Hippocampal alpha5 subunit-containing GABA(A) receptors are involved in the development of the latent inhibition effect. *Neurobiol. Learn. Mem.* 89, 87-94 (2008).

116. Hauser, J. *et al.* Hippocampal alpha5 subunit-containing GABAA receptors modulate the expression of prepulse inhibition. *Mol. Psychiatry* 10, 201-207 (2005).

117. Ludewig, S., Ludewig, K., Geyer, M. A., Hell, D. & Vollenweider, F. X. Prepulse inhibition deficits in patients with panic disorder. *Depress. Anxiety.* 15, 55-60 (2002).

118. Shalev, A. Y., Bloch, M., Peri, T. & Bonne, O. Alprazolam reduces response to loud tones in panic disorder but not in posttraumatic stress disorder. *Biol. Psychiatry* 44, 64-68 (1998).

119. Constantinou, C., Bolaris, S., Valcana, T. & Margarity, M. Diazepam affects the nuclear thyroid hormone receptor density and their expression levels in adult rat brain. *Neurosci. Res.* 52, 269-275 (2005).

120. Rosenberg, G. The mechanisms of action of valproate in neuropsychiatric disorders: can we see the forest for the trees? *Cell Mol. Life Sci.* 64, 2090-2103 (2007).

121. Arulmozhi, D. K., Veeranjaneyulu, A. & Bodhankar, S. L. Migraine: current therapeutic targets and future avenues. *Curr. Vasc. Pharmacol.* 4, 117-128 (2006).

122. Pande, A. C., Crockatt, J. G., Janney, C. A., Werth, J. L. & Tsaroucha, G. Gabapentin in bipolar disorder: a placebo-controlled trial of adjunctive therapy. Gabapentin Bipolar Disorder Study Group. *Bipolar. Disord.* 2, 249-255 (2000).

123. Hansen, H. C. Interstitial cystitis and the potential role of gabapentin. *South. Med. J.* 93, 238-242 (2000).

124. Sasaki, K. *et al.* Oral gabapentin (neurontin) treatment of refractory genitourinary tract pain. *Tech. Urol.* 7, 47-49 (2001).

125. Book, S. W. & Myrick, H. Novel anticonvulsants in the treatment of alcoholism. *Expert. Opin. Investig. Drugs* 14, 371-376 (2005).

126. Miller, J. L. *et al.* Pituitary abnormalities in Prader-Willi syndrome and early onset morbid obesity. *Am. J. Med. Genet. A* (2007).

127. Perna, G., Bertani, A., Caldirola, D. & Bellodi, L. Family history of panic disorder and hypersensitivity to CO2 in patients with panic disorder. *Am. J. Psychiatry* 153, 1060-1064 (1996).

128. Perna, G., Cocchi, S., Bertani, A., Arancio, C. & Bellodi, L. Sensitivity to 35% CO2 in healthy first-degree relatives of patients with panic disorder. *Am. J. Psychiatry* 152, 623-625 (1995).

129. Reschke, A. H. *et al.* Sodium lactate response and familial risk for panic disorder. *Am. J. Psychiatry* 152, 277-279 (1995).

130. Maddock, R. J. The lactic acid response to alkalosis in panic disorder : an integrative review. *J. Neuropsychiatry Clin. Neurosci.* 13, 22-34 (2001).

131. Klein, D. F. False suffocation alarms, spontaneous panics, and related conditions. An integrative hypothesis. *Arch. Gen. Psychiatry* 50, 306-317 (1993).

132. Wible, J. H., Jr., Luft, F. C. & DiMicco, J. A. Hypothalamic GABA suppresses sympathetic outflow to the cardiovascular system. *Am. J. Physiol* 254, R680-R687 (1988).

133. Sajdyk, T. J. & Shekhar, A. Sodium lactate elicits anxiety in rats after repeated GABA receptor blockade in the basolateral amygdala. *Eur. J. Pharmacol.* 394, 265-273 (2000).

134. Lisa, M., Marmo, E., Wible, J. H., Jr. & DiMicco, J. A. Injection of muscimol into posterior hypothalamus blocks stress-induced tachycardia. *Am. J. Physiol* 257, R246-R251 (1989).

135. Liebowitz, M. R. *et al.* Effects of intravenous diazepam pretreatment on lactate-induced panic. *Psychiatry Res.* 58, 127-138 (1995).

136. Concas, A. *et al.* Carbon dioxide inhalation, stress and anxiogenic drugs reduce the function of GABAA receptor complex in the rat brain. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 17, 651-661 (1993).

137. Aronson, P. R. Evaluation of psychotropic drug therapy in chronic hyperventilation syndrome: intensive study design. *J. New Drugs* 6, 305-307 (1966).

138. Kim, C. *et al.* Midazolam depresses carotid body chemoreceptor activity. *Acta Anaesthesiol. Scand.* 50, 144-149 (2006).

139. Weissman, M. M. *et al.* Potential panic disorder syndrome: clinical and genetic linkage evidence. *Am. J. Med. Genet.* 96, 24-35 (2000).

140. Matthew, E. *et al.* Benzodiazepine receptors mediate regional blood flow changes in the living human brain. *Proc. Natl. Acad. Sci. U. S. A* 92, 2775-2779 (1995).

141. Welch, K. M., Chabi, E., Bartosh, K., Achar, V. S. & Meyer, J. S. Cerebrospinal fluid gamma aminobutyric acid levels in migraine. *Br. Med. J.* 3, 516-517 (1975).

142. Russo, L. *et al.* A new susceptibility locus for migraine with aura in the 15q11-q13 genomic region containing three GABA-A receptor genes. *Am. J. Hum. Genet.* 76, 327-333 (2005).

143. Netzer, C. *et al.* Genetic association studies of the chromosome 15 GABA-A receptor cluster in migraine with aura. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 147B, 37-41 (2007).

144. Oswell, G. *et al.* No association of migraine to the GABA-A receptor complex on chromosome 15. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 147B, 33-36 (2007).

145. Silberstein, S. D. Migraine. *Lancet* 363, 381-391 (2004).

146. Gupta, S. *et al.* Potential role of female sex hormones in the pathophysiology of migraine. *Pharmacol. Ther.* 113, 321-340 (2007).

147. Xilouri, M., Avlonitis, N., Calogeropoulou, T. & Papazafiri, P. Neuroprotective effects of steroid analogues on P19-N neurons. *Neurochem. Int.* 50, 660-670 (2007).

148. Schwartz, R. D., Yu, X., Wagner, J., Ehrmann, M. & Mileson, B. E. Cellular regulation of the benzodiazepine/GABA receptor: arachidonic acid, calcium, and cerebral ischemia. *Neuropsychopharmacology* 6, 119-125 (1992).

149. Rogers, M. P. *et al.* Prevalence of medical illness in patients with anxiety disorders. *Int. J. Psychiatry Med.* 24, 83-96 (1994).

150. Wiens, S. C. & Trudeau, V. L. Thyroid hormone and gamma-aminobutyric acid (GABA) interactions in neuroendocrine systems. *Comp Biochem. Physiol A Mol. Integr. Physiol* 144, 332-344 (2006).

151. Utiger, R. D. Effects of smoking on thyroid function. *Eur. J. Endocrinol.* 138, 368-369 (1998).

152. Martin, J. V., Williams, D. B., Fitzgerald, R. M., Im, H. K. & Vonvoigtlander, P. F. Thyroid hormonal modulation of the binding and activity of the GABAA receptor complex of brain. *Neuroscience* 73, 705-713 (1996).

153. Maksay, G. & Ticku, M. K. Diazotization and thiocyanate differentiate agonists from antagonists for the high- and low-affinity receptors of gamma-aminobutyric acid. *J. Neurochem.* 43, 261-268 (1984).

154. Roman, G. C. Autism: transient in utero hypothyroxinemia related to maternal flavonoid ingestion during pregnancy and to other environmental antithyroid agents. *J. Neurol. Sci.* 262, 15-26 (2007).

155. Breslau, N. & Klein, D. F. Smoking and panic attacks: an epidemiologic investigation. *Arch. Gen. Psychiatry* 56, 1141-1147 (1999).

156. Yamamoto, S. *et al.* Insertion of alpha7 nicotinic receptors at neocortical layer V GABAergic synapses is induced by a benzodiazepine, midazolam. *Cereb. Cortex* 17, 653-660 (2007).

157. Berman, J. A., Roberts, J. L. & Pritchett, D. B. Molecular and pharmacological characterization of GABAA receptors in the rat pituitary. *J. Neurochem.* 63, 1948-1954 (1994).

158. Fritschy, J. M., Paysan, J., Enna, A. & Mohler, H. Switch in the expression of rat GABAA-receptor subtypes during postnatal development: an immunohistochemical study. *J. Neurosci.* 14, 5302-5324 (1994).

159. Danglot, L., Triller, A. & Bessis, A. Association of gephyrin with synaptic and extrasynaptic GABAA receptors varies during development in cultured hippocampal neurons. *Mol. Cell Neurosci.* 23, 264-278 (2003).

160. McKernan, R. M. & Whiting, P. J. Which GABAA-receptor subtypes really occur in the brain? *Trends Neurosci.* 19, 139-143 (1996).

161. Acsády, L., Kamondi, A., Sik, A., Freund, T. & Buzsaki, G. GABAergic cells are the major postsynaptic targets of mossy fibers in the rat hippocampus. *J. Neurosci.* 18, 3386-3403 (1998).

162. Christie, S. B. & De Blas, A. L. alpha5 Subunit-containing GABA(A) receptors form clusters at GABAergic synapses in hippocampal cultures. *Neuroreport* 13, 2355-2358 (2002).

163. Serwanski, D. R. *et al.* Synaptic and nonsynaptic localization of GABAA receptors containing the alpha5 subunit in the rat brain. *J. Comp Neurol.* 499, 458-470 (2006).

164. Caraiscos, V. B. *et al.* Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by alpha5 subunit-containing gamma-aminobutyric acid type A receptors. *Proc. Natl. Acad. Sci. U. S. A* 101, 3662-3667 (2004).

165. Bai, D. *et al.* Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by gamma-aminobutyric acid(A) receptors in hippocampal neurons. *Mol. Pharmacol.* 59, 814-824 (2001).

166. Stell, B. M. & Mody, I. Receptors with different affinities mediate phasic and tonic GABA(A) conductances in hippocampal neurons. *J. Neurosci.* 22, RC223 (2002).

167. Goddard, A. W. *et al.* Reductions in occipital cortex GABA levels in panic disorder detected with 1h-magnetic resonance spectroscopy. *Arch. Gen. Psychiatry* 58, 556-561 (2001).

168. Roy-Byrne, P. P. The GABA-benzodiazepine receptor complex: structure, function, and role in anxiety. *J. Clin. Psychiatry* 66 Suppl 2, 14-20 (2005).

169. Nutt, D. J. & Malizia, A. L. New insights into the role of the GABA(A)-benzodiazepine receptor in psychiatric disorder. *Br. J. Psychiatry* 179, 390-396 (2001).

170. Cameron, O. G. *et al.* Reduced gamma-aminobutyric acid(A)-benzodiazepine binding sites in insular cortex of individuals with panic disorder. *Arch. Gen. Psychiatry* 64, 793-800 (2007).

171. Bremner, J. D. *et al.* SPECT [I-123]iomazenil measurement of the benzodiazepine receptor in panic disorder. *Biol. Psychiatry* 47, 96-106 (2000).

172. Kaschka, W., Feistel, H. & Ebert, D. Reduced benzodiazepine receptor binding in panic disorders measured by iomazenil SPECT. *J. Psychiatr. Res.* 29, 427-434 (1995).

173. Kuikka, J. T. *et al.* Abnormal regional benzodiazepine receptor uptake in the prefrontal cortex in patients with panic disorder. *Nucl. Med. Commun.* 16, 273-280 (1995).

174. Malizia, A. L. *et al.* Decreased brain GABA(A)-benzodiazepine receptor binding in panic disorder: preliminary results from a quantitative PET study. *Arch. Gen. Psychiatry* 55, 715-720 (1998).

175. Eser, D. *et al.* Panic induction with cholecystokinin-tetrapeptide (CCK-4) Increases plasma concentrations of the neuroactive steroid 3alpha, 5alpha tetrahydrodeoxycorticosterone (3alpha, 5alpha-THDOC) in healthy volunteers. *Neuropsychopharmacology* 30, 192-195 (2005).

176. Kash, S. F., Tecott, L. H., Hodge, C. & Baekkeskov, S. Increased anxiety and altered responses to anxiolytics in mice deficient in the 65-kDa isoform of glutamic acid decarboxylase. *Proc. Natl. Acad. Sci. U. S. A* 96, 1698-1703 (1999).

177. Strohle, A. *et al.* Induced panic attacks shift gamma-aminobutyric acid type A receptor modulatory neuroactive steroid composition in patients with panic disorder: preliminary results. *Arch. Gen. Psychiatry* 60, 161-168 (2003).

178. Goddard, A. W. *et al.* Impaired GABA neuronal response to acute benzodiazepine administration in panic disorder. *Am. J. Psychiatry* 161, 2186-2193 (2004).

179. Purdy, R. H., Morrow, A. L., Moore, P. H., Jr. & Paul, S. M. Stress-induced elevations of gamma-aminobutyric acid type A receptor-active steroids in the rat brain. *Proc. Natl. Acad. Sci. U. S. A* 88, 4553-4557 (1991).

180. Barbaccia, M. L. *et al.* Time-dependent changes in rat brain neuroactive steroid concentrations and GABAA receptor function after acute stress. *Neuroendocrinology* 63, 166-172 (1996).

181. Strohle, A. *et al.* GABA(A) receptor-modulating neuroactive steroid composition in patients with panic disorder before and during paroxetine treatment. *Am. J. Psychiatry* 159, 145-147 (2002).

182. Reddy, D. S. Is there a physiological role for the neurosteroid THDOC in stress-sensitive conditions? *Trends Pharmacol. Sci.* 24, 103-106 (2003).

183. Stoffel-Wagner, B. Neurosteroid biosynthesis in the human brain and its clinical implications. *Ann. N. Y. Acad. Sci.* 1007, 64-78 (2003).

184. Kumar, S., Fleming, R. L. & Morrow, A. L. Ethanol regulation of gamma-aminobutyric acid A receptors: genomic and nongenomic mechanisms. *Pharmacol. Ther.* 101, 211-226 (2004).

185. Boshuisen, M. L., Ter Horst, G. J., Paans, A. M., Reinders, A. A. & den Boer, J. A. rCBF differences between panic disorder patients and control subjects during anticipatory anxiety and rest. *Biol. Psychiatry* 52, 126-135 (2002).

186. Lepola, U., Nousiainen, U., Puranen, M., Riekkinen, P. & Rimon, R. EEG and CT findings in patients with panic disorder. *Biol. Psychiatry* 28, 721-727 (1990).

187. Knott, V. J., Bakish, D., Lusk, S., Barkely, J. & Perugini, M. Quantitative EEG correlates of panic disorder. *Psychiatry Res.* 68, 31-39 (1996).

188. Dantendorfer, K. *et al.* High frequency of EEG and MRI brain abnormalities in panic disorder. *Psychiatry Res.* 68, 41-53 (1996).

189. Williams, L. M. *et al.* Arousal dissociates amygdala and hippocampal fear responses: evidence from simultaneous fMRI and skin conductance recording. *Neuroimage.* 14, 1070-1079 (2001).

190. Michael, T., Blechert, J., Vriends, N., Margraf, J. & Wilhelm, F. H. Fear conditioning in panic disorder: Enhanced resistance to extinction. *J. Abnorm. Psychol.* 116, 612-617 (2007).

191. Hoehn-Saric, R., McLeod, D. R., Funderburk, F. & Kowalski, P. Somatic symptoms and physiologic responses in generalized anxiety disorder and panic disorder: an ambulatory monitor study. *Arch. Gen. Psychiatry* 61, 913-921 (2004).

192. Bystritsky, A., Craske, M., Maidenberg, E., Vapnik, T. & Shapiro, D. Autonomic reactivity of panic patients during a CO2 inhalation procedure. *Depress. Anxiety.* 11, 15-26 (2000).

193. Porges, S. W. Vagal tone: a physiologic marker of stress vulnerability. *Pediatrics* 90, 498-504 (1992).

194. Ross, D. C., Preter, M. & Klein, D. F. Hematologic alterations and CO(2) hypersensitivity in male panic disorder patients and normal controls:

similarities to high-altitude hypoxia and chronic lung disease. *Depress. Anxiety.* 14, 153-154 (2001).

195. Papp, L. A. *et al.* Respiratory psychophysiology of panic disorder: three respiratory challenges in 98 subjects. *Am. J. Psychiatry* 154, 1557-1565 (1997).

196. Sheikh, J. I., Leskin, G. A. & Klein, D. F. Gender differences in panic disorder: findings from the National Comorbidity Survey. *Am. J. Psychiatry* 159, 55-58 (2002).

197. Wilson, M. A. Influences of gender, gonadectomy, and estrous cycle on GABA/BZ receptors and benzodiazepine responses in rats. *Brain Res. Bull.* 29, 165-172 (1992).

198. Cosgrove, K. P., Mazure, C. M. & Staley, J. K. Evolving knowledge of sex differences in brain structure, function, and chemistry. *Biol. Psychiatry* 62, 847-855 (2007).

199. Maayan, R. *et al.* Influence of 17beta-estradiol on the synthesis of reduced neurosteroids in the brain (in vivo) and in glioma cells (in vitro): possible relevance to mental disorders in women. *Brain Res.* 1020, 167-172 (2004).

200. Maguire, J. & Mody, I. Neurosteroid synthesis-mediated regulation of GABA(A) receptors: relevance to the ovarian cycle and stress. *J. Neurosci.* 27, 2155-2162 (2007).

201. Sigmon, S. T. *et al.* Psychophysiological, somatic, and affective changes across the menstrual cycle in women with panic disorder. *J. Consult Clin. Psychol.* 68, 425-431 (2000).

202. Turgeon, L., Marchand, A. & Dupuis, G. Clinical features in panic disorder with agoraphobia: a comparison of men and women. *J. Anxiety. Disord.* 12, 539-553 (1998).

203. Yonkers, K. A. *et al.* Is the course of panic disorder the same in women and men? *Am. J. Psychiatry* 155, 596-602 (1998).

204. Yonkers, K. A., Bruce, S. E., Dyck, I. R. & Keller, M. B. Chronicity, relapse, and illness--course of panic disorder, social phobia, and generalized anxiety disorder: findings in men and women from 8 years of follow-up. *Depress. Anxiety.* 17, 173-179 (2003).

205. Eaton, W. W., Kessler, R. C., Wittchen, H. U. & Magee, W. J. Panic and panic disorder in the United States. *Am. J. Psychiatry* 151, 413-420 (1994).

206. Joyce, P. R., Bushnell, J. A., Oakley-Browne, M. A., Wells, J. E. & Hornblow, A. R. The epidemiology of panic symptomatology and agoraphobic avoidance. *Compr. Psychiatry* 30, 303-312 (1989).

207. Green, M. A. & Halliwell, R. F. Selective antagonism of the GABA(A) receptor by ciprofloxacin and biphenylacetic acid. *Br. J. Pharmacol.* 122, 584-590 (1997).

208. Kavvadias, D. *et al.* The flavone hispidulin, a benzodiazepine receptor ligand with positive allosteric properties, traverses the blood-brain barrier and exhibits anticonvulsive effects. *Br. J. Pharmacol.* 142, 811-820 (2004).

209. Charlton, M. E. *et al.* Chronic ethanol administration regulates the expression of GABAA receptor alpha 1 and alpha 5 subunits in the ventral tegmental area and hippocampus. *J. Neurochem.* 68, 121-127 (1997).

210. Bailey, C. D., Brien, J. F. & Reynolds, J. N. Chronic prenatal ethanol exposure alters the proportion of GABAergic neurons in layers II/III of the adult guinea pig somatosensory cortex. *Neurotoxicol. Teratol.* 26, 59-63 (2004).

211. Zimmerberg, B., Drucker, P. C. & Weider, J. M. Differential behavioral effects of the neuroactive steroid allopregnanolone on neonatal rats prenatally exposed to alcohol. *Pharmacol. Biochem. Behav.* 51, 463-468 (1995).

212. Orchinik, M., Weiland, N. G. & McEwen, B. S. Chronic exposure to stress levels of corticosterone alters GABAA receptor subunit mRNA levels in rat hippocampus. *Brain Res. Mol. Brain Res.* 34, 29-37 (1995).

213. Saxe, M. D. *et al.* Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proc. Natl. Acad. Sci. U. S. A* 103, 17501-17506 (2006).

214. Sanchez, M. M., Ladd, C. O. & Plotsky, P. M. Early adverse experience as a developmental risk factor for later psychopathology: evidence from rodent and primate models. *Dev. Psychopathol.* 13, 419-449 (2001).

215. Liu, D., Caldji, C., Sharma, S., Plotsky, P. M. & Meaney, M. J. Influence of neonatal rearing conditions on stress-induced adrenocorticotropin responses and norepinepherine release in the hypothalamic paraventricular nucleus. *J. Neuroendocrinol.* 12, 5-12 (2000).

216. Genest, S. E., Balon, N., Laforest, S., Drolet, G. & Kinkead, R. Neonatal maternal separation and enhancement of the hypoxic ventilatory response in rat: the role of GABAergic modulation within the paraventricular nucleus of the hypothalamus. *J. Physiol* 583, 299-314 (2007).

217. Lee, H. J. *et al.* Fluoxetine enhances cell proliferation and prevents apoptosis in dentate gyrus of maternally separated rats. *Mol. Psychiatry* 6, 610, 725-610, 728 (2001).

218. Raskin, M., Peeke, H. V., Dickman, W. & Pinsker, H. Panic and generalized anxiety disorders. Developmental antecedents and precipitants. *Arch. Gen. Psychiatry* 39, 687-689 (1982).

219. Nusser, Z. & Mody, I. Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. *J Neurophysiol*. 87, 5, 2624-2628 (2002).

220. Shumyatsky, G. P. *et al.* Identification of a signaling network in lateral nucleus of amygdala important for inhibiting memory specifically related to learned fear. *Cell* 111, 905-918 (2002).

221. Ko, L. J. & Engel, J. D. DNA-binding specificities of the GATA transcription factor family. *Mol. Cell Biol.* 13, 4011-4022 (1993).

222. Shimizu, H. *et al.* Identification of an optimal Ncx binding sequence required for transcriptional activation. *FEBS Lett.* 475, 170-174 (2000).

223. Dorfman, D. M., Wilson, D. B., Bruns, G. A. & Orkin, S. H. Human transcription factor GATA-2. Evidence for regulation of preproendothelin-1 gene expression in endothelial cells. *J. Biol. Chem.* 267, 1279-1285 (1992).

224. Iitsuka, Y. *et al.* An enhancer element for expression of the Ncx (Enx, Hox11L1) gene in neural crest-derived cells. *J. Biol. Chem.* 274, 24401-24407 (1999).

225. Herberth, B., Minko, K., Csillag, A., Jaffredo, T. & Madarasz, E. SCL, GATA-2 and Lmo2 expression in neurogenesis. *Int. J. Dev. Neurosci.* 23, 449-463 (2005).

226. Nardelli, J., Thiesson, D., Fujiwara, Y., Tsai, F. Y. & Orkin, S. H. Expression and genetic interaction of transcription factors GATA-2 and GATA-3 during development of the mouse central nervous system. *Dev. Biol.* 210, 305-321 (1999).

227. Crowe, R. R., Noyes, R., Pauls, D. L. & Slymen, D. A family study of panic disorder. *Arch. Gen. Psychiatry* 40, 1065-1069 (1983).

228. Solis-Anez, E. *et al.* Molecular analysis of the GABRB3 gene in autistic patients: an exploratory study. *Invest Clin.* 48, 225-242 (2007).

229. Samaco, R. C., Hogart, A. & LaSalle, J. M. Epigenetic overlap in autism-spectrum neurodevelopmental disorders: MECP2 deficiency causes

reduced expression of UBE3A and GABRB3. *Hum. Mol. Genet.* 14, 483-492 (2005).

230. Chamary, J. V. & Hurst, L. D. Evidence for selection on synonymous mutations affecting stability of mRNA secondary structure in mammals. *Genome Biol.* 6, R75 (2005).

231. Hatfield, D. & Rice, M. Aminoacyl-tRNA(anticodon): codon adaptation in human and rabbit reticulocytes. *Biochem. Int.* 13, 835-842 (1986).

232. Duan, J. *et al.* Synonymous mutations in the human dopamine receptor D2 (DRD2) affect mRNA stability and synthesis of the receptor. *Hum. Mol. Genet.* 12, 205-216 (2003).

233. Liu, H. X., Cartegni, L., Zhang, M. Q. & Krainer, A. R. A mechanism for exon skipping caused by nonsense or missense mutations in BRCA1 and other genes. *Nat. Genet.* 27, 55-58 (2001).

234. Shabalina, S. A., Ogurtsov, A. Y. & Spiridonov, N. A. A periodic pattern of mRNA secondary structure created by the genetic code. *Nucleic Acids Res.* 34, 2428-2437 (2006).

235. Lacerda, A. L., Caetano, D. & Keshavan, M. S. Urinary pH in panic disorder. *Psychiatry Res.* 134, 199-203 (2005).

236. Martin, V.T. & Behbehani, M. Ovarian Hormones and Migraine Headache: Understanding Mechanisms and Pathogenesis—Part I. Headache: The Journal of Head and Face Pain 46 (1), 3–23 (2006).

237. Dudbridge F. Pedigree disequilibrium test for multilocus haplotypes. *Genet. Epidemiol.* 25, 115-121 (2003).

238. Blatt, G. J. *et al.* Density and distribution of hippocampal neurotransmitter receptors in autism: an autoradiographic study. *J. Autism Dev. Disord.* 31, 537-543 (2001).

239. Dhossche, D. *et al.* Elevated plasma gamma-aminobutyric acid (GABA) levels in autistic youngsters: stimulus for a GABA hypothesis of autism. *Med. Sci. Monit.* 8, R1-R6 (2002).

240. Aldred, S., Moore, K. M., Fitzgerald, M. & Waring, R. H. Plasma amino acid levels in children with autism and their families. *J. Autism Dev. Disord.* 33, 93-97 (2003).

241. Iida K., and Akashi H. A test of translational selection at ‘silent’ sites in the human genome: base composition comparisons in alternatively spliced genes. *Gene.* 261, 93–105 (2000).

242. Pierson RC, Lyons AM, and Greenfield LJ, Jr. Gonadal steroids regulate GABAA receptor subunit mRNA expression in NT2-N neurons. *Brain Res.Mol.Brain Res.* 138 (2), 105-115 (2005).

243. Dean B, Scarr E & McLeod M. Changes in hippocampal GABAA receptor subunit composition in bipolar 1 disorder. *Brain Res Mol Brain Res*. 138 (2):145-55 (2005).

244. Gerasimov M.R. and Dewey S.L. Development of a GABAergic treatment for substance abuse using PET. *Drug Development Research* 59 (2):240-248 (2003).

245. Lutz P.L. and Leone-Kabler S.L. Upregulation of the GABAA/benzodiazepine receptor during anoxia in the freshwater turtle brain. *Am.J.Physiol* 268 (5 Pt 2):R1332-R1335 (1995).

246. Nackley A.G. *et al.* Human catechol-O-methyltransferase haplotypes modulate protein expression by altering mRNA secondary structure. *Science* 314 (5807):1930-1933 (2006).

3.8. Figures and tables

Figure 3.1. Two point linkage results for GABRB3 in 120 PD pedigrees
Phenotypic diagnostic categories for panic disorder (PD) are broad (b), intermediate (i), narrow (n), PD with syndromic features (s), or PD with bladder problems (bl), for dominant (dom) or recessive (rec) modes of inheritance. LOD = logarithm of odds, Het = locus heterogeneity, Hom = locus homogeneity, SNP = single nucleotide polymorphism.

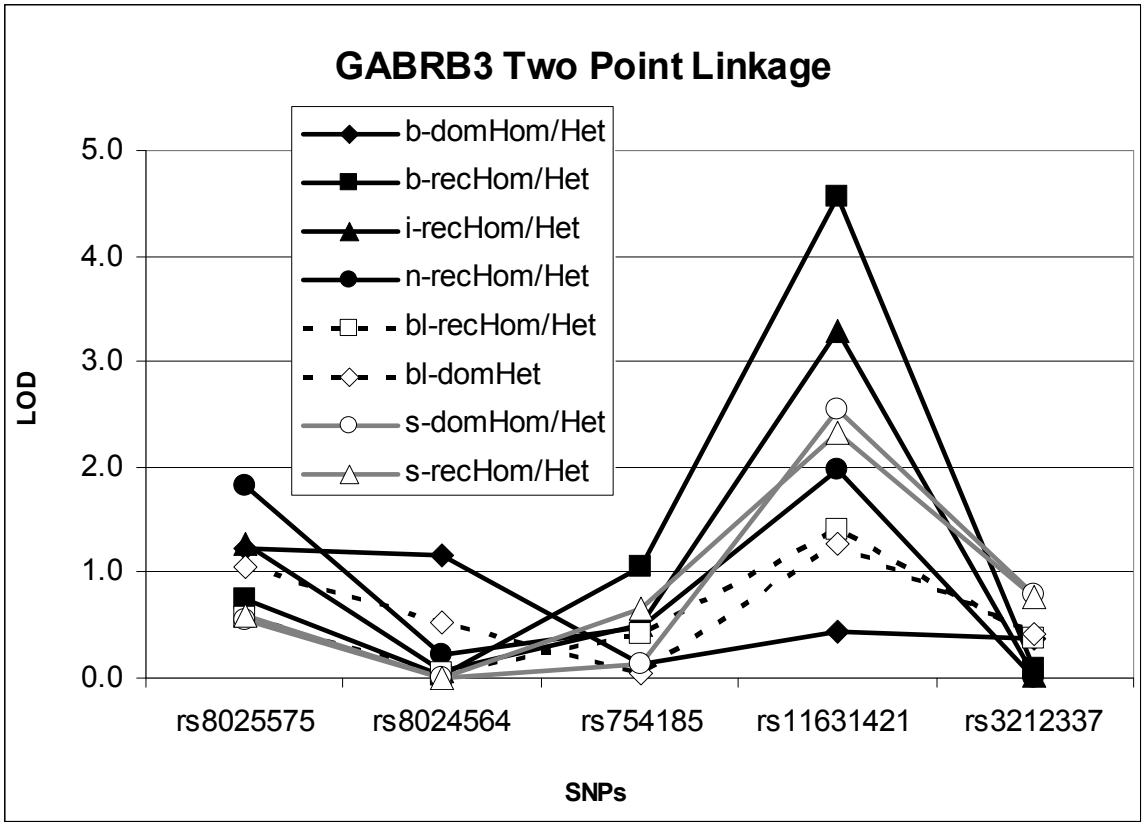

Figure 3.2. Two point linkage results for GABRA5 in 120 PD pedigrees
Phenotypic diagnostic categories for panic disorder (PD) are broad (b), intermediate (i), narrow (n), or PD with bladder problems (bl), for dominant (dom) mode of inheritance. LOD = logarithm of odds, Het = locus heterogeneity, Hom = locus homogeneity, SNP = single nucleotide polymorphism.

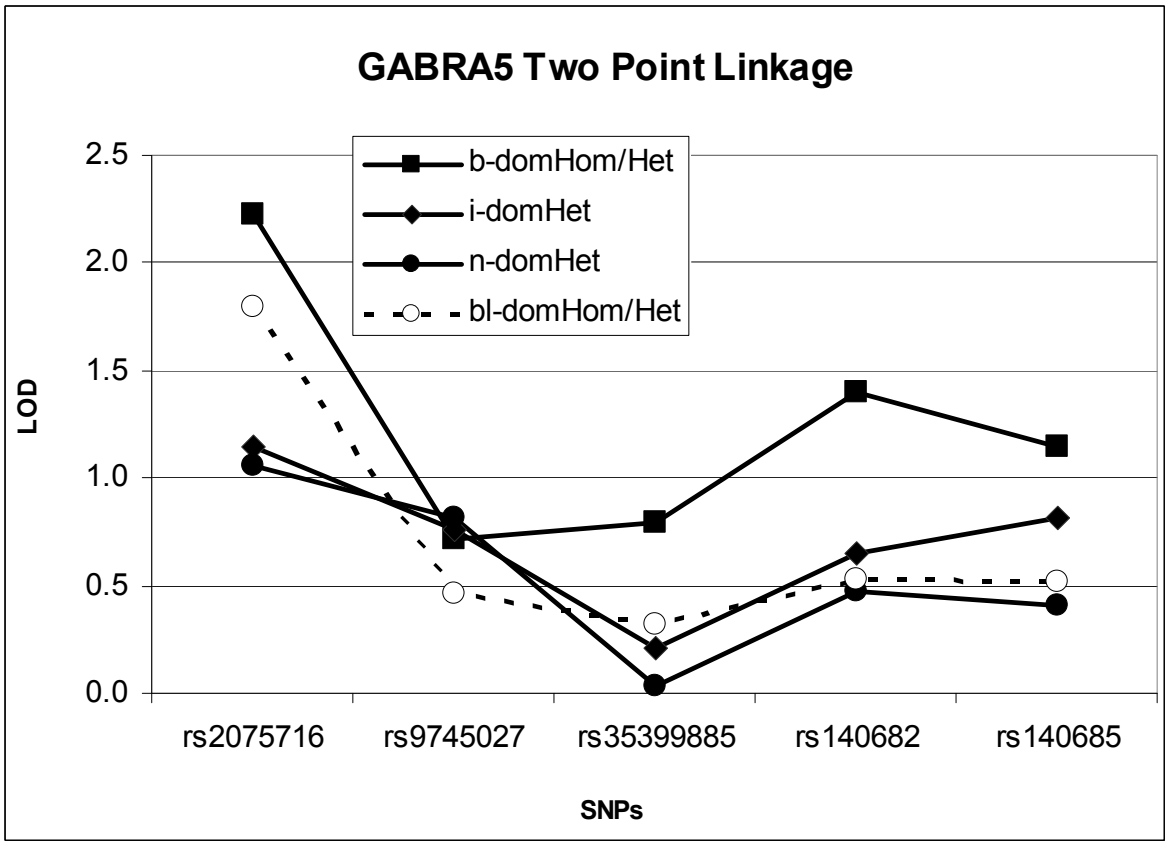

Figure 3.3. Multipoint linkage results for GABRB3 in 120 PD pedigrees

Phenotypic diagnostic categories for panic disorder (PD) are broad, intermediate (intermed), narrow PD, PD with syndromic features (syndrome), and PD with bladder problems (bladder), for dominant (d) or recessive (r) modes of inheritance. Single nucleotide polymorphisms markers are the denotedon the x-axis. LOD = logarithm of odds, Het = locus heterogeneity, cM = centiMorgans. Locus homogeneity scores were negative.

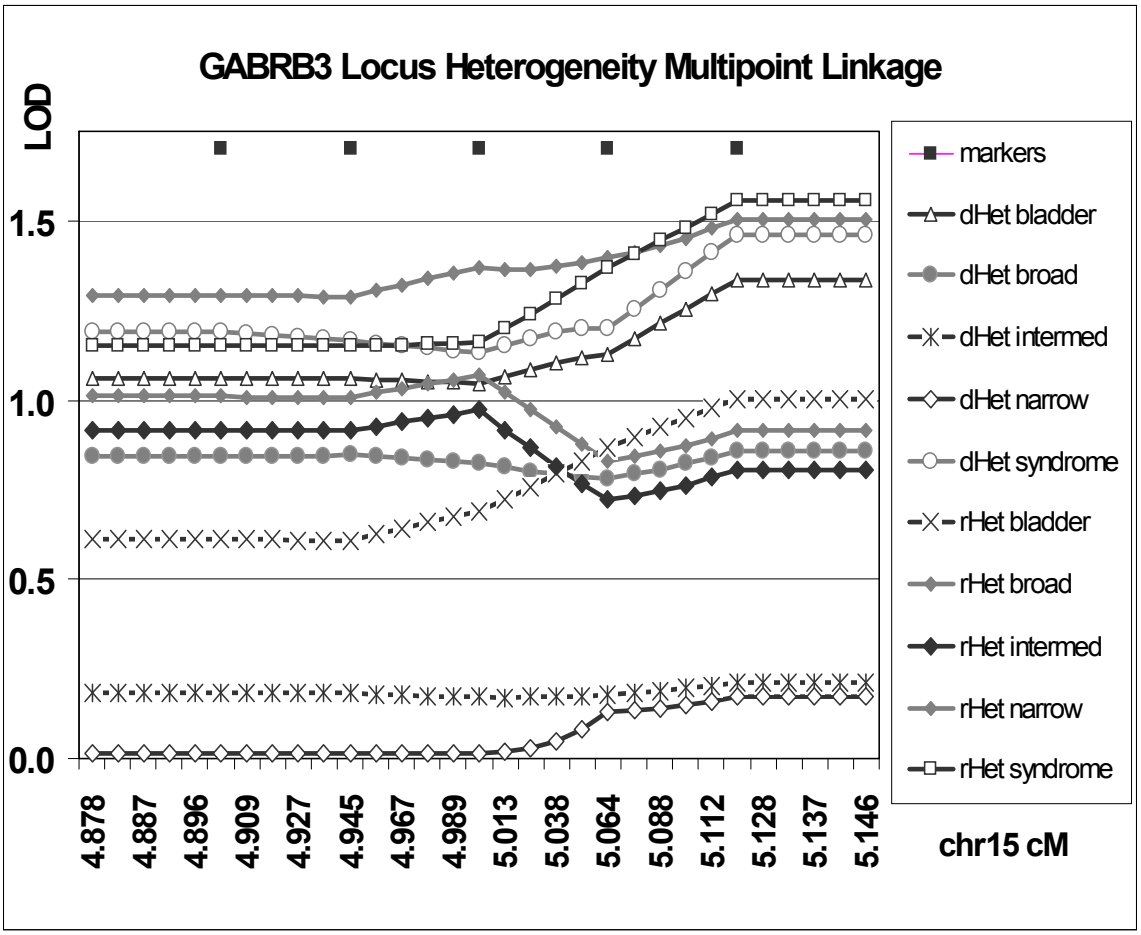

Figure 3.4. Multipoint linkage results for GABRA5 in 120 PD pedigrees

Phenotypic diagnostic categories for panic disorder (PD) are broad, intermediate (intermed), narrow, PD with syndromic features (syndrome), or PD with bladder problems (bladder), for dominant (d) or recessive (r) modes of inheritance. Single nucleotide polymorphisms markers are the denoted on the x-axis. LOD = logarithm of odds, Het = locus heterogeneity, cM = centiMorgans. Locus homogeneity scores were negative.

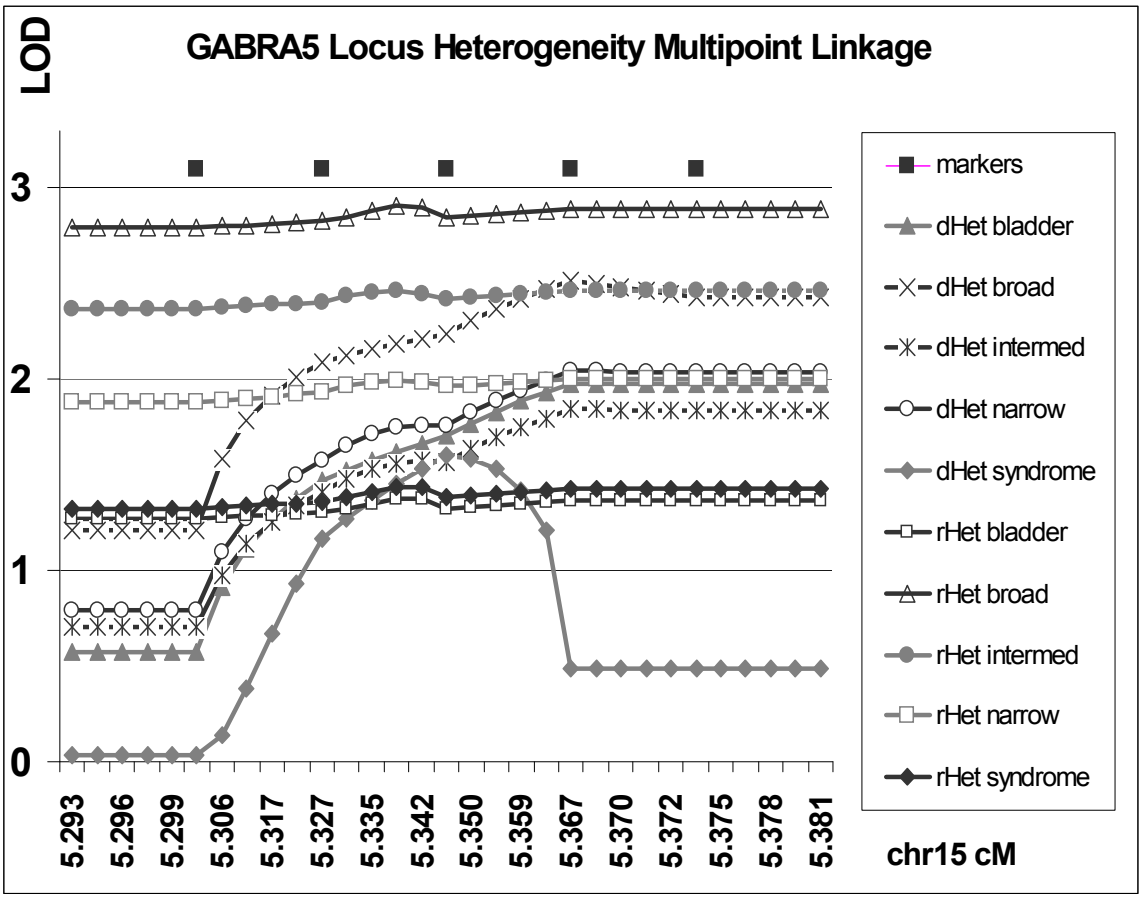

Table 3.1. GABRB3 and GABRA5 TaqMan reagent information

ABI = Applied Biosystems Incorporated.

Marker ID	Variant	Location	ABI Assay ID	Celera ID
GABRB3-01	rs8025575	863bp 3'	C_2911914_10	hCV2911914
GABRB3-02	rs8024564	intronic	C_2901104_10	hCV2901104
GABRB3-03	rs754185	intronic	C_2901163_10	hCV2901163
GABRB3-04	rs11631421	intronic	C_245488_10	hCV245488
GABRB3-05	rs3212337	intronic	C_218360_10	hCV218360
GABRA5-01	rs2075716	intronic	C_1843341_1	hCV1843341
GABRA5-02	rs9745027	intronic	C_27725_10	hCV27725
GABRA5-03	rs35399885	intronic	C_42974_10	hCV42974
GABRA5-04	rs140682	synonymous	C_1028938_1	hCV1028938
GABRA5-05	rs140685	synonymous	C_1028939_1	hCV1028939

Table 3.2. Sequence primer information for GABRB3 and GABRA5 mutation screens

Numerical rows indicate amplicon number, corresponding to table 3.3.

	Location	Chr15 May 2004 NCBI position	Forward Primer	Reverse Primer
1	GABRB3 3'UTR	24339689-24340395	cataacccaaaagggcatgt	tccacctgaaaaccagttca
2	GABRB3 3'UTR	24340330-24341166	gtttcaccaattgttcagttttg	attctgaacccggacctctgac
3	GABRB3 3'UTR	24341076-24341784	tccccaatgataacgtttctg	tggacaaaaacagtcaatggtc
4	GABRB3 3'UTR	24341597-24342276	atccagaaaaccttgggaag	gaggatgcgtgtggtgtg
5	GABRB3 exon 9	24342168-24342852	tgggttttgaactgttgcag	catcctcatgcctttatttctg
6	GABRB3 exon 9	24342709-24343405	ctcctttgcctcagagaacg	ggcagtgtgcttacctgttg
7	GABRB3 exon 9	24343242-24343943	gaagctggatccactccaag	acacacaagcagacacacaca
8	GABRB3 exon 9	24343729-24344423	actttctttaatatgcatcctgtgg	gaaattatgctgctggctgtc
9	GABRB3 exon 8	24357103-24357798	tcatgcaagtaaatgcagtagc	tgctaaattctgggcttattttt
10	GABRB3 exon 7	24363323-24364021	tccagctgtcaaataaccaatg	tctgccatgttgtttctcca
11	GABRB3 exon 6	24376234-24376923	ctgcctccaaggtcatcaat	ccagcctctcgacttttcag
12	GABRB3 exon 5	24379030-24379749	cctgatatgccaggaggtgt	gttcccacaggggcttactc
13	GABRB3 exon 4	24417275-24417983	ccatcctgagggaatctgtg	tgacacacacagactcatgtacc
14	GABRB3 intron 3	24427299-24428020	ggaggcaaggaaagtattgc	gggaggagagttggttgtga
15	GABRB3 intron 3	24462161-24462815	gaaatggcacgtgtggtaag	gtcaggctctcagtggcttg
16	GABRB3 intron 3	see primer 3	ggctgcaaaattatgggaaa	cactcacacacatgcacagg
17	GABRB3 exon 2, 3, partial 1	24568571-24569179	actgtggacgcctgtgat	gcttttcggcatcttctcg
18	GABRB3 exon 3	24568586-24568736	gatcgccgtgtcctgtgctt	gatcccagacagcgggcc
19	GABRB3 exon 1a, 5'	24569856-24570059	gggggtaggggcggggatc	tgcagaacgccgggaagcc
20	5' of GABRB3	24570431-24571127	tcattagaaggctactggcgcactgg	ccagcagggcatttctccaaaagaac
21	5' of GABRB3	24570661-24571249	catcaagctcgtttttgtgttc	tgtaatgtggctttgcaagtct
22	GABRA5 5'UTR	24663078-24663818	ctatcacccgactgctgacact	cagtcgccagggtaaagtttg

	Location	Chr15 May 2004 NCBI position	Forward Primer	Reverse Primer
23	GABRA5 5'UTR	24664932-24665357	tgtgcttttccccacttctt	cctggaatcaaatcacactgc
24	GABRA5 exon 1, 5'UTR	24665352-24665854	tccaggaactgtgacacaaca	tcacagctgactcggatatcat
25	GABRA5 exon 2	24676965-24677655	tgttctcagatcctcgtctca	aaggatgactgcattccaca
26	GABRA5 exon 5	24742482-24743178	ccgaagtgctgagtagatttga	cacagcaatgattacagcacaa
27	GABRA5 exon 6	24764928-24765661	tccaaccaccctatgcattt	ttgagagcatctgcttccttc
28	GABRA5 exon 7	24767473-24768236	ggggaggctaaatcctgaaat	ggcaatgtcaaaccacacaa
29	GABRA5 exon 8	24770882-24771651	cacaagaatcgcttgaacca	accctgcatggtgaaatga
30	GABRA5 exon 9, 3'UTR	24775789-24776495	caaatggcgtgtccttacatt	ttcactgggaaaatttgtatgaaa
31	GABRA5 3'UTR	24776419-24777156	tttttaactgcttcaagtgttacct	tcaggaaatgagtccaccaa

Table 3.3. GABRB3 and GABRA5 mutation screen sequencing reactions conditions

TD = touchdown, cyc = cycles, s = seconds, m = minutes, C = Celsius, numerical rows indicate amplicon number, corresponding to table 3.2.

	dNTP μM	Salt	Adjuncts	Primer μM	Polymerase	DNA ng/μL	PCR conditions
1	50	1.5mM $MgCl_2$	1.5M betaine	0.2	0.05U/uL Plat Taq	4	Usual TD method
2	200	1.5mM $MgCl_2$	1.5M betaine	0.2	0.05U/uL Plat Taq	3	Usual TD method with 42 post-TD cyc
3, 5-7, 10, 13-15	50	1.5mM $MgCl_2$	1.5M betaine	0.2	0.05U/uL Plat Taq	2	Usual TD method
4	200	2.5mM $MgCl_2$	1.5M betaine	0.4	0.05U/uL Plat Taq	2.5	94Cx3m hotstart; 7cyc: 94Cx30s melt, 65-59C TDx30s at -1C/cyc anneal, 72Cx30s extend; 42cyc: 94Cx30s melt, 58Cx30s anneal, 72Cx30s extend; 72Cx10m extend
8	200	2.5mM $MgCl_2$	1.5M betaine	0.2	0.05U/uL Plat Taq	2	94Cx5m hotstart; 45cyc: 90Cx30s melt, 58Cx30s anneal, 72Cx30s extend; 72Cx10m extend
9	50	1.5mM $MgCl_2$	1.5M betaine	0.2	0.05U/uL Plat Taq	2	94Cx5m hotstart; 45cyc: 90Cx30s melt, 58Cx30s anneal, 72Cx30s extend; 72Cx10m extend
11-12, 16	200	1.5mM $MgCl_2$	1.5M betaine	0.2	0.05U/uL Plat Taq	2	Usual TD method
17	200	1x conc LA buffer	1.5M betaine	0.5	0.04 U/uL AccuTaq LA	2	94Cx1m hotstart; 45cyc: 94Cx30s melt, 60Cx15s anneal, 68Cx5m extend; 68Cx30m extend
18	200	2.5 mM GeneAmp GOLD	none	0.5	0.03U/uL Ampli Taq GOLD pol	2	95Cx12m hotstart; 5cyc: 95Cx15s, 72-68C TDx20s at -1C/cyc; 33cyc: 95Cx15s melt, 68x15s anneal, 72x30s extend

	dNTP μM	Salt	Adjuncts	Primer μM	Polymerase	DNA ng/μL	PCR conditions
19	200	2.5 mM GeneAmp GOLD	none	0.5	0.03U/uL Ampli Taq GOLD pol	5	95Cx12m hotstart; 5cyc: 95Cx15s, 72-68C TDx20s at -1C/cyc; 33cyc: 95Cx15s melt, 68x15s anneal, 72x30s extend
20	250	2.75mM BD mix $Mg(OAc)_2$	1.25M BD GC-Melt	0.25	1x conc BD Adv pol mix	2	95Cx1m hotstart; 40cyc x 95Cx20s melt, 65Cx30s anneal, 68Cx4m extend; 68Cx3m extend
21	200	1.5mM $MgCl_2$	1.5M betaine	0.2	0.05U/uL Plat Taq	2	94Cx5m hotstart; 45cyc: 90Cx30s melt, 58Cx30s anneal, 72x30s extend, 72Cx10m extend
22	100	1.5mM $MgCl_2$	1.25M betaine, 5% DMSO	0.2	0.075U/uL Plat Taq	2.5	95Cx5m hotstart; 10cyc: 94Cx30s melt, 65-60.5C TDx30s at -0.5C/cyc, 72Cx45s extend; 38cyc: 94Cx30s melt, 60Cx30s anneal, 72Cx45s exten; 72Cx10m extend
23, 25-30	50	1.5mM $MgCl_2$	1.0M betaine	0.2	0.05U/uL Plat Taq	2	Usual TD method
24	50	1.5mM $MgCl_2$	1.5M betaine	0.15	0.05U/uL Plat Taq	2	Usual TD method
31	100	1.5mM $MgCl_2$	1.5M betaine	0.4	0.05U/uL Plat Taq	2	94Cx3m hotstart; 45cyc: 90x30s melt, 62x30s anneal, 72x45s extend; 72Cx10m extend

Table 3.4. Association results ($p \leq 0.05$) for GABRB3 and GABRA5 SNPs in 120 PD pedigrees

Phenotypic diagnostic categories for panic disorder are broad, intermediate, or narrow. NS = non-significant, FBAT = family-based association test.

Association *p*-values from FBAT Ananlysis				
Marker ID	**Variant**	**Broad**	**Intermediate**	**Narrow**
GABRB3-01	rs8025575	0.02	NS	NS
GABRB3-02	rs8024564	0.005	0.02	0.03
GABRA5-03	rs35399885	0.05	NS	NS

Table 3.5. Association results ($p \leq 0.05$) for two-marker GABRB3 haplotypes, protective against PD.

Haplotype ID	Variant 1	Variant 2	Global *p*-value	Haplotypic *p*-value	D'	Phenotype
GABRB3-01,-02	rs8025575	rs8024564	0.03	0.003	0.89	Broad
GABRB3-01,-05	rs8025575	rs3212337	0.05	0.005	0.31	Broad
GABRB3-03,-05	rs754185	rs3212337	0.05	0.011	0.06	Broad

Table 3.6. Mutation screen results for GABRB3

Variations observed in a mutation screen of GABRB3 in 26-92 PD probands depending upon the amplicon. MAF = minor alllele frequency.

GABRB3 Mutation Screen				
May 2004 NCBI position	**Variant**	**Location**	**Alleles**	**PD MAF**
chr15:24568629	rs8179186	intronic	C>T	0.17
chr15:24427342	rs751994	intronic	G>A	0.30
chr15:24462207	novel	intronic	T>C	0.01
chr15:24462234	novel	intronic	G>A	0.06
chr15:24462237	rs11161328	intronic	T>C	0.22
chr15:24462683	rs7181175	intronic	C>T	0.01
chr15:24462711	novel	intronic	C>G	0.02
chr15:24462768	rs4567674	intronic	G>A	0.03
chr15:24471663	rs7164718	intronic	C>T	0.39
chr15:24569890	rs20318	synonymous	C>T	0.18
chr15:24570030	rs20317	intronic	C>G	0.32
chr15:24417406	rs5811434	intronic	del>T	0.14
chr15:24417831	novel	intronic	G>A	0.50
chr15:24417301	novel	intronic	C>G	0.02
chr15:24417317,8	novel	intronic	TA>del	0.35
chr15:24417359	novel	intronic	del>T	0.50
chr15:24379094	rs2033420	intronic	G>A	0.01
chr15:24379208	novel	intronic	A>C	0.01
chr15:24379261	novel	intronic	G>A	0.01
chr15:24379341	novel	intronic	C>T	0.01
chr15:24357157	rs3751582	intronic	A>G	0.15
chr15:24357453	novel	intronic	C>G	0.02
chr15:24357486	rs3751583	intronic	A>G	0.06
chr15:24357752	rs11631853	intronic	C>T	0.41
chr15:24343508	rs11637141	3'UTR	C>T	0.21
chr15:24341934	novel	3'UTR, NCX TFBS	G>A	0.01
chr15:24341930	novel	3'UTR, GATA TFBS	C>T	0.01
chr15:24340255	rs2017247	3'UTR	G>A	0.25

Table 3.7. Mutation screen results for GABRA5

Variations observed in a mutation screen of GABRA5 in 38-92 PD probands depending upon the amplicon. MAF = minor alllele frequency.

GABRA5 Mutation Screen				
May 2004 NCBI position	**Variant**	**Location**	**alleles**	**PD MAF**
chr15:24663219	rs8036439	5'UTR	G>C	0.24
chr15:24663263	rs25411	5'UTR	G>A	0.36
chr15:24663255	rs25410	5'UTR	G>A	0.36
chr15:24663615	rs8041106	intronic	G>C	0.23
chr15:24664985	novel	5'UTR	G>T	0.002
chr15:24665132	novel	intronic	A>G	0.003
chr15:24665214	novel	intronic	T>C	0.003
chr15:24665298	novel	intronic	A>G	0.003
chr15:24677377	rs9286393	intronic	A>C	0.49
chr15:24677361	novel	intronic	A>G	0.01
chr15:24677001	novel	intronic	G>A	0.01
chr15:24765544	rs11630089	intronic	A>G	0.09
chr15:24765402	novel	intronic	T>C	0.49
chr15:24765103	rs140682	synonymous	G>A	0.49
chr15:24764958	rs140681	intronic	C>T	0.10
chr15:24768103	novel	intronic	C>T	0.13
chr15:24770953	novel	intronic	G>A	0.04
chr15:24771081	rs140683	intronic	A>T	0.38
chr15:24771099	rs140684	intronic	T>G	0.12
chr15:24771205	rs140685	synonymous	A>G	0.49
chr15:24771246	novel	synonymous	G>A	0.01
chr15:24771450	novel	intronic	G>A	0.01
chr15:24771551	novel	intronic	A>G	0.01
chr15:24776393	novel	3'UTR	C>T	0.05
chr15:24776355	novel	3'UTR	A>G	0.01
chr15:24776075	novel	3'UTR	T>C	0.01
chr15:24775871	rs2229942	synonymous	T>C	0.07
chr15:24775813	novel	intronic	T>C	0.01
chr15:24776533	novel	3'UTR	T>C	0.02

Table 3.8. Genotypic association ($p \leq 0.05$) of GABRB3 expression in 35 unphenotyped Caucasians, given 10,000 permutations

Cell lines from 35 unphenotyped Caucasians consisted of 6 dyads, 1 trio, and 20 additional unrelateds individuals for analysis. MAF = minor allele frequency, r^2 = measure of linkage disequilibrium between variants.

Variant	May 2004 NCBI position	Location	$-\log_{10}p$ permuted	Variants captured $r^2=1.0$, MAF≥0.05
rs2315904	chr15:24518365	GABRB3 intron	4.0	rs2315903
rs10519566	chr15:24526713	GABRB3 intron	3.2	N/A
rs6576613	chr15:24585909	5' of GABRB3	2.2	rs12595843, rs7497801
rs7183628	chr15:24546570	GABRB3 intron	2.1	N/A

4. Chapter Four. The Genetic Determinants of COMT Expression*

4.1. Abstract

Catechol-*O*-methyltransferase (COMT) is a ubiquitous enzyme that mediates neurotransmission, autonomic homeostasis, and xenobiotic/endobiotic elimination via phase II biotransformation of substrates, such as the catecholamines and catechol estrogens. A convergence of positional and biological evidence implicates COMT in the susceptibility of panic disorder (PD). Our lab previously showed genetic linkage to several variants within and near the COMT locus in seventy PD pedigrees. Including our study, the non-synonymous single nucleotide polymorphism (SNP), rs4680, has been significantly associated to PD in five independent study populations, although the valence of allelic association is inconsistent. For example, rs4680 has been associated with PD in different studies, although populations differ in their association to the valine or methionine allele. The SNP rs4680 is a natural candidate for phenotypic association to COMT-related disorders due to its correlation with variation in COMT activity, however, the literature is replete with contradictory data, such as with PD. Population-specific patterns of linkage disequilibrium (LD), contributing to large ethnic haplotype diversity, could inform, or explain the discordant data. Allelic association to opposite alleles of the same variant in different studies may be explained by a causal, pan-ethnic variant that is harbored on the population-specific haplotype. Our approach is to characterize the pattern of COMT

* The material in this chapter is in preparation for publication (Hodges LM *et al*.)

expression in a control Caucasian population for association with potential *cis* regulatory variants, with the goal of identifying COMT mutations imparting potential functional consequences on COMT activity. Our analysis reveals that variation in steady-state expression of COMT is heritable. The variants found to cosegregate with COMT expression patterns may be used for comparison in other ethnic groups and/or disease populations to pinpoint etiological factors in COMT dysregulation, and perhaps PD, that are generalizable to all populations.

4.2. Background

4.2.1. COMT function and distribution

Catechol-*O*-methyltransferase (COMT) is a key deactivating enzyme that catalyzes the transfer of a methyl group from S-adenosyl-L-methionine (SAM) coenzyme to the *meta*- or *para*- hydroxyl substituents of catechol substrates. COMT terminates dopaminergic and noradrenergic neurotransmission, mediates physiological processes, and biotransforms xenobiotic and endobiotic toxins[2]. It is distributed throughout the human brain[3] at neuronal and extraneuronal sites, in the cortex, striatum, and spinal sensory ganglia[4,5] to mediate cognition, information processing, behavioral affect, and nociception. Expression of COMT in the hypothalamus, thyroid, adrenal gland, pituitary, pancreas, and kidney serves to mediate autonomic homeostasis, such as salt-water balance and blood pressure control[6]. In the gut, liver, lung, and kidney, COMT serves both roles of detoxification and physiological regulation. The role of COMT in blood/spinal fluid-brain barriers (cerebromicrovascular smooth muscle, epithelial capillaries of

the choroid plexus, ependymal cells of the ventricles)[7,8], blood-gut barrier (epithelia of stomach, intestinal mucosa, duodenum, and ileum), uterus[7], and placenta[9] protects against toxins. COMT distribution in somatostatin- and enkephalin-secreting cells reflects its endocrine function. Examples include anxiety reduction[10], mediation of autonomic signaling, nociception, and learning and memory[11]. Due to its ubiquity, dysregulation of COMT is associated with pleiotropic systemic and neurological disorders.

4.2.2. The valine to methionine SNP, rs4680

4.2.2.1. Variation in COMT activity correlated to rs4680

The much studied non-synonymous single nucleotide polymorphism (SNP), rs4680 (formerly rs165688, also val158met, val108met), is associated with variation in COMT activity in humans[12] and is therefore a natural candidate for COMT dysfunction. The rs4680 G>A nucleotide transition in the fourth exon of COMT, results in a valine (val) to methionine (met) residue change that exhibits greater protein thermolability[13]. This SNP is associated with 70% to 90% reduced protein in liver[14], and 40% to 80% (1.6 to 5-fold) less activity in red blood cells, liver, kidney and the duodenum[13-17]. A parallel decrease of ~40% (1.6-fold less) activity for the rs4680 A (met) allele is seen in lymphocytes and in the brain in Caucasians and African Americans[18]. Kinetic studies *in vitro* using heterogously expressed recombinant COMT and protein fractions from different tissues, show that the rs4680 SNP is the principal genetic determinant of a trimodal distribution of high, intermediate, and low COMT activity, correlated with

the G/G (val/val), G/A (val/met), and A/A (met/met) genotypes, respectively[12].

4.2.2.2. Other contributions to COMT activity

Segregation analysis attributes only 59% of the variance in COMT activity to a biallelic major gene locus[19], with an additional 27% due to polygenic inheritance[20], and 14% undetermined. Allele-specific expression studies in heterozygous individuals show that the rs4680 A (met) allele is overexpressed relative to the rs4680 G (val) allele, which might represent a compensatory mechanism for low activity[21,22]. *In vitro* studies also show that the more thermolabile rs4680 A (met) allele can be stabilized by SAM coenzyme, suggesting that COMT kinetics may depend upon the physiological availability of SAM[13]. Conformational changes caused by the rs4680 A (met) allele is predicted to expose a number reactive cysteines in the protein, allowing for greater inactivation by exogenous hormones[23] and protein-glutathione adduct formation under oxidative conditions[24]. So, a portion of the variability in COMT activity is owed to allele-specific transcriptional regulation, exogenous and endogenous stabilizing or inactivating factors, and physiological conditions associated with the rs4680 A (met) allele. Finally, there are significant gender differences in COMT activity that are unexplained by rs4680, and certain ethnic groups are nearly monomorphic at this locus. Thus, the presumption of COMT activity being a direct reflection of the rs4680 genotype may be an oversimplification of the true *in vivo* situation.

4.2.3. COMT substrates, metabolites, and inhibitors

4.2.3.1. The catecholamines

The catecholamines (dopamine, norepinephrine, and epinephrine) are substrates for COMT that are stored in vesicles, each with signature effects[25] upon their release or leakage from neuronal and endocrine cells[26]. Dopamine (DA) in the basal ganglia is associated with cognition, emotions, motor control, learning, pleasure and drive. Central DA levels are elevated in schizophrenia (SCZ) and low in Parkinson's disease. Peripheral DA acts as a natriuretic hormone to regulate blood pressure, diuresis, and natriuresis[25,27]. Norepinephrine (NE) contained in neurons of the locus coeruleus, project to the cortex, hippocampus, thalamus and midbrain, to mediate attention and arousal, potentiated by amphetamines. In the peripheral sympathetic nervous system (SNS), NE induces vasoconstriction, blood pressure elevation, and bladder relaxation. NE levels rise with anoxia, migraine[28], aggression[29], "fight or flight" stress, and accumulates with cardiac ischemia (hypoxia)[30] and injury. COMT is critical in clearing ischemic accumulation of NE[30].

Tricyclic antidepressants (TCA) used to treat PD, enuresis, migraine, and neuropathic pain, paradoxically cause NE levels to rise, although side effects include tachycardia and anxiogenesis, due to dose-sensitive noradrenergic effects. The symapthomimetic, cocaine, which causes accumulation of NE, DA, and the indole monoamine, serotonin (5HT) by non-specific blockade of their respective reuptake transporters, can trigger panic attacks in chronic users[31]. Epinephrine (E) is tightly regulated by the hypothalamic-pituitary-adrenal (HPA) axis, and disproportionally increases over NE levels[32] in response to

hypoglycemia and symptoms reminiscent of panic attacks, such as asphyxiation, fainting, fear, and emotional distress. Endocrine effects of E include increased heart rate, vasodilation, and activation of energy stores, with spillover associated with panic attacks, severe exercise, and essential hypertension in the heart[33]. Paroxysmal hypertension and panic (anxiety, palpitations, and dyspnea) are symptomatic of E-secreting adrenal tumors[25].

Comorbidities of PD, such as substance abuse, and syndromic features of PD, such as migraine, enuresis, bladder or renal problems with secondary hypertension, mitral valve prolapse[34], and cardiovascular disorders[35], overlap with catecholaminergic effects, including downstream symapthomimetic effects[36] of the *meta* metabolites of COMT. Meta metabolites of COMT have high affinity for the trace amine associated receptor (TAAR1, formerly TAR1)[37], which is implicated in migraine[197], and is the target of substances of abuse (e.g. ecstasy, amphetamines, lysergic acid diethylamide-like hallucinogens[38]), and is hypothesized to be essential for cocaine sensitivity[39]. This may explain the mechanisms underlying the propensity for substance abuse in subjects with PD.

4.2.3.2. Other COMT substrates

Other COMT substrates include L-3,4-dihydroxyphenylalanine (L-DOPA)[40], and alpha-methyldopa[41]. Compounds that act as substrate and inhibitor include dietary flavonoids (e.g. in green and black tea) with both anti-oxidant/cytoprotective[42] and cancer-promoting[43] effects, as well as oncogenic catechol estrogens[7], and the COMT pro-oxidant reaction by-product, homocysteine[44], which is implicated in cardiovascular disease, Parkinsonism,

and Alzheimer's disease. Variation in COMT is associated with altered antioxidant capacity and redox homeostasis in autism[194], and cancer risk due to reduced ability to clear mitogenic catechol estrogens in hormone replacement drugs[1,152,193-196]. COMT inhibitors, such as tolcapone and entacapone, are used as adjuncts to potentiate levodopa and reduce anxiety in Parkinsonism[45], induce natriuresis with intrarenal DA increases[46], reduce levodopa-induced elevations in plasma homocysteine[47], and have been shown to enhance avoidance memory and learning among the male rats[48]. And anxiolytic properties of COMT inhibitor, *Rhodiola rosea*, occur with increased attention span, memory, and 60% decreased COMT activity[49]. Thus, COMT contributes to salt-water balance, memory, anxiety, and cellular oxidative homeostasis implicated in neurological and cardiovascular disease.

4.2.3.3. Abnormal COMT metabolite levels in PD

Altered steady-state levels of COMT metabolites are associated with PD probands at baseline, and with PD drugs that alter catecholaminergic tone, by shunting the system reaction pathway in favor of COMT. For example, drugs that target the alpha-2-adenoreceptor (ADRA2A) alter NE levels (with agonism resulting in feedback inhibition of NE release), and TCAs enhance DA, NE, and 5HT by inhibiting reuptake transporters for NE and 5HT (i.e. NET, SERT). TCAs cause DA accumulation by inhibiting NET, which has higher affinity for DA than NE[50]. The TCA desipramine, used to treat PD, enuresis, neuropathic pain, and phobic disorder, elevates DA and NE in the frontal cortex[50]. In subjects with comorbid PD and social anxiety, lower baseline levels of HVA in the

cerebrospinal fluid (CSF) are observed relative to controls[51]. Anxiety scores for people with PD are modestly correlated to COMT metabolite levels in the CSF[52], and significantly decreased with anxiolytic TCAs[53]. Administration of NE-reducing ADRA2A agonists, such as clonidine, (anxiolytic, antimigraine, antihypertensive), and NE-enhancing ADRA2A antagonists, like panicogenic yohimbine, in subjects with PD with or without agoraphobia reveals significantly exaggerated decreases and increases in plasma COMT metabolites relative to controls, respectively[54-56]. An herbal anxiolytic, Kampo, which has shown efficacy in a small trial of PD subjects[57], increases cerebral COMT metabolite levels, with concomitant diminution of striatal DA flux in mice[58]. Thus, catecholaminergic tone is important for endogenous and pharmacological control of anxiety and other symptoms associated with PD.

Catecholaminergic effects, seen by changes in COMT metabolite levels, follow “fear network” relays in the brain. Electrical stimulation of NE flux in the locus coeruleus (the panic/stress and main homeostatic control center) causes increases in DA and COMT metabolites in the hippocampus (memory center). Although COMT prevents the entry of substrates from the blood into the brain[8], plasma COMT metabolite ratios parallel central COMT activity[59], via communication between the central and peripheral compartments. For example, enkephalin-mediated changes in catecholaminergic tone in the periphery sends autonomic signals to the brain to influence learning and memory[11] via sensory ganglia and circumventricular organs. Ideal for the study of psychiatric

phenotypes, COMT activity and expression in peripheral blood lymphocytes reflects central COMT.

4.2.4. Compensatory mechanisms to COMT

Postsynaptic and glial COMT activity in the central nervous system (CNS) is tempered by the contribution of multiple pathway genes for the biosynthesis, release and clearance of COMT substrates. Key catecholaminergic pathway genes include synthesizing tyrosine hydroxylase (TH), storage and release by vesicular monoamine transporters (VMATs) and chromogranin (CHGA), extracellular clearance by high affinity (synaptic) or high capacity (glial) DA and NE reuptake transporters (DATs, NETs), and mitochondrial deamination by monoamine oxidases (MAOs) at presynaptic (MAO-A) and glial (MAO-B) sites[60]. Thus, pharmacological blockade or targeted deletion of COMT causes no gross deleterious consequences[61], and few changes in catecholamine levels at baseline. COMT is of particular importance in regulating dopaminergic tone in the prefrontal cortex (PFC) [62,63] due to a regional paucity of DAT expression[64,65], where postsynaptic *O*-methylation of DA to 3-*O*-methoxydopa comprises >60% of total PFC DA metabolism[66]. In the PFC, NET[67], with greater affinity for DA than DAT[50], also contributes. As a result, administration of COMT and NET inhibitors, tolcapone and desipramine, raise DA and NE in the PFC.

Feedback regulation of other pathway genes in response to COMT also highlights the relative impact of COMT in the system. In COMT null mice little compensatory regulation of pathway genes was observed[62]. However, in

humans, increased TH mRNA expression was observed for in brain tissue of healthy controls with a COMT SNP associated with higher *O*-methylation activity, as a putative feed-forward mechanism[68]. Individuals with the rs4680 G/G (val/val) genotype showed a 42% increase in TH expression in neurons projecting to the striatum, and a 48-72% increase in regions of the substantia nigra, relative to the rs4680 G/A (val/met) genotype[68], suggesting a heritable influence on homeostasis of catecholaminergic tone. In individuals with SCZ and BP (highly comorbid with PD), excess brain expression of the membrane-bound COMT (MB-COMT) isoform (see COMT mRNA isoforms and kinetics) is inversely correlated with DA receptor 1 (DRD1), in contrast to the opposite relative expression (DRD1>MB-COMT) in controls[69]. Similar gene-gene interaction occurs in the periphery to regulate blood pressure and salt-water balance, as when the COMT inhibitor, entacapone, causes intra-renal DA elevation and induction of DRD1-mediated natriuresis[46].

4.2.5. Lessons from animal models of COMT function

In multiple experimental animal paradigms, COMT deficiency alters dopaminergic metabolism, although studies differ for other endpoints according to gender and breed. COMT knockout results in a two-to-three-fold increase in DA accumulation in the PFC in males only, of a mixed 129Sv/6J-C57BL genetic background[29], and in both sexes of COMT knockouts of the more homogenous C57BL/6J strain[63]. Other studies revealed no PFC accumulation of DA in the C57BL/6J strain at baseline[63], but did show brain DA accumulation when

overcoming the capacity for DA storage and catabolism with a DAT inhibitor[63], or with administration of levodopa and the DA decarboxylase (DDC) inhibitor, carbidopa[62]. Conversion of dihydroxyphenylacetic acid (DOPAC) to homovanillic acid (HVA) is a COMT-specific reaction[71]. Elevation in the DOPAC/HVA ratio is observed with COMT inhibitor drugs[72], and in COMT-deficient animals. Baseline elevation in the DOPAC/HVA ratio in both genders of COMT knockouts was observed for the C57BL/6J[62] and 129Sv/6J /C57BL[29] strains.

Ethological tests revealed heightened anxiety in COMT null (-/-) females, and heightened aggression in heterozygous-deficient (+/-) males of the mixed 129Sv/6J /C57BL background[29], but not in the C57BL/6J strain for either gender or genotype[61]. Using the Affymetrix microarray system, variation in COMT expression was inversely correlated (0.9, $p<0.05$) with eight inbred mouse strains, ranked by intensity of aggression as a phenotype[73], in agreement with the knockout study by Gogos *et al.*[29]. High COMT expression in the C57BL/6J strain was associated with low aggression[73], in agreement with the lack of aggression in the knockout study by Hassio *et al.* [61]. In dogs, the aggression phenotype of territorial defensiveness was associated with one missense and one synonymous SNP in the canine homolog for COMT (84% homologous with human COMT), at sites predicted to be responsible for magnesium and SAM binding[74], which authors suggests may influence phenotypic variation. And distinctive morphine responsiveness in the C57BL mouse correlated with strain differences in striatal COMT expression, by Affymetrix microarray and quantitative real time-polymerase chain reaction (qRT-PCR), although with

opposite expression valences by technique[75]. Interestingly, the PD candidate gene, $GABR_A$, was also among the 3% of 39,000 transcripts showing baseline expression differences in this study.

Similar to animals, human anxiety[76], harm avoidance in women[77], female phobic avoidance[78], panic attacks in females[79], opioid response[80], and aggression[81,82] are associated with the rs4680 A (met) allele in COMT activity or allele frequency.

4.2.6. Pleiotropic and sexual dimorphic effects of COMT

Relevant to our interest in pathological effects of genes in common between PD and related features of PD, COMT variants have been significantly associated with comorbid, syndromic, and other features of PD, such as migraine[83], alcoholism[84,85], prefrontal working memory[86], and bladder/renal problems (in men)[87]. COMT dysfunction in animals is reminiscent of the bladder/renal conditions used to categorize our PD/bladder families during subject recruitment (see section 2.3.1), such as renal insufficiency with secondary hypertension. Mechanistically, variation in COMT activity significantly alters renal salt-water balance[88] and blood pressure[6] via peripheral DA actions. For example, spontaneously hypertensive rats have lower renal COMT activity[88] and CHGA overexpression[89] (see section 4.2.4.), resulting in less vesicular release and turnover of catecholamines than in the normotensive rat; and knockout of COMT in normotensive rats results in resistance to salt-induced hypertension[90]. Reduced COMT expression in the hypertensive Dahl salt-

sensitive (DS) rat is thought to arise from a palindromic insertion in the 3' untranslated region (UTR) of the gene, which may promote mRNA instability[91].

The DS rat also exhibits cognitive impairment[92]. Prepulse inhibition (PPI) response, which is a highly heritable (50-70%) cognitive index of sensorimotor gating[93] is altered in PD[105-107], and normalized with DA-interfering drugs[87,94,95]. Similar to the animal models, COMT-related phenotypes, such as PD, BP, SCZ, OCD[96], and pain sensitivity[97], have distinctive gender effects at the molecular level[98], in symptomology, prevalence, and/or in the pattern of genetic association. In fact, as a class, the biogenic amines show highly conserved sexually dimorphic effects from fruit flies to humans[99]. Thus, sexually dimorphic and pleiotropic COMT effects are conserved among species.

4.2.7. COMT mRNA isoforms and kinetics

COMT exhibits different kinetic profiles from two different messenger ribonucleic acid (mRNA) isoforms, transcribed from alternative start sites on a single gene[9]. The isoforms show different substrate affinities[100] and tissue-specific distribution and abundance in the CNS and periphery[9]. The membrane-bound isoform (MB-COMT) has greater affinity for the SAM coenzyme[13], NE, and DA, than does the soluble isoform (S-COMT)[13,101]. Therefore, despite greater mRNA and protein abundance of MB-COMT in lymphocytes[18,102] and the brain[3,9,101,103], S- and MB-COMT kinetics depend more on substrate affinity and sub-cellular localization than on their relative abundance. High-affinity MB-COMT kinetics predominate in the CNS, where it efficiently catalyzes ambient,

nanomolar concentrations of catecholamines[13]. Alternatively, high substrate concentrations in the periphery (e.g. liver, kidney, duodenum) that saturate the MB-COMT isoform, leave the high capacity S-COMT isoform to predominate in clearance of peripheral substrates[9].

Due to structural constraints, MB-COMT exhibits greater reaction regioselectivity resulting in a higher meta-to-para methylation ratio[13,101], relevant to the aforementioned TAAR1 in its ligand affinity for the symapthomimetic meta-methylated metabolites of DA and NE[37]. In the CNS, MB-COMT lies preferentially just at the inner surface of post-synaptic neurons for neurotransmission[3], as well as non-neuronal sites in the glia (astrocytes, oligodendrocytes, and microglia)[4,5] and capillary walls[12]. Central S-COMT localizes mostly to the cytoplasmic and nuclear compartments of glia[24]. Both S- and MB-COMT require internalization of substrates for catechol-O-methylation[107]. MB-COMT-mediated cognitive processes are thought to reflect a human-specific evolutionary change, since this high affinity isoform is all but absent in the rodent brain[18]. In vitro studies show higher COMT activity in the rat compared to humans[18], suggesting that modulation of a finer dynamic range in COMT activity is an adaptation of humans. Indeed, brain imaging in humans suggests that cognitive performance follows an inverted-U curve of dopaminergic tone in the PFC, where optimal functioning occurs at mid-range DA levels, causing pharmacogenetic differences in amphetamine drug response[108]. Homozygotes for the “low-activity” rs4680 A/A (met/met) genotype outperform G/G (val/val) homozygotes at baseline, but monoamine potentiation with cognition-enhancing

amphetamines is shown to enhance cognitive performance in rs4680 G/G (val/val) homozygotes, but cause deleterious effects in rs4680 A/A (met/met) homozygotes[108]. Because of this dose-dependent effect in catecholaminergic tone, genotype-phenotype associations to COMT-related dysfunction may likely show non-linear correlation.

4.2.8. Lessons from COMT gene structure

Structural features of the COMT gene highlight important functional attributes. The 27 kb gene on chromosome 22q11.2 contains six exons, including two non-coding exons, and two ATG start codons for initiating the translation of the 1.5 kb and 1.3 kb transcripts, for MB- and S-COMT, respectively[9]. A proximal "P1" promoter, situated between the two start codons (200 bp upstream of the MB-ATG start codon), controls S-COMT expression, while the distal "P2" promoter regulates MB-COMT expression[9]. The 271-residue MB-COMT isoform contains fifty extra amino acids at the amino terminus to anchor the protein into the membrane[109] of the endoplasmic reticulum and at the plasma membrane[107]. One potential source of discordance between isoform-specific mRNA and protein levels is attributed to a leaky translation-initiation scanning mechanism whereby the 1.5 kb mRNA can generate the S-COMT protein[9]. However, because the MB-ATG site is more favorably located for the ribosomal machinery, this mechanism is thought to play a minor role[3].

4.2.8.1. Epigenetic regulation of COMT expression

An important genetic feature of the COMT locus is the preponderance of nucleotide repeats, making the region susceptible to deoxyribonucleic acid (DNA) methylation at regulatory CpG dinucleotide repeats in promoter regions, and chromosomal rearrangements causing megabase microdeletions or amplification. DNA methylation of the COMT gene is common and rarely dissimilar in blood and brain tissue of healthy controls[110], and chromosomal rearrangements occur in asymptomatic people. However, aberrant COMT methylation and/or copy number is observed in a number of COMT-related psychiatric and systemic disorders. There is also evidence that varied DNA methylation states may serve to compensate for copy number aberrations.

Hemizygous microdeletion of chromosome 22q11, found in 1 in 4,000 individuals, reduces the copy number for COMT, and is associated with disorders having related etiology[111] or comorbidity with PD[112], such as schizophrenia (SCZ) [113], bipolar disorder (BP), obsessive-compulsive disorder (OCD)[114], and attention-deficit hyperactivity disorder (ADHD) [69,114]. A study of chromosome 22q11 deletion syndrome mice revealed decreased expression of COMT (0.6-fold, $p<0.0005$), the adjacent gene, TXNRD2 (0.73-fold, $p<0.0005$), and increased expression of GRP (1.69-fold, $p<0.005$) (PD candidate gene on chr18) in the hippocampus compared to wild-type mice[115]. Rarer microduplication, triplication, or tetrasomic chr22q11 is observed in Cat Eye[116] and other syndromes[117] with phenotypic effects ranging from below detection, to measurably impairment in cognition, sleep, abnormal electroencephalogram, and

behavioral problems (e.g. aggression), some of which overlap with PD.

Methylcytosine, the product of DNA methylation, which occurs at 60-90% of CpG sites, is considered the 'fifth base' of the genome[118], with significant epigenetic effects that occur irrespective of the target DNA sequence. Aberrant DNA hypermethylation (gene silencing) of MB-COMT is observed in endometrial cancer[119]. Interestingly, there may be generational effects that are transmitted by epigenetic modifications since risk of estrogen-related cancer is transmitted from mothers with the rs4680 A (met) allele to daughters, irrespective of the daughter's genotype[120]. This potential epigenetic effect certainly would hamper traditional initiatives to identify genetic determinants of COMT-related disease using a case-control design, where segregation patterns are not observed.

Furthermore, observations suggest a possible dynamic interplay between epigenetic mechanisms to normalize COMT function. Perhaps as compensation for low copy number due to microdeletion, Abdolmaleky *et al.* showed that brain samples from subjects with SCZ and BP show a two-fold increase in MB-COMT expression associated with *hypo*methylation of the COMT promoter region[69]. As well, a subset of SCZ and BP subjects who were taking psychiatric medication, or who had a history of comorbid alcohol abuse, exhibited greater COMT methylation as compared with those who were unmedicated[69]. We are reminded that alcohol and substance abuse is highly comorbid with PD[121]. Thus, DNA methylation of COMT may mechanistically explain efficacy of drugs and motives to self-medicate to bring about changes in brain chemistry. Abdolmaleky *et al.* also showed that MB-COMT methylation was found to be approximately 2.7-fold

greater in the left hemisphere of the brains of healthy controls compared to SCZ and BP patients[69]. Similarly, strong right lateralization in PFC activation during cognitive tests are associated with COMT haplotypes in healthy controls[86], and asymmetric hippocampal activation via limbic relays occurs in PD patients in response to threat words[122]. Thus, complex, region-specific regulation of COMT by DNA methylation may contribute to modulating emotional and cognitive neurocircuits.

4.2.8.2. Hormonal influences on COMT

Human COMT activity is widely reported to be lower in females compared to males, in erythrocytes[15,123,124], liver[16], and the PFC[18]. However, studies in Asian[125] and mixed ethnic groups[18] showed non-significant differences in COMT activity in lymphoblastoid cells, and one study showed higher brain mRNA expression in females compared to males[126]. Sex differences in COMT activity have been attributed to sex-specific hormonal regulatory differences[127], which may explain the conflicting data, since hormone status was not monitored in these studies. Mechanisms of hormonal regulation of COMT include less transient DNA hypermethylation of MB-COMT in estrogenic cancer[119], as well as more cyclic changes with fluctuations in hormones. Both the P1 and P2 promoters contain progesterone[128] and estrogen[129,130] response elements for the upregulation[128] and downregulation[128-130] of COMT, depending upon tissue-specific abundance of nuclear hormone receptors[128,131]. Estradiol binds the estrogen receptor (ESR) in the COMT promoter to suppress COMT activity and protein levels, which is reversed by ESR inhibitor ICI 128,780[129]. Progesterone

with the progesterone receptor (PR) upregulates uterine COMT during implantation of the fertilized egg, which is reversed by the PR antagonist, RU-486 (mifepristone)[132]. Variants, such as the P2 promoter SNP, rs2097603, associated with lower lymphoblastoid COMT activity[18] and ethnic differences in hormone levels[133], are additional variables in hormonal control of COMT. Thus, physiological and disease-related changes in hormones modulate COMT expression at promoter regions (1) via nuclear receptor transactivation or transrepression, which may be influenced by mutations in regulatory regions, and/or (2) by DNA methylation, which is independent of target gene sequence, but may be influenced by variation in hormone levels.

Neurobehavioral phenotypes are partly mediated by hormones. Reduction in hypothalamic (but not hepatic or frontal cortex) COMT protein is observed in estrogen-deficient, aromatase knockouts, as well as OCD-like behaviors in males that can be reversed with exogenous estrogen administration[134]. In humans, OCD also presents sex-specific symptomology and association to COMT[135,136]. Estrogen is protective against OCD in women[137], such that hormonal changes during pregnancy and postpart are associated with episodic OCD[138]. Pro-dopaminergic effects of estrogen significantly reduce threshold doses of levodopa required to treat Parkinson's patients who lack DA[139], and exacerbates symptoms of SCZ[140], which results from excess DA.

4.2.9. Important population differences in COMT allele frequencies

The frequency of the rs4680 A (met) allele ranges between <1% (near fixation of the G (val) allele) to 62% among different ethnic populations, reflecting divergent microevolution at the human COMT locus. Variation in rs4680 is therefore a poor candidate for explaining COMT-related phenotypes across populations. Asians and African Americans have roughly half the minor allele frequency (MAF) of Caucasians of the rs4680 met (A) allele (~25% vs. ~50%)[141], giving a four-fold difference in the frequency of A/A (met/met) homozygotes between populations (~6% vs. ~25%, respectively). Among seventy-five different ethnic groups in AL*elle* FRE*quency* D*atabase* (ALFRED, www://alfred.med.yale.edu)[142], the frequency of rs4680 A/A (met/met) homozygotes varies substantially world-wide (0.008-9% in six South American, 0.003-14% in fifteen African, 2-20% in twenty-one Asian/East Asian, 11-38% in seventeen European populations, respectively). Similarly, the heterozygosity for a seven-marker haplotype containing rs4680 ranges between 0.25-0.94 in thirty-eight ethnic populations, with ten European populations ranging between 0.79-0.91 alone[141]. Thus, genotype-phenotype associations need be controlled for population stratification by ethnicity.

The low-activity rs4680 A (met) allele is up to ten-times less frequent in Asians compared to Caucasians, however the rs6267 G (ala)> T (ser) SNP in this population explains additive reduction in COMT activity[125], increased protein thermolability, and susceptibility to inactivation by hormone drugs[139]. The rs6267 T/T (ser/ser) genotype was significantly associated SCZ in Koreans[125], in contrast to the Ashkenazi Jewish population, which is monomorphic at this

site[143]. Thus, requisite differences in population-specific associations to COMT are borne out of ethnic allele frequency differences.

In addition to ethnic differences, significant gender differences in allele frequency has been reported in healthy Ashkenazi Jewish replicate populations, where the G allele of the 3'UTR A>G SNP, rs165599, was significantly less frequent in females, compared to males[143], and out of Hardy-Weinberg equilibrium[86], which begs the question of fitness issues associated with COMT alleles in certain populations, as speculated by Shifman *et al*. Therefore, matching case and control subjects by ethnicity and gender is important for this locus due to the unusual population effects.

4.2.10. Utility in multimarker haplotypes

Despite the increase in statistical tests with multiple haplotypes, its advantage over single-marker analysis is finer definition of the architecture of the genetic background in which a phased causative variant lies[144]. The COMT SNP, rs4680, has shown controversially inter-study association to PD (see COMT association to PD) and other COMT-related phenotypes, by ethnic group or gender, for SCZ, pain sensitivity[80,145-148], and OCD[149]. Large genetic background differences are presumed to cause population-specific genotype-phenotype associations.

Haplotype information can explain seemingly confounding effects of a single marker, for example, if the same allele of a single marker resides on different haplotype backgrounds with opposing phenotypic associations, thus

haplotypes can pinpoint the contribution from cis variants. For example, analysis of a two-marker (rs6267-rs4680) diplotype parses out the confounding effect of the rs4680 G (val) allele, and reveals the contribution of both the rs4680 A (met) allele and rs6267 C (ser) allele with reduced COMT activity and SCZ in Korean subjects[125].

In a study of female subjects with pain from temporomandibu disorder by Diatchencko *et al*., a multimarker COMT haplotype (rs6269-rs4633-rs4818-rs4680) was associated with low (G-C-G-Gval), average (A-T-C-Amet) and high (A-C-C-Gval) pain sensitivity (LPS, APS, HPS, respectively)[97], in which the rs4680 G (val) allele was found at opposite extremes of the pain phenotype. Therefore, the rs4680 allele alone does not explain the association. Using three of these markers (rs4633-rs4818-rs4680) in haplotypes corresponding to LPS, APS, and HPS, Nackley et al. showed that in silico differences in the mRNA stem loop structure, local to rs4818 and rs4680, alters mRNA stability (calculated by Gibbs free energy), and observed enzyme activity and protein levels *in vitro*[150]. This highlights important structural and functional contributions from synonymous SNPs, such as rs4818 in combination with the non-synonymous rs4680, in generating the conditions in which the same rs4680 allele could mechanistically explain opposing phenotypes. In this case, haplotypic analysis is better than a single functional variant in predicting functional variability.

A study by Meyer-Lindenberg *et al*. of prefrontal working memory in Caucasian controls was significantly associated with a COMT haplotype formed from the P2 promoter variant, rs2097603, with rs4680, and 3'UTR SNP,

rs165599, and not with any of numerous single markers tested[86]. Thus, contribution from cis variants can improve the power to detect association. Ying-yang haplotypes (in which each haplotype is composed of entirely opposite alleles) in this population exhibited the largest difference in PFC activation, and in a separate study by Michaelovsky *et al*. were also shown to be significantly associated with opposing risk to OCD (deleterious, protective) in the single COMT copy from individuals with hemizygous chromosome 22q11 microdeletions[114].

Multimarker studies such as those by Lee *et al*., Diatchencko *et al*., and Nackley *et al*., help discern the contribution of markers other than rs4680, such as the non-synonymous rs6267 and synonymous rs4818, in COMT activity related to SCZ and pain. Studies by Meyer-Lindenberg *et al*. and Michaelovsky *et al*. are shown to correlate common COMT haplotypes with phenotypes such as prefrontal cognition and OCD susceptibility. Thus, *cis* variants help to pinpoint complex COMT associations with COMT-related phenotypes in different populations. Despite the improvement of multimarker analysis over single-marker analysis[151], haplotypic associations are still prone to population differences as haplotype frequencies also vary dramatically by population[141]. This is reflected by recombination hot spots that reduce LD in the region[152].

To illustrate, the A-A-A haplotype for rs737865-rs4680-rs165599 was associated with protection against OCD, but population background differences in haplotype frequencies cause discordant results for risk of OCD. For example, the converse G-G-G haplotype was overtransmitted among OCD probands in a

family-based study population, while the A-G-G haplotype showed significant overtransmission in the unrelated cases of OCD due to the relative rarity of the G-G-G haplotype in the case-control OCD study population. The same three-marker haplotype was studied in SCZ wth similar results. SCZ was associated with the G-G-G haplotype in Ashkenazi Jews[143], whereas in U.S. Caucasians, a haplotype with the opposite alleles in a four-marker haplotype with rs2097603 was significantly associated with reduced risk of psychotic-affective disorders[154]. Interestingly, the A-A-A haplotype for rs737865-rs4680-rs165599 is undertransmitted in two PD study populations (family-based and case-control) of predominantly Caucasian Canadians[153].

An explanation for potential discord in study population results is reflected in large population-specific differences in LD patterns. For example, Samaritan and Ashkenazi Jewish populations show high pairwise LD for the two-marker haplotype, rs2097603-rs737865, (Xi, ξ >0.6-0.8, and > 0.4, respectively). In contrast, the Pima, Micronesian, Cambodian, Ibo, Yoruban and Biaka populations, show no LD for these markers, and thus would be not be expected to carry similar multimarker association[141]. Genetic background differences among study populations warrant further characterization of *cis* variants in homogeneous populations to discern the genetic factors influencing COMT function. LD blocks up to ~46kb, generated from the HapMap variants for the Caucasian population for the ~480kb region investigated in this work, are outlined in the results (see figure 4.6.; tables 4.1.-4.4.; section 4.5.4.).

4.2.11. Linkage and association of COMT to PD

COMT was significantly associated and linked to PD in a study of seventy Caucasian, multiplex PD pedigrees[155], with the highest logarithm of odds score, assessing locus heterogeneity (HLOD), = 2.93 for a microsatellite, D22S944, which lies approximately 339 kb centromeric to the COMT locus. The non-synonymous and synonymous SNPs, rs4680 and rs4633, also had maximal HLODs of 2.88 and 2.62, respectively. Using sibling pairs, non-parametric linkage (NPL) scores were significant for D22S944 (p=0.00008-0.02), rs4680 (p=0.001-0.004), rs4633 (p=0.006-0.01), and for an intronic tetranucleotide repeat, (AAAT)n, (p=0.02-0.04). The transmission disequilibrium test (TDT) in triads revealed significant allelic association to D22S944 (p=0.0001–0.0003), and the rs4680 G (val) allele (p=0.02-0.03), improved when stratified for gender (females p=0.0003-0.0004). Mild association was also observed for rs769224 (p=0.03, 0.05 in females, males). Significant female-specific associations were observed for a number of genic SNPs, including rs4680, using gender-based TDT and haplotype-based haplotype relative risk (HHRR) (p=0.0003-0.03) tests. In the family-based sample, female probands were three-fold greater in number than male probands across diagnostic phenotype definitions, thus better-powered for female-specific association. Significant multimarker associations included the D22S944-rs4680-rs4633 haplotype (global p=0.005), in which the rs4680 G (val) allele was present, and D22S944-rs362204 (p=0.0005), capturing the two farthest spaced markers for the locus. LD estimates revealed high correlation between a genic (AAAT)n tetranucleotide repeat and SNPs, but not between D22S944 with the SNPs. However LD between the D22S944 and

(AAAT)n was significant. Interestingly, D22S944 was significantly associated to SCZ in Caucasians from the United Kingdom and Ireland[156], as well as in female Ashkenazi Jews[157]. Thus, our investigation of COMT expression extends to a ~480 kb region around COMT that encompasses the proximal D22S944 microsatellite to test for possible long-range *cis*-acting mutations contributing to COMT-related phenotypes (see Methods).

4.2.11.1. Replication of COMT association studies in Caucasian PD subjects

Three out of four PD studies by other investigators showed association of the rs4680 G (val) allele, with female-specific effects in probands of primarily Caucasian ethnicity. In a case-control study by Domschke *et al*. using 115 Caucasian probands and 115 ethnically and gender-matched controls from Germany, the rs4680 G (val) allele was associated (p=0.04) to PD, with female-specific effects (p=0.01 in females, p=0.1 in males), given only 1.8 times more females in the study[158]. Rothe and colleagues showed similar association to the rs4680 G (val) allele in Canadian probands in two independent studies, using a family-based study design and a case-control study design with gender and ethnicity-matched subjects[153]. The rs4680 G (val) allele was significantly associated with PD in the case-control study (p<0.01), as well as in the family-based study (p=0.004) using FBAT, and overtransmitted to children affected with PD from heterozygous parents in nuclear families (p=0.005) using TDT. When stratified by gender, significant association to rs4680 G (val) was found only in the female probands (genotypic p=0.014, allelic p=0.008) in the case-control

study. After combining the z-scores from the family-based and case-control studies, the significance of the association improved (p=0.00002). No association was found for the MB-COMT intronic SNP, rs737865, and the 3'UTR SNP, rs165599. In trios, there was little LD among the three SNPs (D'=0.01-0.27), but in the case-control sample good LD was observed between each of the other variants and rs4680: rs787365-rs4680 (D'=0.72) and rs4680-rs165599 (D'=0.62). Population-specific allele frequency and LD patterns at the COMT locus are known to vary greatly, even between Caucasian groups (see section 4.2.8.). According to the different LD patterns observed for the two above mentioned PD study cohorts, different three-marker haplotypes formed by the SNPs (rs737865-rs4680-rs165599) were associated with PD, although the valence of the rs4680 allelic association remained consistent. In the case-control study, rs4680 G (val)-harboring haplotypes (A-G-G and A-G-A) were significantly overrepresented (p=0.002 and p=0.039, respectively), while a rs4680 A (met)-harboring haplotype (A-A-A) was underrepresented (p=0.039) in PD probands. In trios, having less inter-marker LD, a different G (val)-harboring haplotype (G-G-G) was overtransmitted (p=0.017), but the same A (met)-harboring haplotype (A-A-A) was undertransmitted (p=0.001). Thus, at least in the study by Rothe the rs4680 G (val) allele consistently segregated with PD, although the haplotypic architecture differed between the family-based and case-control study populations. Interestingly, Rothe *et al.* showed significant parental bias in rs4680 allelic transmission (maternal p=0.02, paternal p=0.0006), such that subjects with PD received the associated allele from fathers more than mothers. Lastly, in a

fourth study of Caucasians, Samochowiec and colleagues, showed no association to rs4680 in a case-control study using 197 controls and ninety-five panickers from Poland, as assessed by the Composite International Diagnostic Interview (CIDI)[159]. However, compared to the other studies of PD, in which the *Diagnostic and Statistical Manual of Mental Disorders* (DSM) was used to define PD, it is unclear how the Polish study compares, given that CIDI diagnostic criteria tend to generate more false negatives[160].

4.2.11.2. Replication studies in Asian PD subjects

In contrast to the major findings in Caucasians, two of three studies in Asian populations reveal association between PD and the rs4680 A (met) allele. Woo and colleagues initially tested fifty-one cases and forty-five controls from Korea, with equal matching for gender, and found significant association to the rs4680 A (met) allele (p=0.005) and A/A (met/met) genotype (p<0.04), as well as poorer response to paroxetine SSRI treatment given the A/A (met/met) genotype (p=0.01)[161]. A independent replication study by Woo *et al*. using separate Korean subjects (178 cases and 182 gender-matched controls) revealed less significant association to the rs4680 A (met) allele (p=0.1), but sustained association to the A/A (met/met) genotype versus the combined alternate genotypes (OR=2.38, p=0.04)[162]. The latter, better-powered study revealed more significant association between the A/A (met/met) genotype and poor paroxetine response (p=0.002), with only a trend toward association in female probands (p=0.063) when stratified by gender. As an aside, and related to the

chromosome 15q12 $GABR_A$ candidate genes for PD (Chapter Three), it is interesting to note that in both Woo *et al.* studies, adjunctive $GABR_A$-targeting benzodiazepines (alprazolam or clonazepam) were coadministered with the SSRI, paroxetine, in a proportion of subjects to control symptoms in the first month of treatment. Given that SSRIs and $GABR_A$-targeting anxiolytics are *both* shown to alter $GABR_A$ expression patterns, there may be interesting gene-gene interaction between these candidate genes (see Chapter Three).

An earlier Asian study by Ohara and colleagues used twenty-nine Japanese probands and 135 ethnic and gender-matched controls and revealed no association to rs4680[163]. However, perhaps due to the relative scarcity (n=3) of probands with the A/A (met/met) genotype compared with the Woo studies (3.3 to 6.3 times more met/met homozygote cases), which appears to account for Asian-specific rs4680 allelic association, the Ohara study may have been underpowered. Ohara *et al*. also noted the number of probands with family history of PD, which would presumably increase the genetic load for PD. They cite only three out of the twenty-nine cases with family history of PD.

4.2.11.3. Contradiction in COMT association studies related to our study aims

To conclude, the contradictory association with the rs4680 G (val) and A (met) alleles in Caucasian and Asian studies, respectively, suggests ethnic-specific causes, which was confirmed by meta analysis of the above-mentioned case-control studies[164], with a significant ethnic effect on the valence of the odds

ratio (OR) (p=0.035) according to Asian or Caucasian ethnicity. Among female probands, the rs4680 G (val) allele was a risk factor in the pooled Caucasian sample (OR=1.54, p=0.04) and protective in the pooled Asian sample (OR=0.61, p=0.02). Observation of dramatic rs4680 A (met) allele frequency differences between the Caucasian (Canadian, German, Polish) controls (0.53-0.63) versus Asian (Japanese, Korean) controls (0.19-0.35) points to large ancestral differences in human microevolution of COMT. Depending upon the population studied, the frequency of the rs4680 A (met) allele was 0.009-0.62 among seventy-five distinct ethnic populations, per ALFRED.

Association studies are prone to spurious association due to population stratification. The meta-analysis of PD studies showed population-specific allele frequency differences in the control subjects that result in a 10-fold difference in the abundance of A/A (met/met) homozygotes between Koreans and Poles at the extreme (0.036 vs. 0.396, respectively). By contrast, the P2 promoter region SNP, rs2097603, exhibits far less variation in allele frequency, perhaps due to functional constraints, since it may be important for the expression of the MB-COMT isoform. The contribution of important *cis* variants, such as this promoter region SNP, relative to functional coding variants, like rs4680, are therefore expected to vary by population and perhaps explain population-specific associations.

4.2.12. COMT expression

Variability in gene expression is a potential source of human disease susceptibility and pharmacogenetic differences. Changes in COMT expression occur in cell lines from individuals with breast cancer[128], with chronic DA-regulating drug exposure[165,166], and during hypoxic events that are implicated in SCZ[167]. A comparison of the variances in COMT expression in lymphoblastoid cell lines from individuals ranging in relatedness provides evidence for heritability of gene expression (variance in unrelated>siblings>twins)[168]. In a genome-wide San Antonio Family heart study of thirty extended families, using the Illumina BeadChip expression platform and 432 microsatellites, both COMT (GI_6466449-A) and MB-COMT (GI_6466451-I) were found to be heritably expressed (heritability = 0.29 and 0.31, respectively)[169]; and the heritable expression of COMT in thyroid cancer has been associated with the *cis* variant, rs165849[170]. Allelic imbalance of gene expression implicates *cis* variants in the mediation of differences in gene expression. A study by Yan *et al.* showed that allele-specific expression (ASE) occurred in lymphoblastoid cell lines for certain genes, including some which showed Mendelian segregation in pedigrees[171]. They did not find significant difference in COMT allelic expression for twenty-one rs4633 heterozygotes; however Bray *et al.* found lower relative expression of the rs4633 C and rs4680 G (val) alleles in twenty-three postmortem control brain samples[21]. In Bray's study, a haplotype formed from the intronic MB-COMT SNP, rs737865, and 3'UTR SNP, rs165599, with rs4680 was correlated with underexpression of the G-G-G (rs737865-rs4680-rs165599) haplotype, which is significantly associated with SCZ in an Ashkenazi Jewish population (OR=1.4)[143].

However, the rs4680 met (A) allele, contained in the opposite A-A-A haplotype was associated with SCZ in Australian Caucasians (OR=3.12)[172]. Thus, Bray's expression study is compelling in light of the controversy over the discordant association of rs4680 to SCZ[145,146,173] because SCZ is hypothesized to result from *hyper*dopaminergic tone[174] due in part to decreased COMT function. This agrees with psychotic symptoms observed in patients with velocardiofacial syndrome (VCFS) and SCZ, who often present with a 1.3-1.5 Mb hemizygous microdeletion in chr22q11 around COMT. Furthermore, DA antagonists are effective antipsychotics, and side effects of excess dopaminergic tone in Parkinson's treatment result in psychosis[68]. Presuming that the rs4680 G (val) allele displays greater COMT activity, it's downregulation could explain the overall loss in activity of the G-G-G haplotype in SCZ in Ashkenazi. Using brain and lymphoblastoid samples from various ethnic controls Zhu *et al*. showed that the rs4680 G (val) and rs4818 G alleles were underexpressed in both tissues[22]. Multimarker rs2097603-rs4680-rs165599 diplotypes (i.e. formed from both chromosomes) were predictive of COMT expression in the brain. The rs165599 G allele most often resided on underexpressed haplotypes harboring the rs4680 G (val) allele, in agreement with Bray *et al*. Characterization of COMT expression patterns in a larger homogenous population should reveal population-specific contributors to COMT expression in order to resolve differing genotypic association with COMT–related disorders.

4.3. Aims

In this study, we sought to characterize COMT expression levels in a population of Caucasian nuclear families from Utah, of Northern and Western European descent, to test for heritability of expression. We selected variants in a 480 kb region encompass both COMT and the microsatellite, D22S944, situated ~339 kb proximal to COMT that was previously linked and associated to PD in multiplex pedigrees. We used variants in this region to test for association with variation in COMT expression. Identification of genetic contributors to variation in COMT expression in this unphenotyped Caucasian population may be used to compare with PD probands and families to isolate loci responsible for the genetic risk of PD.

4.4. Methods

4.4.1. Samples

Ninety Epstein-Barr virus-transformed lymphoblastoid cell lines (LCLs) from thirty Caucasian nuclear families (mother-father-child trios) were purchased from the Coriell sample repository for expansion under uniform cell culturing conditions as described in Chapter Three. Association of variants with expression levels was carried out for thirty trios (n=90 LCLs), as well as for the unrelated parents (n=60 LCLs).

4.4.2. Cell culture, RNA prep, and heritable expression analysis

Cell culture, RNA preparation, and heritable RNA expression analysis using the Illumina BeadChip assay were performed as described in Chapter Three (sections 3.4.7. and 3.4.8). Reference genes were empirically selected from a pilot study (see Chapter Three) to normalize intra-sample variability in target gene expression due to biological and procedural sources. Briefly, these reference genes were empirically selected from a subset of five cell lines in which replicate culturing, harvesting, and RNA preparation was performed over multiple days. Reference gene expression was within a five-fold signal intensity of the target gene expression.

Two transcripts for COMT were detected using different 50-mer hybridization probes on an Illumina expression array (HumanRef-8 v1 Expression BeadChip, ~ 24,000 transcripts). MB-COMT expression was detected by a probe complementary to a non-coding exonic region specific to the MB-COMT isoform (GI_6466451-I). Global COMT expression (thus forth called COMT) was detected by a probe complementary to the 3'UTR region, common to both the S-COMT and MB-COMT isoforms (GI_6466449-A) in all ninety LCLs. Ten reference genes for COMT were selected (FLJ21128 (GI_19923612-S), RING1 (GI_11863157-S), IDH3B (GI_28178815-A), LOC375391 (GI_37540588-S), LOC115098 (GI_34147541-S), NDUFV3 (GI_21361323-S), FLJ20422 (GI_8923393-S), DT1P1A10 (GI_24308387-S), FLJ10581 (GI_21359921-S), and DEPC-1 (GI_21040274-S)), and averaged for each cell line before used in the denominator in a ratio of COMT over reference gene. Four reference genes were empirically selected for MB-COMT (M11S1 (GI_42558249-I), TANK

(GI_19743570-A), MED6 (GI_42544154-S), and PDCD10 (GI_22538793-A)).

Expression of the MB-COMT isoform was detected in eighty-eight of the ninety samples. MB-COMT for two cell line samples (#4, GM07029 1340-01, and #77, GM12716 1358-11) was below the limit of detection. Selection of reference genes for MB-COMT was carried out in eleven out of the fifteen harvest replicates (three triplicates and one duplicate harvest in four cell lines) due to suboptimal signal-to-noise ratio in four samples. To summarize, rather than apply externally derived reference genes to normalize our expression data, we empirically derived our normalization genes. We utilized the available transcriptome data *en masse* to identify co-expressed transcripts which could reproducibly reduce the intra-sample variation in MB-COMT and global COMT expression due to artifacts of cell culture and sample processing procedures.

Genetic markers spanning ~480 kb around the COMT locus (18.3 Mb), encompassing the D22S944 (17.9 Mb) microsatellite previously linked to PD, were used for analysis. Genotypes annotated by the International HapMap Project website (phase II data from NCBI build 35) (www.hapmap.org), for 302 non-redundant SNPs (the majority SNPs with correlation of r^2=1.0 were discarded), plus coding COMT SNPs of interest, with at least 2% minor allele frequency (MAF), and the microsatellite 217xF4 from the Mammalian Genotyping Service database (research.marshfieldclinic.org) were used for quantitative trait association testing. The genotypic information for 217xF4 (16 Mb) was available for twenty of the thirty trios. All positional data is given per NCBI genome browser website annotation from May 2004.

Using UNPHASED statistical software, the relative ratio of COMT to the average of the reference genes was assessed in a quantitative transmission disequilibrium test (QTDT)[175]. Genotype-phenotype association was performed using the quantitative transmission disequilibrium test (QTDT) in informative trios, and unrelated parents (from the same ninety LCLs). Permutation analysis of 10,000 tests was performed for data points that reached nominal significance ($p \leq 0.05$) by a single test.

4.5. Results

4.5.1. Characteristics of the sample set data

Six thousand two hundred fifty-five transcripts (13% of HumanRef-8 v1 Expression BeadChip transcripts) were reliably detected ($p<0.05$) in all ninety cell lines using the Illumina BeadChip Gene Expression system. The distribution of the average percent standard deviation, or coefficient of variation (CV), for each transcript across the ninety cell lines was relatively flat among expressed transcripts (figure 4.1.). The minimum CV was 15%, with ninety percent of the transcripts below 41% CV, and 57% CV at the 99th percentile. An outlier with 142% CV for the heat shock protein 1B gene, HSPA1B was detected among the ninety samples. HSPA1B lies in the major histocompatibility complex class III region, involved in the cellular protection from ischemic shock. CVs for the global COMT (S- and MB-COMT) and isoform-specific MB-COMT transcripts were average for all transcripts, as 26% and 23%, with 821 and 180 average arbitrary signal intensity, respectively. The mean transcript level across the ninety cell

lines ranged from 142 to 35,942, with 90% of the data at a median level of 1,841, and only a slight increase in CV according to increase in signal strength (figure 4.2.). The mean fold change (MFC), defined as the ratio of maximum to minimum expression in the entire sample set, is a measure of expression variability. MFC values across all expressed transcripts were relatively flat, with the minimum MFC at 2, the maximum at 69, and the 90th percentile at 8 MFC. The top 5% of expressed transcripts (n=311) showed greater than 10.6 MFC (figure 4.3.). The MFC for COMT and MB-COMT was 3.2 and 4.8, respectively.

4.5.2. Determination of BeadChip assay variability

Four cell lines were used in a preliminary analysis to determine the inherent variability in the Illumina BeadChip analysis procedure using the Illumina 48K HumanWG-6 BeadChip. Each cell line was split into duplicate samples at the point of hybridization to different Illumina BeadChips for detection of gene expression. Inter-sample variation of gene expression exceeded the intra-sample variation. Regression of the detected transcripts from duplicate hybridizations of the same cell line shows very high intra-sample correlation of expression (r^2=0.97) as compared to different cell lines. Within-sample correlation of all expressed transcripts for duplicate hybridizations was 0.987, 0.997, and 0.995 by logarithmic, linear, and power methods, respectively. Specifically, the average percent standard deviation (%CV) between duplicates was 11% and 7% for COMT and MB-COMT, respectively. Comparatively, the inter-sample %CV was 18% and 14% for COMT and MB-COMT, respectively,

among three cell line samples. Similar correlation (r^2=0.96-0.99) among replicate samples between and within BeadChips was observed by Stranger *et al*. for sixty parental cell lines among the same HapMap sample set[176]. Thus, procedural variability in the Illumina assay was minimal.

4.5.3. Determination of intra-sample variability in replicate sample preps

A pilot study was carried out for five cell lines in triplicate to determine the contribution of biological and procedural variability *within* a cell line to COMT expression. The maximum observed *intra*-sample %CV in triplicate cell line preparations for the Illumina BeadChip assay was 37% for COMT before normalization. This exceeded the *inter*-sample variation of 26% among the entire sample set of ninety cell lines. Normalization of target gene expression to a selection of reference genes significantly reduced the *within*-sample variation 4.6-fold to a maximum of 8%CV for all five cell lines prepared and analyzed in triplicate. To minimize inflation of variance with signal intensity, reference genes were within five-fold of target gene signal intensity. After normalization, *inter*-sample variability in COMT expression in the entire ninety cell lines was reduced to 12%CV, which exceeded the *within*-sample variability of 8%CV, and allowed for sufficient signal-to-noise to detect differences in COMT expression between samples. *Inter*-sample variation for normalized MB-COMT expression in eighty-eight cell lines (21%CV) likewise exceeded the maximum *intra*-sample variation in a subset of harvest replicates (7%CV). Prior to normalization, variability in MB-COMT was between 15 and 42%CV among replicate harvests.

In contrast, the commonly used housekeeping gene, beta-actin (ACTB), performed poorly as a reference gene for COMT in the Illumina BeadChip assay and in a quantitative real-time PCR (qRT-PCR) assay. In the Illumina assay, ACTB had a mean intensity value at 40 times that of COMT (33,454 arbitrary units), with 2.2 MFC, and 16%CV. In the Illumina BeadChip assay ACTB normalization caused two-fold greater intra-sample %CV in COMT expression values. Using qRT-PCR, relative quantitation of COMT against ACTB likewise introduced greater intra-sample variability in triplicate samples (results not shown). This was in agreement with data from 13,629 human gene Affymetrix array samples, in which ACTB was found unsuitable as a housekeeping gene due to wide variability in expression[185]. The qRT-PCR assay is limited in the number of genes able to be simultaneously detected within a sample, thus normalization is highly dependent on the reference gene selected. Two studies attempting to cross-validate COMT expression results in microarray and qRT-PCR technologies, were unable to do so[75,177]. One study showed opposite correlation, while the other showed a lack of correlation thought to be due to poor signal strength by qRT-PCR.

Data from comparable studies is often log-transformed, however our COMT expression data did not contain outliers, uneven or non-normal distribution, unequal variation (SD) across data points (after normalization), or greater than 3 MFC[178]. By contrast, microarray data in the meta-analysis by DeJonge *et al*., taken from different tissue types and experimental conditions, reported three orders of magnitude difference in the COMT expression intensities

(93 to 176,310), which necessitated log-transformation of the raw data. Our Illumina data showed a relatively flat distribution in COMT expression across samples, thus no transformation was applied. This is one way in which our analysis differs from other studies.

Data normalization techniques also differed. Quantile normalization in which the entire distribution of the expression data is compared pairwise between samples is often used[179]. Illumina BeadStudio software applies iterative pairwise selection of an average of the top least-variably expressed transcripts as internal control to target gene expression. However, this procedure does not apply the same reference transcripts to the entire data set of ninety samples, thus lacks uniformity. Our study used evidence-based selection of reference genes to normalize the *intra*-sample variation from biological and technical sources of cell culturing and sample preparation differences prior to detection using the Illumina platform, while other studies do not address this variability, and focus on controlling for variability in hybridization by performing multiple hybridizations of the same sample. Our preliminary tests showed that variability in hybridization steps and BeadChip arrays for the same sample was less than the *intra*-sample variation generated by day-to-day differences in cell culture and sample preparation. Correction for this external variability successfully dampened the spurious variability in target gene expression that would otherwise be falsely attributed to sample-to-sample differences. Similar expression studies in cultured lymphoblastoid cell lines do not address this issue.

4.5.4. Global COMT expression

We determined the association between DNA variants and a quantitative COMT expression phenotype, first for global COMT (soluble and membrane-bound isoforms, combined), then for the membrane-bound form, alone. Three hundred and two SNPs with at least 2% MAF, and most with less than perfect linkage disequilibrium (LD) to one another (r^2<1.0), as well as two microsatellites were tested for association to global COMT expression (S-COMT and MB-COMT) in lymphoblastoid cell lines (LCLs) from ninety Caucasians cell lines. The variants covered a 480 kb region around COMT and microsatellite D22S944, which was previously linked and associated with PD[155]. Eight genes, besides COMT, lie in this region: septin (SEPT5), platelet glycoprotein Ib (GP1BB), a transcription factor (TBX1), a guanine nucleotide binding protein (GNB1L), hypothetical protein (C22ORF25), thioredoxin reductase (TXNRD2), a catentin family gene (AVRCF), and a gene associated with DiGeorge microdeletion syndrome (DGCR8) (see figures 4.4.-4.7.).

Among the SNPs tested for association to global COMT expression, none met the level of Bonferroni correction at p=0.05 significance for 302 tests (p=0.00017, or 3.8 $-\log_{10}p$-value) in trios or unrelated individuals. At the significance level of $p \leq 0.05$, twenty SNPs were associated in thirty nuclear families (figure 4.4.), and twelve in sixty unrelated parents (figure 4.5.). Several of the SNPs that showed a significance level of $p \leq 0.05$ sustained significant association upon 10,000 permutations. Seven SNPs were significantly associated in thirty nuclear families (table 4.1.), and nine in sixty unrelated parents (table 4.2.). Results from the trios gives information as to the

transmission of alleles associated with variable COMT expression (n=30 trio tests), while results from the parents has slightly more power to detect strict association of alleles with variable COMT expression (n=60 unrelated sample tests). There was little correlation between the top best-associated SNPs (r^2<0.2).

Four of the variants associated with global COMT expression in nuclear families and unrelated parents lay in a gene-poor region within ~0.5-19 kb of the microsatellites D22S943 and D22S944. Predicted TFBSs for TCF4, NKX3A, NKX22, and SOX9 in this region occur with high sequence conservation across species. There is a gene poor region greater than 183 kb around rs9617813, although a 2,026 bp LD block formed ($r^2 \geq 0.8$) with nearby variants, spans potentially important D22S944 repeat and the predicted TFBSs for SOX9 and TST1 (chr22:17,983,211-17,985,236 per May 2004 NCBI build 35) according to the HapMap browser for these Caucasian cell lines.

Other variants associated with global COMT expression were found in the introns of ARVCF, DGCR8, TXNRD2, TBX1, and COMT itself (rs740603). Several predicted TFBSs were found near association signals, or implicated indirectly through LD relationships with other variants (see tables 4.1.-4.2.). Regression of the rs4680 variant in COMT expression by gender showed no significant association in our control sample. Regression of the rs4680 genotype against COMT expression in sixty unrelated parents was also insignificant. There was no significant difference in the mean expression COMT in 44 males was 1.10 (0.15 standard deviation (SD)), and in 46 females was 1.12 (0.11 SD).

The intronic variant rs2871047 in TXNRD2 associated with COMT expression in trios gave one of the highest scores ($-\log_{10}p$=4.0). This SNP is in high LD with another SNP, rs10483103, at a predicted ESR1 TFBS, which may potentially be important for hormonal control of COMT expression (see section 4.2.8.2.).

About half of the variants associated with global COMT expression in nuclear families lay in a gene-poor region within ~1-19 kb of the D22S943 and D22S944 microsatellites. SNP rs2078747, located within 3.7kb of D22S943, was significantly associated ($p \leq 0.001$) to COMT expression in trios. Predicted TFBSs for TCF4, NKX3A, and NKX22 in this region occur with high sequence conservation across species. Variant rs9617813, associated in unrelated individuals, lies ~500bp from D22S944. There are no genes in greater than 183 kb around rs9617813, although a 2,026 bp LD block formed (r^2>0.8) with nearby variants spans D22S944 and the predicted TFBSs for SOX9 and TST1. These TFBSs reside in sequence with very high species conservation according to the 7-way regulatory potential (comparing human, chimp, macaque, dog, cow, rat, and mouse). The SOX9 TFBS also shows high phylogenetic sequence conservation across 17 vertebrates (LOD=36), as well as in a 28-way vertebrate (LOD=55) and mammal (LOD=30) comparisons, as annotated in the UCSC Genome Browser on Human March 2006 Assembly.

Other variants associated with global COMT expression were found in the introns of ARVCF, DGCR8, TBX1, and COMT itself (rs740603). Several predicted TFBSs were found near association signals, or implicated indirectly through LD relationships with other variants (see tables 4.1.-4.2.). In the subset

of LCL samples with available genotypes for microsatellite 217xF4, there were no significant associations to global COMT expression (p>0.1). Regression of the rs4680 variant in COMT expression by gender showed no significant association in the total sample set. Regression of the rs4680 genotype against COMT expression in sixty unrelated parents was also insignificant (r^2=0.04). The mean expression COMT in 44 males was 1.10 (0.15 SD), and in 46 females was 1.12 (0.11 SD), a non-significant difference.

4.5.5. MB-COMT Expression

All but one SNP from the variants tested for global COMT expression was available in the smaller sample set that showed reliable MB-COMT expression. Among those 302 SNPs tested for association to MB-specific COMT expression (with MAF≥0.02, most with r^2<1.0), and combining the sample data sets for twenty-eight nuclear families and fifty-nine unrelated parents, thirty-nine SNPs were nominally associated with MB-COMT expression at significance of p≤0.05 (figures 4.6. and 4.7.). Among those SNPs nominally associated, twenty-three sustained significant association to MB-COMT expression after 10,000 permutation tests (table 4.3.-4.4.). More than half of the permuted associations occur in a gene-poor region near the D22S944 and D22S943 microsatellites, including the top three associated (p=0.001-0.005) SNPs (rs9617813, rs10854554, and rs13054962) (MAF=0.22-0.24) in parents. These three SNPs are in high LD with each other (r^2≥0.8), as well as with another SNP, rs10854553 (r^2>0.8), which lies within 100bp of the D22S944 $(CA)_n$ microsatellite sequence.

This LD relationship implicates a 2 kb LD block ($r^2 \geq 0.8$) spanning microsatellite D22S944, SOX9 and TST1 TFBSs, and including rs10854553 within the (CA)n sequence for D22S944 with high LD to rs10854554 (r2 > 0.9). This is intriguing since the best linkage score in pedigrees with PD was for the D22S944 marker, some ~340 kb away from COMT.

SNPs associated with MB-COMT expression were also found in GNB1L, TXNRD2, and DGCR8. Interestingly, GNB1L has been associated with SCZ[189]. None of the exonic SNPs tested for COMT (rs4680, rs4818, and rs4633) showed association to global COMT, or MB-COMT-specific expression.

In trios, SNP rs11912807, ~2,900 bp from D22S943, showed significant association after 10,000 permutations. This is intriguing since the best linkage score in PD pedigrees was for the D22S944 marker, some ~340 kb away from COMT (see section 4.2.12. linkage and association of COMT to PD), followed by SNP markers at the COMT locus[155]. None of the exonic SNPs tested for COMT (rs4680, rs4818, and rs4633) showed association to global COMT, or MB-COMT-specific expression.

The MB-COMT isoform showed nominal association to the 217xf4 microsatellite in twenty trios (p=0.04) and and non-significant association in forty unrelated parents (p=0.06), however their significance p-values were not sustained in 1,000 permutations (p>0.1).

Despite the reported predominance of the MB-COMT mRNA form in lymphocytes[18,102], the MB-COMT isoform showed very low expression, at the limit of detection, in our Illumina assay. This may have been an artifact of the

oligomer probe design, since the sequence contains two low-frequency SNPs and a nucleotide mismatch, according to the reference sequence on the NCBI genome browser website. However, we would expect minimal contribution of the SNPs in hybridization efficiency due to their rarity (figure 4.8.). Despite this, the global COMT and MB-COMT expression results had five SNPs in common at the level of 10,000 permutation tests (rs2078747, rs2157732, rs2871047, rs9617813, and rs11912807). Both MB-COMT and global COMT expression results were significant for SNPs in TXNRD2 and DGCR8. SNP rs2078747 in the first intron of TXNRD2, which showed significant association (p = 0.0001) to both global COMT and isoform-specific MB-COMT in trios, and rs2157732 likewise showed overlapping significance for MB-COMT and COMT expression. The SNP, rs10854554, which is significantly associated to MB-COMT expression in unrelateds by permutation, is in high LD with rs9617857 (also named rs12168204), which was associated to MB-COMT expression in trios. Both MB-COMT and global COMT expression results were significant for SNPs in TXNRD2 introns. So, there is some overlap in markers and heritable expression between parents, families and the two COMT isoforms.

4.6. Discussion

4.6.1. Comparison with other expression studies

In a genome-wide association study in fifty-seven of our sixty unrelated parental lymphoblastoid cell lines by Affymetrix platform, Cheung and Spielman *et al.* observed significant association to *trans* or *cis* variants in fifteen of the top

forty transcripts having the greatest variability in gene expression[180]. (COMT was not among the top most variably expressed genes tested.) Our results for six of these genes (CTSH, DDX17, EIF3S8, IL16, VAMP8, and SMARCB1), in the same fifty-seven cell lines revealed only nominal association (p<0.02) for two transcripts (CTSH and EIF3S8), and a trend for association (p=0.06) in one transcript (IL16). Another gene, cystatin B (CSTB), was significantly associated to different *cis* variants (rs880987, rs2838386, rs1041456, rs26539999) in four expression studies, using Affymetrix and Illumina detection platforms in more than one population of of Caucasian and Asian ethnicity[176,180-182]. Given the convergence of evidence for CSTB, we tested three of the variants in our sample as a positive control. Genotype information for the chimpanzee-specific SNP, rs26539999, reported by Morley *et al*. was unavailable. Perhaps the annotation for this variant was updated since its analysis, or there is yet unresolved information for this SNP. Our data showed CSTB to have greater expression variability (5.6 MFC) than COMT and MB-COMT, thus calculations were made for absolute and log-transformed expression values alike. Using the quantitative transmission disequilibrium test (QTDT) in UNPHASED, we observed nominal association of CSTB expression to the three variants (p=0.0004-0.02) in sixty unrelated parents, which was sustained by permutation testing (10,000 permutations). However, no association was observed in the thirty trios ($p\geq$0.4). Log-transformation of the CSTB expression values did not significantly improve p-values, and regression of the natural log of CSTB against genotype was also insignificant. Thus, although we did not replicate the previously reported results

to the same level of significance, we saw a trend for association in these variants as determinants of CSTB expression in our hands.

4.6.2. Normalization techniques for gene expression

In traditional quantitative and semi-quantitative gene expression assays, correction for sources of technical and biological variation has been addressed by the use of stably expressed housekeeping genes, or by averaging results from replicates of the same sample. It is recommended to select reference genes that control for sample variation empirically, in order to address study-specific variables[183]. Despite wide use of various constitutively expressed reference genes, such as ACTB, GAPDH, hypoxanthine–guanine phosphoribosyltransferase (HPRT), β2-microglobulin (β2M), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and glucose 6-phosphate dehydrogenase (G6PDH), systematic evaluation of these transcripts by quantitative real-time polymerase chain reaction (qRT-PCR) showed significant inter-study variation leading to dramatic misinterpretation of the data, which is particularly problematic for low abundance transcripts[184]. In our Illumina results, MB-COMT was among the lowest abundance of the transcripts in our assay, with an average absolute signal (180 average signal, GI_6466451-I) just above the limit of detection. MB-COMT expression in two of the ninety cell lines (parental samples GM12873, 1459-10, and GM11829, 1350-10) was undetected (detection *p*-value >0.05). In contrast, ACTB was three orders of magnitude greater (33,454 average signal, GI-5016088-S), being the second highest expressed transcript out of 6,255 total

detected probes in the Illumina BeadChip assay. We saw similar disparity in reference (ACTB) and target (COMT) gene expression was observed in a pilot qRT-PCR study. Due to the larger variation in ACTB than COMT in samples analyzed in triplicate, ACTB was discarded as an internal control for target gene expression (data not shown). Ribosomal RNA is likewise unsuitable as a reference for mRNA samples and poly-A tail-primed reverse transcription products, and also shows large transcriptional variation[184].

Meta analysis of more than 13,000 human samples from different tissues and experiments, assayed by Affymetrix array technology, identified the top most stably expressed housekeeping genes (e.g. RPS13, RPL27, RPS20 and OAZ1), which were recommended to use as internal controls for target gene expression normalization[185]. In this study selection criteria included low absolute variation in expression, as determined by the <2 mean fold change (MFC), and <4% coefficient of variation (CV). However, Affymetrix and Illumina data are not directly comparable. For example, the minimum MFC and %CV seen in our lymphoblastoid cell lines by Illumina was 2 MFC and 15%. In addition, the recommended housekeeping genes by De Jonge *et al*. were not among the most stably expressed transcripts in our Illumina BeadChip-based data, with fourteen of the fifteen top suggested housekeeping genes (RPL9 was not available) having an average of four-fold absolute variation in expression across sample cell lines (range 2.3-6.0) (see Characteristics of the sample set data). As the authors suggest, and as is observed here, reference genes are not universally applicable to all experiments across different assay platforms, which underscores

the importance of empiric validation for each study.

Vandesompele *et al*.[186] suggest taking the average of a number of reference genes to reduce the risk of spurious effects of a single gene. The Illumina BeadStudio software offers a number of normalization procedures to apply to the data. None, however, apply the same reference genes calculation across all samples in the sample set, but instead performs iterative, pairwise comparisons throughout the sample set. Therefore, in order to apply a uniform correction for *intra*-sample variation, we empirically selected reference genes from a subset of harvest replicates to minimize spurious variation in target gene expression. The probability of randomly finding these reference genes is highly unlikely, as it would translate into the probability of finding by chance the same proportion of genes in all five cell lines, or a probability of $(0.05)^5 = 3 \times 10^{-7}$.

4.6.3. Expression profiling studies

4.6.3.1. Long-range associations

Significant association of gene expression with long-range markers has been found in regions with greater than 100 kb to the nearest transcribed gene[187], and within 5 Mb of target genes[169,182]. A study by Davidson *et al.* exploited the "phylogenetic footprint" of multiple species to pinpoint a highly conserved sequence with multiple transcription factor binding sites (TFBSs) as a potential long-range enhancer of the tachykinin (substance P neuropeptide) gene, lying 158 kb away[188]. They generated transgenic mice with the conserved TFBS sequence in an expression reporter gene construct, and showed region-

specific colocalization with tachykinin in the amygdala, supporting the hypothesis of a remote enhancer for tachykinin expression. In addition, both TAC1 and TACR1 contain nearby MEIS1 TFBSs.

A number of the SNPs most significantly associated to COMT expression in the unphenotyped population were to distant markers, in a region surrounding the D22S944 and D22S943 microsatellites, ~340 kb upstream of COMT (tables 4.1.-4.4.). Interestingly, the D22S944 marker in this region is also associated and linked to PD[155] in our PD pedigrees, and SCZ in different populations[156,157]. Perhaps variation in this proximal region, which is implicated in normal COMT and MB-COMT expression, may be the source of dysregulation in PD. This is the first link between the regulation of normal COMT expression and PD. Interestingly GNB1L, which harbors SNPs associated with COMT expression in this study, is also associated with SCZ[189].

4.6.3.2. Phylogenetic footprinting and TFBSs

How might a distant region, some ~340 kb away, be correlated with COMT expression? The phylogenetic footprint generated by multi-species sequence alignment[190] in a 150 kb region around D22S944 and D22S943 reveals discrete regions of highly conserved sequence, with the most significant alignment among twenty-eight vertebrates near three predictied TFBSs. They are TCF4 (also named ITF-2, SEF2, E2-2, and ME2), NKX22, and NKX3A. Because TCF4 binds glucocorticoid response elements (GREs)[191], which are also located in COMT, this raises the possibility of TCF4 regulation of COMT. Binding sites for

the more ubiquitous NKX family members of transcription factors, including NKX22, NKX3A, NKX25 and NKX61, were observed at candidate genes for PD (TAC1, TACR1, GABRB3, GRP, GRPR, and COMT). TFs implicated in COMT and MB-COMT expression have potentially important functions, including neurological or other related phenotypic relationships to PD and anxiety processes. For example, EGR2 is shown to regulate genes critical for myelination of neurons, and is disrupted in patients with peripheral neuropathies[202]. Pulmonary and repiratory dysfunction is also observed in patients with EGR2 mutations[203].

In a case-control study of PD, Philibert *et al*. observed approximately three-fold greater COMT expression in lymphoblastoid cell lines from males relative to females with PD[177]. Thus, disease state may exacerbate gender differences. Interestingly, the same study found in male probands nearly two-fold greater expression of the transcription factor SP1, which regulates COMT expression. SP1 TFBSs are present in the second intron of COMT, in the region of the S-COMT promoter[9]. The SP1 TFBS also serves as a site for the estrogen receptor alpha (ESR1)[131], which downregulates COMT expression in the presence of estrogen. Variability in ESR1 and SP1 transcription factor expression, altering the availability of these TFs, could speculatively impact the epigenetic regulation of target genes by competitive binding to common TFBSs.

Variants associated with COMT expression lying in regions of predicted TFBSs suggest that there may be possible coregulation of these TFs with COMT. Alternatively, variation within or near these predicted TFBSs may impact TF-

mediated regulation of COMT. Other definitive functional studies could include chromatin imunoprecipitation (ChIP) and electrophoretic mobility gel shift (EMSA) assays to show the binding of these TFs to regions of chromosome 22 associated with COMT expression. Additionally, reporter assays could be exploited to show direct regulation of COMT, as Xie *et al*. and Jiang *et al*. did for estrogenic downregulation of COMT via estrogen response elements[129,130].

Many interesting hypotheses may be generated from the interaction of these TFs in the putative dysregulation of multiple candidate genes for PD. A number of phenotypes reflecting symptoms and syndromic features of PD are observed for these TFs. Both TCF4 and NKX2-2 are known to mediate neuronal development[204,205]. TCF4 is also implicated in adult neuronal plasticity[205], bipolar disorder[206], and Pitt-Hopkins syndrome, which are characterized by symptoms that include severe autonomic dysfunction and intermittent hyperventilation[207]. Target genes for TCF4 include the glucocorticoid receptor (GR)[191] and somatostatin 2 receptor (SSTR2)[208,209], expressed in the brain among other tissues. Downstream effects of glucocorticoid stress hormones impinge on all of our candidate genes for PD, including negative feedback on hypothalamic corticotropin-releasing hormone (CRH) and pro-opiomelanocortin (POMC). Somatostatin is co-localized with COMT in the brain and periphery, and is observed to suppress anxiety in rats[10] as well as have anxiolytic properties in a trial of subjects with panic-like symptoms[210]. Somatostatin suppresses the release of two panicogenic compounds: gastrin (pentagastrin) and choleocystokinin (CCK). Their respective receptors are targets of a patented

anxiolytic antagonist in Europe. The somatostatin 3 receptor (SSTR3) family member, with 46% identity to SSTR2, which is shown *in vitro* to inhibit dopaminergic effects[211], resides at a significant linkage peak (D22S445, LOD=2.93) in PD families with syndromic features. The NKX2-5 transcription factor has homeostatic roles, including atrial natriuresis, which is important for modulating cardiac function[212] and inflammation associated with bladder disorders via TACR1 actions[213]. Therefore, given the potential mechanistic overlap in these candidate genes, one can envision multiple endocrine and autonomic effects by their coordinate regulation of TCF4 and NKX TFs.

Higher density genotyping in the proximal and other region associated with COMT expression would provide finer positional information. Comparison of the heritability of COMT expression in PD probands versus the HapMap sample described here would be a natural next step. About a third of the seventy PD pedigrees in the initial genome scan study showed linkage to the proximal variant, D22S944. Since the previous genome scan, an additional fifty PD pedigrees have been added to our sample collection for investigation. The proximal region around D22S944 is susceptible to chromosomal abberations that could be assayed for copy number variants in control and affected individuals. Although the D22S944 marker is approximately 1 Mb away from the nearest annotated gene, according to the NCBI Genome Browser, a study in cancer patients illustrates the relevance of this gene-poor region. Colon cancer patients show an increase in microsatellite instability in this region that disrupts the TCF4 TFBS and causes downstream dysregulation of target genes and signalling

pathway partners of the TCF4 transcription factor[192]. Thus, non-genic regions of the genome have important roles in disease states via long-range *cis* and *trans* regulation of expression of target genes.

Sex differences in gene expression in lymphoblastoid cell lines have been observed in clinically important genes, including CYP3A4, which is responsible for the metabolic turnover of approximately 60% of prescribed drugs[214]. Hormonal control of the expression of PD candidate genes is a common theme, based on observed phenotypic changes correlated with hormonal fluctuations. Functional studies show that estrogen and progesterone regulate transcription of COMT[128-130]. We, however, saw no inherent gender difference in COMT expression. Since the lymphoblastoid cell lines in this study are expected to be devoid of estrogen, hormonal regulation of COMT should not be operant in this assay. However, a microarray expression study by Philibert *et al*., which compared the expression profiles of lymphoblastoid cell lines in controls and PD probands, showed that COMT was significantly overexpressed in males relative to females with PD. Thus, it appears that gender differences in COMT expression may result from other, non-hormonal causes.

Expression profiling studies suggests that approximately 10-30% of genes tested are heritably expressed[201]. Here we present data in support of heritable factors associated with the expression of COMT in cell lines from an unphenotyped Caucasian population. Genetic variants in COMT have been linked and/or association with PD in five studies, with controversial results based upon the different populations, albeit with some controversy as to the ethnic-

specific valence of association to alleles. In order to map the causative mutation(s) that may contribute to the clinical phenotype of PD in our own affected Caucasian pedigrees, we characterized the natural variation in COMT expression as a molecular phenotype of COMT function. In this way, inherited COMT expression patterns can be used as a molecular phenotype for COMT regulation, operant in a control Caucasian population. It is intriguing to observe the same proximal region to the COMT, which was associated and linked to PD, is also associated with COMT expression in an otherwise healthy population. Perhaps this is evidence of a link between a potential regulatory element in control subjects that may be dysregulated in PD probands.

4.7. Future directions

Gene expression may be influenced by short-range and long-range regulatory factors, mRNA splice sites, as well as alterations in RNA stability. To better understand the role of COMT in PD across different ethnic populations, future studies may want to investigate the variables that may underlie the contradictory allelic association of COMT variants with PD in different study populations. A study by Spielman *et al.*, showed that about a quarter of the heritably expressed genes in Caucasians were differentially expressed in Asians, in which about half of the ethnic expression differences was accounted for by allele frequency differences in *cis* regulatory variants[181]. The SNP rs12168204 (or rs9617857), associated with with COMT expression in this study, for example, has 19.2% MAF in Caucasians, 6-7% MAF in Asians, and 47.5% in Yorubans. Thus, comparison of the heritable expression pattern of COMT across ethnic

groups would be helpful to resolve differences that may be due to disparity in allele frequency. Since the prevalence of PD across countries is relatively similar, one might expect a unifying reason for the involvement of COMT in different populations, despite the vast allele frequency differences by ethnic group.

Functional studies would ultimately be needed to show the unambiguous role of associated variants in COMT expression. For example, binding assays (e.g., chromatin immunoprecipitation (ChIP) with specific transcription factors), DNase I hypersensivity, and transfection assays for reporter gene assays may be used to demonstrate a mechanism behind the genetic association.

Comparative expression profiling in disease states could be used to identify changes in gene expression in response to physiological challenges. For example, perivascular microglia (i.e. brain macrophage cells) are activated with injury and microenvironmental changes, causing induction of COMT expression and activity, which is implicated in pathological inflammatory responses leading to cardiovascular and neurological disease[200]. Dysregulation of more than one gene pathway, such as the GABAergic and catecholaminergic pathways, are interesting to note in the literature as well. For example, chronic and subchronic administration of L-DOPA, which mimics catecholamine-overexpressing adrenal pheochromocytoma[199], significantly increases GABA levels in the substantia nigra in rats[198]. Thus, comparative expression profiling in PD subjects and controls could identify gene networks purported to be dysregulated in PD.

4.8. References

1. Li Y *et al. (*2004) Equine catechol estrogen 4-hydroxyequilenin is a more potent inhibitor of the variant form of catechol-O-methyltransferase. *Chem.Res.Toxicol.* 17 (4):512-520.

2. Hegedus ZL (2000) The probable involvement of soluble and deposited melanins, their intermediates and the reactive oxygen side-products in human diseases and aging. *Toxicology* 145 (2-3):85-101.

3. Matsoto M *et al. (*2003) Catechol O-methyltransferase mRNA expression in human and rat brain: evidence for a role in cortical neuronal function. *Neuroscience* 116 (1):127-137.

4. Karhunen T *et al. (*1995) Catechol-O-methyltransferase (COMT) in rat brain: immunoelectron microscopic study with an antiser against rat recombinant COMT protein. *Neurosci.Lett.* 187 (1):57-60.

5. Karhunen T *et al. (*1995) Neuronal and non-neuronal catechol-O-methyltransferase in primary cultures of rat brain cells. *Int.J.Dev.Neurosci.* 13 (8):825-834.

6. Hirano Y *et al. (*2007) Suppression of catechol-O-methyltransferase activity through blunting of alpha2-adrenoceptor can explain hypertension in Dahl salt-sensitive rats. *Hypertens.Res.* 30 (3):269-278.

7. Raftogianis R *et al. (*2000) Estrogen metabolism by conjugation. *J.Natl.Cancer Inst.Monogr* (27):113-124.

8. Spatz M *et al. (*1986) The presence of catechol-o-methyltransferase activity in separately cultured cerebromicrovascular endothelial and smooth muscle cells. *Brain Res.* 381 (2):363-367.

9. Tenhunen J *et al. (*1994) Genomic organization of the human catechol O-methyltransferase gene and its expression from two distinct promoters. *Eur.J.Biochem.* 223 (3):1049-1059.

10. Meis S *et al. (*2005) Mechanisms of somatostatin-evoked responses in neurons of the rat lateral amygdala. *Eur.J.Neurosci.* 21 (3):755-762.

11. Schulteis G and Martinez JL, Jr. (1992) Peripheral modulation of learning and memory: enkephalins as a model system. *Psychopharmacology (Berl)* 109 (3):347-364.

12. Mannisto PT and Kaakkola S (1999) Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. *Pharmacol.Rev.* 51 (4):593-628.

13. Lotta T *et al. (*1995) Kinetics of human soluble and membrane-bound catechol O-methyltransferase: a revised mechanism and description of the thermolabile variant of the enzyme. *Biochemistry* 34 (13):4202-4210.

14. Shield AJ *et al. (*2004) Human catechol O-methyltransferase genetic variation: gene resequencing and functional characterization of variant allozymes. *Mol.Psychiatry* 9 (2):151-160.

15. Boudikova B *et al. (*1990) Human liver catechol-O-methyltransferase pharmacogenetics. *Clin.Pharmacol.Ther.* 48 (4):381-389.

16. De Santi C *et al. (*1998) Catechol-O-methyltransferase: variation in enzyme activity and inhibition by entacapone and tolcapone. *Eur.J.Clin.Pharmacol.* 54 (3):215-219.

17. Lachman HM *et al. (*1996) Human catechol-O-methyltransferase pharmacogenetics: description of a functional polymorphism and its potential application to neuropsychiatric disorders. *Pharmacogenetics* 6 (3):243-250.

18. Chen J *et al. (*2004) Functional analysis of genetic variation in catechol-O-methyltransferase (COMT): effects on mRNA, protein, and enzyme activity in postmortem human brain. *Am.J.H.Genet.* 75 (5):807-821.

19. Goldin LR (1985) Segregation analysis of dopamine-beta-hydroxylase (DBH) and catechol-O-methyltransferase (COMT): identification of major locus and polygenic components. *Genet.Epidemiol.* 2 (3):317-325.

20. Goldin LR *et al. (*1982) Segregation and linkage studies of plasma dopamine-beta-hydroxylase (DBH), erythrocyte catechol-O-methyltransferase (COMT), and platelet monoamine oxidase (MAO): possible linkage between the ABO locus and a gene controlling DBH activity. *Am.J.H.Genet.* 34 (2):250-262.

21. Bray NJ *et al. (*2003) A haplotype implicated in schizophrenia susceptibility is associated with reduced COMT expression in human brain. *Am.J.H.Genet.* 73 (1):152-161.

22. Zhu G *et al. (*2004) Differential expression of human COMT alleles in brain and lymphoblasts detected by RT-coupled 5' nuclease assay. *Psychopharmacology (Berl)* 177 (1-2):178-184.

23. Li Y *et al. (*2004) Equine catechol estrogen 4-hydroxyequilenin is a more potent inhibitor of the variant form of catechol-O-methyltransferase. *Chem.Res.Toxicol.* 17 (4):512-520.

24. Cotton NJ, Stoddard B, and Parson WW (2004) Oxidative inhibition of human soluble catechol-O-methyltransferase. *J.Biol.Chem.* 279 (22):23710-23718.

25. Goldstein DS, Eisenhofer G, and Kopin IJ (2003) Sources and significance of plasma levels of catechols and their metabolites in humans. *J.Pharmacol.Exp.Ther.* 305 (3):800-811.

26. Eisenhofer G, Kopin IJ, and Goldstein DS (2004) Catecholamine metabolism: a contemporary view with implications for physiology and medicine. *Pharmacol.Rev.* 56 (3):331-349.

27. Contreras F *et al. (*2002) Dopamine, hypertension and obesity. *J.H.Hypertens.* 16 Suppl 1:S13-S17.

28. Nagel-Leiby S *et al. (*1990) Event-related slow potentials and associated catecholamine function in migraine. *Cephalalgia* 10 (6):317-329.

29. Gogos JA *et al. (*1998) Catechol-O-methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behavior. *Proc.Natl.Acad.Sci.U.S.A* 95 (17):9991-9996.

30. Fujii T *et al. (*2004) Extraneuronal enzymatic degradation of myocardial interstitial norepinephrine in the ischemic region. *Cardiovasc.Res.* 64 (1):125-131.

31. O'Brien MS, Wu LT, and Anthony JC (2005) Cocaine use and the occurrence of panic attacks in the community: a case-crossover approach. *Subst.Use.Misuse.* 40 (3):285-297.

32. Pacak K *et al. (*1998) Heterogeneous neurochemical responses to different stressors: a test of Selye's doctrine of nonspecificity. *Am.J.Physiol* 275 (4 Pt 2):R1247-R1255.

33. Esler M (2000) The sympathetic system and hypertension. *Am.J.Hypertens.* 13 (6 Pt 2):99S-105S.

34. Weissman MM *et al. (*2000) Potential panic disorder syndrome: Clinical and genetic linkage evidence. *American Journal of Medical Genetics* 96 (1):24-35.

35. Weissman MM *et al. (*1990) Panic disorder and cardiovascular/cerebrovascular problems: results from a community survey. *Am.J.Psychiatry* 147 (11):1504-1508.

36. Berry MD (2004) Mammalian central nervous system trace amines. Pharmacologic amphetamines, physiologic neuromodulators. *J.Neurochem.* 90 (2):257-271.

37. Bunzow JR *et al. (*2001) Amphetamine, 3,4-methylenedioxymethamphetamine, lysergic acid diethylamide, and metabolites of the catecholamine neurotransmitters are agonists of a rat trace amine receptor. *Mol.Pharmacol.* 60 (6):1181-1188.

38. Miller GM *et al. (*2005) Primate trace amine receptor 1 modulation by the dopamine transporter. *J.Pharmacol.Exp.Ther.* 313 (3):983-994.

39. McClung C and Hirsh J (1999) The trace amine tyramine is essential for sensitization to cocaine in Drosophila. *Curr.Biol.* 9 (16):853-860.

40. Maruyama W, Naoi M, and Narabayashi H (1996) The metabolism of L-DOPA and L-threo-3,4-dihydroxyphenylserine and their effects on monoamines in the human brain: analysis of the intraventricular fluid from parkinsonian patients. *J.Neurol.Sci.* 139 (1):141-148.

41. Berdeaux A and Giudicelli JF (1987) Antihypertensive drugs and baroreceptor reflex control of heart rate and blood pressure. *Fundam.Clin.Pharmacol.* 1 (4):257-282.

42. Spencer JP (2003) Metabolism of tea flavonoids in the gastrointestinal tract. *J.Nutr.* 133 (10):3255S-3261S.

43. Nagai M, Conney AH, and Zhu BT (2004) Strong inhibitory effects of common tea catechins and bioflavonoids on the O-methylation of catechol estrogens catalyzed by human liver cytosolic catechol-O-methyltransferase. *Drug Metab Dispos.* 32 (5):497-504.

44. Zhu BT (2002) On the mechanism of homocysteine pathophysiology and pathogenesis: a unifying hypothesis. *Histol.Histopathol.* 17 (4):1283-1291.

45. Richard IH, Schiffer RB, and Kurlan R (1996) Anxiety and Parkinson's disease. *J.Neuropsychiatry Clin.Neurosci.* 8 (4):383-392.

46. Odlind C *et al. (*1999) Regulation of dopamine-induced natriuresis by the dopamine-metabolizing enzyme catechol-O-methyltransferase. *Exp.Nephrol.* 7 (4):314-322.

47. Nissinen E *et al. (*2005) The COMT inhibitor, entacapone, reduces levodopa-induced elevations in plasma homocysteine in healthy adult rats. *J.Neural Transm.* 112 (9):1213-1221.

48. Khromova I *et al. (*1997) Effects of selective catechol-O-methyltransferase inhibitors on single-trial passive avoidance retention in male rats. *Behav.Brain Res.* 86 (1):49-57.

49. Blum K *et al. (*2007) Manipulation of catechol-O-methyl-transferase (COMT) activity to influence the attenuation of substance seeking behavior, a subtype of Reward Deficiency Syndrome (RDS), is dependent upon gene polymorphisms: a hypothesis. *Med.Hypotheses* 69 (5):1054-1060.

50. Moron JA *et al. (*2002) Dopamine uptake through the norepinephrine transporter in brain regions with low levels of the dopamine transporter: evidence from knock-out mouse lines. *J.Neurosci.* 22 (2):389-395.

51. Johnson MR *et al. (*1994) Plasma and CSF HVA levels in panic patients with comorbid social phobia. *Biol.Psychiatry* 36 (6):425-427.

52. Lepola U *et al. (*1990) Cerebrospinal fluid monoamine metabolites and neuropeptides in patients with panic disorder. *Ann.Med.* 22 (4):237-239.

53. Eriksson E *et al. (*1991) Cerebrospinal fluid levels of monoamine metabolites in panic disorder. *Psychiatry Res.* 36 (3):243-251.

54. Charney DS *et al. (*1987) Neurobiological mechanisms of panic anxiety: biochemical and behavioral correlates of yohimbine-induced panic attacks. *Am.J.Psychiatry* 144 (8):1030-1036.

55. Charney DS and Heninger GR (1986) Abnormal regulation of noradrenergic function in panic disorders. Effects of clonidine in healthy subjects and patients with agoraphobia and panic disorder. *Arch.Gen.Psychiatry* 43 (11):1042-1054.

56. Charney DS, Heninger GR, and Breier A (1984) Noradrenergic function in panic anxiety. Effects of yohimbine in healthy subjects and patients with agoraphobia and panic disorder. *Arch.Gen.Psychiatry* 41 (8):751-763.

57. Mantani N *et al. (*2002) Four cases of panic disorder successfully treated with Kampo (Japanese herbal) medicines: Kami-shoyo-san and Hange-koboku-to. *Psychiatry Clin.Neurosci.* 56 (6):617-620.

58. Kaneko A *et al. (*2005) Hange-koboku-to, a Kampo medicine, modulates cerebral levels of 5-HT (5-hydroxytryptamine), NA (noradrenaline) and DA (dopamine) in mice. *Phytother.Res.* 19 (6):491-495.

59. Kopin IJ (1985) Catecholamine metabolism: basic aspects and clinical significance. *Pharmacol.Rev.* 37 (4):333-364.

60. Trendelenburg U (1990) The interaction of transport mechanisms and intracellular enzymes in metabolizing systems. *J.Neural Transm.Suppl* 32:3-18.

61. Haasio K *et al. (*2003) Tissue histopathology, clinical chemistry and behaviour of adult Comt-gene-disrupted mice. *J.Appl.Toxicol.* 23 (4):213-219.

62. Huotari M *et al. (*2002) Brain catecholamine metabolism in catechol-O-methyltransferase (COMT)-deficient mice. *Eur.J.Neurosci.* 15 (2):246-256.

63. Yavich L *et al. (*2007) Site-specific role of catechol-O-methyltransferase in dopamine overflow within prefrontal cortex and dorsal striatum. *J.Neurosci.* 27 (38):10196-10209.

64. Lewis DA *et al. (*2001) Dopamine transporter immunoreactivity in monkey cerebral cortex: regional, laminar, and ultrastructural localization. *J.Comp Neurol.* 432 (1):119-136.

65. Sesack SR *et al. (*1998) Dopamine axon varicosities in the prelimbic division of the rat prefrontal cortex exhibit sparse immunoreactivity for the dopamine transporter. *J.Neurosci.* 18 (7):2697-2708.

66. Karo F, Chrapusta SJ, and Egan MF (1994) 3-Methoxytyramine is the major metabolite of released dopamine in the rat frontal cortex: reassessment of the effects of antipsychotics on the dynamics of dopamine release and metabolism in the frontal cortex, nucleus accumbens, and striatum by a simple two pool model. *J.Neurochem.* 63 (3):972-979.

67. Moll GH *et al. (*2000) Age-associated changes in the densities of presynaptic monoamine transporters in different regions of the rat brain from early juvenile life to late adulthood. *Brain Res.Dev.Brain Res.* 119 (2):251-257.

68. Akil M *et al. (*2003) Catechol-O-methyltransferase genotype and dopamine regulation in the human brain. *J.Neurosci.* 23 (6):2008-2013.

69. Abdolmaleky HM *et al. (*2006) Hypomethylation of MB-COMT promoter is a major risk factor for schizophrenia and bipolar disorder. *H.Mol.Genet.* 15 (21):3132-3145.

70. Huotari M *et al. (*2002b) Effect of dopamine uptake inhibition on brain catecholamine levels and locomotion in catechol-O-methyltransferase-disrupted mice. *J.Pharmacol.Exp.Ther.* 303 (3):1309-1316.

71. Boulton AA and Eisenhofer G (1998) Catecholamine metabolism. From molecular understanding to clinical diagnosis and treatment. Overview. *Adv.Pharmacol.* 42:273-292.

72. Oechsner M *et al. (*2002) COMT-inhibition increases ser levels of dihydroxyphenylacetic acid (DOPAC) in patients with advanced Parkinson's disease. *J.Neural Transm.* 109 (1):69-75.

73. Fernandes C *et al. (*2004) Hippocampal gene expression profiling across eight mouse inbred strains: towards understanding the molecular basis for behaviour. *Eur.J.Neurosci.* 19 (9):2576-2582.

74. Masuda K *et al. (*2004) Breed differences in genotype and allele frequency of catechol O-methyltransferase gene polymorphic regions in dogs. *J.Vet.Med.Sci.* 66 (2):183-187.

75. Korostynski M *et al. (*2006) Gene expression profiling in the striatum of inbred mouse strains with distinct opioid-related phenotypes. *BMC.Genomics* 7:146.

76. Shulman R, Griffiths J, and Diewold P (1978) Catechol-O-methyl transferase activity in patients with depressive illness and anxiety states. *Br.J.Psychiatry* 132:133-138.

77. Enoch MA *et al. (*2003) Genetic origins of anxiety in women: a role for a functional catechol-O-methyltransferase polymorphism. *Psychiatr.Genet.* 13 (1):33-41.

78. McGrath M *et al. (*2004) Association Between Catechol-O-Methyltransferase and Phobic Anxiety. *American Journal of Psychiatry* 161 (9):1703-1705.

79. Olsson CA *et al. (*2005) Association between the COMT Val158Met polymorphism and propensity to anxiety in an Australian population-based longitudinal study of adolescent health. *Psychiatr.Genet.* 15 (2):109-115.

80. Rakvag TT *et al. (*2005) The Val158Met polymorphism of the human catechol-O-methyltransferase (COMT) gene may influence morphine requirements in cancer pain patients. *Pain* 116 (1-2):73-78.

81. Jones G *et al. (*2001) Aggressive behaviour in patients with schizophrenia is associated with catechol-O-methyltransferase genotype. *Br.J.Psychiatry* 179:351-355.

82. Lachman HM *et al. (*1998) Association between catechol O-methyltransferase genotype and violence in schizophrenia and schizoaffective disorder. *Am.J.Psychiatry* 155 (6):835-837.

83. Emin EM *et al. (*2001) Significance of the catechol-O-methyltransferase gene polymorphism in migraine. *Brain Res.Mol.Brain Res.* 94 (1-2):193-196.

84. Enoch MA (2006) Genetic and environmental influences on the development of alcoholism: resilience vs. risk. *Ann.N.Y.Acad.Sci.* 1094:193-201.

85. Sery O *et al. (*2006) The association between high-activity COMT allele and alcoholism. *Neuro.Endocrinol.Lett.* 27 (1-2):231-235.

86. Meyer-Lindenberg A *et al. (*2006) Impact of complex genetic variation in COMT on human brain function. *Mol.Psychiatry* 11 (9):867-77, 797.

87. Kamide K *et al. (*2007) Association of genetic polymorphisms of ACADSB and COMT with human hypertension. *J.Hypertens.* 25 (1):103-110.

88. Odlind C *et al. (*2001) The role of dopamine-metabolizing enzymes in the regulation of renal sodium excretion in the rat. *Pflugers Arch.* 442 (4):505-510.

89. Wen G *et al. (*2004) Both rare and common polymorphisms contribute functional variation at CHGA, a regulator of catecholamine physiology. *Am.J.H.Genet.* 74 (2):197-207.

90. Helkamaa T *et al. (*2003) Resistance to salt-induced hypertension in catechol-O-methyltransferase-gene-disrupted mice. *J.Hypertens.* 21 (12):2365-2374.

91. Kajimoto K *et al. (*2007) Exclusion of the catechol-o-methyltransferase gene from genes contributing to salt-sensitive hypertension in dahl salt-sensitive rats. *Hypertens.Res.* 30 (5):459-467.

92. Terry AV, Jr., Hernandez CM, and Buccafusco JJ (2001) Dahl salt-sensitive and salt-resistant rats: examination of learning and memory performance, blood pressure, and the expression of central nicotinic acetylcholine receptors. *Neuroscience* 103 (2):351-363.

93. Anokhin AP *et al. (*2003) Genetic influences on prepulse inhibition of startle reflex in humans. *Neurosci.Lett.* 353 (1):45-48.

94. Hagen K *et al. (*2007) High systolic blood pressure is associated with Val/Val genotype in the catechol-o-methyltransferase gene. The Nord-Trondelag Health Study (HUNT). *Am.J.Hypertens.* 20 (1):21-26.

95. Savitz J, Solms M, and Ramesar R (2006) The molecular genetics of cognition: dopamine, COMT and BDNF. *Genes Brain Behav.* 5 (4):311-328.

96. Karayiorgou M *et al. (*1999) Family-based association studies support a sexually dimorphic effect of COMT and MAOA on genetic susceptibility to obsessive-compulsive disorder. *Biol.Psychiatry* 45 (9):1178-1189.

97. Diatchenko L *et al. (*2005) Genetic basis for individual variations in pain perception and the development of a chronic pain condition. *H.Mol.Genet.* 14 (1):135-143.

98. Zubieta JK *et al. (*2002) mu-opioid receptor-mediated antinociceptive responses differ in men and women. *J.Neurosci.* 22 (12):5100-5107.

99. Yellman C *et al. (*1997) Conserved and sexually dimorphic behavioral responses to biogenic amines in decapitated Drosophila. *Proc.Natl.Acad.Sci.U.S.A* 94 (8):4131-4136.

100. Jeffery DR and Roth JA (1984) Characterization of membrane-bound and soluble catechol-O-methyltransferase from human frontal cortex. *J.Neurochem.* 42 (3):826-832.

101. Reenila I and Mannisto PT (2001) Catecholamine metabolism in the brain by membrane-bound and soluble catechol-o-methyltransferase (COMT) estimated by enzyme kinetic values. *Med.Hypotheses* 57 (5):628-632.

102. Sladek-Chelgren S and Weinshilboum RM (1981) Catechol-o-methyltransferase biochemical genetics: human lymphocyte enzyme. *Biochem.Genet.* 19 (11-12):1037-1053.

103. Hong J *et al. (*1998) Distribution of catechol-O-methyltransferase expression in human central nervous system. *Neuroreport* 9 (12):2861-2864.

104. Ghisolfi ES *et al. (*2006) P50 sensory gating in panic disorder. *J.Psychiatr.Res.* 40 (6):535-540.

105. Ludewig S *et al. (*2002) Prepulse inhibition deficits in patients with panic disorder. *Depress.Anxiety.* 15 (2):55-60.

106. Ludewig S *et al. (*2005) Information-processing deficits and cognitive dysfunction in panic disorder. *J.Psychiatry Neurosci.* 30 (1):37-43.

107. Ulmanen I *et al. (*1997) Expression and intracellular localization of catechol O-methyltransferase in transfected mammalian cells. *Eur.J.Biochem.* 243 (1-2):452-459.

108. Mattay VS *et al. (*2003) Catechol O-methyltransferase val158-met genotype and individual variation in the brain response to amphetamine. *Proc.Natl.Acad.Sci.U.S.A* 100 (10):6186-6191.

109. Bertocci B *et al. (*1991) Human catechol-O-methyltransferase: cloning and expression of the membrane-associated form. *Proc.Natl.Acad.Sci.U.S.A* 88 (4):1416-1420.

110. Murphy BC, O'Reilly RL, and Singh SM (2005) Site-specific cytosine methylation in S-COMT promoter in 31 brain regions with implications for studies involving schizophrenia. *Am.J.Med.Genet.B Neuropsychiatr.Genet.* 133 (1):37-42.

111. Heun R, & Maier W. (1995) Relation of schizophrenia and panid disorder: evidence from a controlled family study. *Am. J. Med. Genet.* 60 (2):127-132.

112. Fones CS *et al. (*2000) History of childhood attention deficit hyperactivity disorder (ADHD) features among adults with panic disorder. *J.Affect.Disord.* 58 (2):99-106.

113. Karayiorgou M *et al. (*1995) Schizophrenia susceptibility associated with interstitial deletions of chromosome 22q11. *Proc.Natl.Acad.Sci.U.S.A* 92 (17):7612-7616.

114. Michaelovsky E *et al. (*2007) Association between a common haplotype in the COMT gene region and psychiatric disorders in individuals with 22q11.2DS. *Int.J.Neuropsychopharmacol.*:1-13.

115. Jurata LW *et al. (*2006) Altered expression of hippocampal dentate granule neuron genes in a mouse model of human 22q11 deletion syndrome. *Schizophr.Res.* 88 (1-3):251-259.

116. Yobb TM *et al. (*2005) Microduplication and triplication of 22q11.2: a highly variable syndrome. *Am.J.H.Genet.* 76 (5):865-876.

117. Ensenauer RE *et al. (*2003) Microduplication 22q11.2, an emerging syndrome: clinical, cytogenetic, and molecular analysis of thirteen patients. *Am.J.H.Genet.* 73 (5):1027-1040.

118. Bird AP (1986) CpG-rich islands and the function of DNA methylation. *Nature* 321 (6067):209-213.

119. Sasaki M *et al. (*2003) Multiple promoters of catechol-O-methyltransferase gene are selectively inactivated by CpG hypermethylation in endometrial cancer. *Cancer Res.* 63 (12):3101-3106.

120. Ahsan H *et al. (*2004) A family-based genetic association study of variants in estrogen-metabolism genes COMT and CYP1B1 and breast cancer risk. *Breast Cancer Res.Treat.* 85 (2):121-131.

121. Kessler RC *et al. (*2006) The epidemiology of panic attacks, panic disorder, and agoraphobia in the National Comorbidity Survey Replication. *Arch.Gen.Psychiatry* 63 (4):415-424.

122. Maddock RJ *et al. (*2003) Brain regions showing increased activation by threat-related words in panic disorder. *Neuroreport* 14 (3):325-328.

123. Fahndrich E *et al. (*1980) Erythrocyte COMT-activity in patients with affective disorders. *Acta Psychiatr.Scand.* 61 (5):427-437.

124. Scanlon PD, Raymond FA, and Weinshilboum RM (1979) Catechol-O-methyltransferase: thermolabile enzyme in erythrocytes of subjects homozygous for allele for low activity. *Science* 203 (4375):63-65.

125. Lee SG *et al. (*2005) Association of Ala72Ser polymorphism with COMT enzyme activity and the risk of schizophrenia in Koreans. *H.Genet.* 116 (4):319-328.

126. Dempster EL *et al. (*2006) The quantification of COMT mRNA in post mortem cerebell tissue: diagnosis, genotype, methylation and expression. *BMC.Med.Genet.* 7:10.

127. Parvez S *et al. (*1978) Activity of catechol-o-methyltransferase in brain regions and adrenal gland during the oestrus cycle. *J.Neural Transm.* 42 (4):305-312.

128. Salama SA *et al. (*2007) Progesterone regulates catechol-O-methyl transferase gene expression in breast cancer cells: distinct effect of progesterone receptor isoforms. *J.Steroid Biochem.Mol.Biol.* 107 (3-5):253-261.

129. Jiang H *et al. (*2003) human catechol-O-methyltransferase down-regulation by estradiol. *Neuropharmacology* 45 (7):1011-1018.

130. Xie T, Ho SL, and Ramsden D (1999) Characterization and implications of estrogenic down-regulation of human catechol-O-methyltransferase gene transcription. *Mol.Pharmacol.* 56 (1):31-38.

131. Klinge CM (2001) Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res.* 29 (14):2905-2919.

132. Inoue K and Creveling CR (1991) Induction of catechol-O-methyltransferase in the linal epithelium of rat uterus by progesterone. *J.Histochem.Cytochem.* 39 (6):823-828.

133. Hong CC *et al. (*2003) Val158Met Polymorphism in catechol-O-methyltransferase gene associated with risk factors for breast cancer. *Cancer Epidemiol.Biomarkers Prev.* 12 (9):838-847.

134. Hill RA *et al. (*2007) Estrogen deficient male mice develop compulsive behavior. *Biol.Psychiatry* 61 (3):359-366.

135. Alsobrook JP *et al. (*2002) Association between the COMT locus and obsessive-compulsive disorder in females but not males. *Am.J.Med.Genet.* 114 (1):116-120.

136. Karayiorgou M *et al. (*1997) Genotype determining low catechol-O-methyltransferase activity as a risk factor for obsessive-compulsive disorder. *Proc.Natl.Acad.Sci.U.S.A* 94 (9):4572-4575.

137. Casas M *et al. (*1986) Antiandrogenic treatment of obsessive-compulsive neurosis. *Acta Psychiatr.Scand.* 73 (2):221-222.

138. Altemus M (2006) Sex differences in depression and anxiety disorders: potential biological determinants. *Horm.Behav.* 50 (4):534-538.

139. Blanchet PJ *et al. (*1999) Short-term effects of high-dose 17beta-estradiol in postmenopausal PD patients: a crossover study. *Neurology* 53 (1):91-95.

140. Salem JE and Kring AM (1998) The role of gender differences in the reduction of etiologic heterogeneity in schizophrenia. Clin.Psychol.Rev. 18 (7):795-819.

141. Palmatier MA *et al. (*2004) COMT haplotypes suggest P2 promoter region relevance for schizophrenia. *Mol.Psychiatry* 9 (9):859-870.

142. Cheung KH *et al. (*2000) ALFRED: an allele frequency database for diverse populations and DNA polymorphisms. *Nucleic Acids Res.* 28 (1):361-363.

143. Shifman S *et al. (*2002) A highly significant association between a COMT haplotype and schizophrenia. *Am.J.H.Genet.* 71 (6):1296-1302.

144. Hennah W *et al. (*2004) Haplotype analysis and identification of genes for a complex trait: examples from schizophrenia. *Ann. Med.* 36 (5):322-331.

145. Fan JB *et al. (*2005) Catechol-O-methyltransferase gene Val/Met functional polymorphism and risk of schizophrenia: a large-scale association study plus meta-analysis. *Biol.Psychiatry* 57 (2):139-144.

146. Glatt SJ, Faraone SV, and Tsuang MT (2003) Association between a functional catechol O-methyltransferase gene polymorphism and schizophrenia: meta-analysis of case-control and family-based studies. *Am.J.Psychiatry* 160 (3):469-476.

147. Hagen K *et al. (*2006) No association between chronic musculoskeletal complaints and Val158Met polymorphism in the Catechol-O-methyltransferase gene. The HUNT study. *BMC.Musculoskelet.Disord.* 7:40.

148. Zubieta JK *et al. (*2003) COMT val158met genotype affects mu-opioid neurotransmitter responses to a pain stressor. *Science* 299 (5610):1240-1243.

149. Pooley EC, Fineberg N, and Harrison PJ (2007) The met(158) allele of catechol-O-methyltransferase (COMT) is associated with obsessive-compulsive disorder in men: case-control study and meta-analysis. *Mol.Psychiatry* 12 (6):556-561.

150. Nackley A.G. *et al.* Human catechol-O-methyltransferase haplotypes modulate protein expression by altering mRNA secondary structure. *Science* 314 (5807):1930-1933 (2006).

151. DeMille MM *et al. (*2002) Population variation in linkage disequilibrium across the COMT gene considering promoter region and coding region variation. *H.Genet.* 111 (6):521-537.

152. Li Y *et al. (*2005) Characterization of two new variants of human catechol O-methyltransferase in vitro. *Cancer Lett.* 230 (1):81-89.[152]

153. Rothe C *et al. (*2006) Association of the Val158Met catechol O-methyltransferase genetic polymorphism with panic disorder. *Neuropsychopharmacology* 31 (10):2237-2242.

154. Funke B *et al. (*2005) COMT genetic variation confers risk for psychotic and affective disorders: a case control study. *Behav.Brain Funct.* 1:19.

155. Hamilton SP *et al. (*2002) Evidence for a susceptibility locus for panic disorder near the catechol-O-methyltransferase gene on chromosome 22. *Biol.Psychiatry* 51 (7):591-601.

156. Williams NM *et al. (*2002) Mutation screening and LD mapping in the VCFS deleted region of chromosome 22q11 in schizophrenia using a novel DNA pooling approach. *Mol.Psychiatry* 7 (10):1092-1100.

157. Horowitz A *et al. (*2005) Further tests of the association between schizophrenia and single nucleotide polymorphism markers at the catechol-O-methyltransferase locus in an Askenazi Jewish population using microsatellite markers. *Psychiatr.Genet.* 15 (3):163-169.

158. Domschke K *et al. (*2004) Association of the functional V158M catechol-O-methyl-transferase polymorphism with panic disorder in women. *Int.J.Neuropsychopharmacol.* 7 (2):183-188.

159. Samochowiec J *et al. (*2004) Association studies of MAO-A, COMT, and 5-HTT genes polymorphisms in patients with anxiety disorders of the phobic spectrum. *Psychiatry Res.* 128 (1):21-26.

160. Means-Christensen A *et al. (*2003) **The Composite International Diagnostic Interview (CIDI-Auto): Problems and Remedies for Diagnosing Panic Disorder and Social Phobia.** International Journal of Methods in Psychiatric Research 12 (4):167-181.

161. Woo JM, Yoon KS, and Yu BH (2002) Catechol O-methyltransferase genetic polymorphism in panic disorder. *Am.J.Psychiatry* 159 (10):1785-1787.

162. Woo JM *et al. (*2004) The association between panic disorder and the L/L genotype of catechol-O-methyltransferase. *J.Psychiatr.Res.* 38 (4):365-370.

163. Ohara K *et al. (*1998) No association between anxiety disorders and catechol-O-methyltransferase polymorphism. *Psychiatry Res.* 80 (2):145-148.

164. Domschke K *et al. (*2007) Meta-analysis of COMT val158met in panic disorder: ethnic heterogeneity and gender specificity. *Am.J.Med.Genet.B Neuropsychiatr.Genet.* 144 (5):667-673.

165. Chen ML and Chen CH (2007) Chronic antipsychotics treatment regulates MAOA, MAOB and COMT gene expression in rat frontal cortex. *J.Psychiatr.Res.* 41 (1-2):57-62.

166. Cheng MC *et al. (*2007) Chronic treatment with aripiprazole induces differential gene expression in the rat frontal cortex. *Int.J.Neuropsychopharmacol.*:1-10.

167. Schmidt-Kastner R *et al. (*2006) Gene regulation by hypoxia and the neurodevelopmental origin of schizophrenia. *Schizophr.Res.* 84 (2-3):253-271.

168. Cheung VG *et al. (*2003) Natural variation in human gene expression assessed in lymphoblastoid cells. Nat.Genet. 33 (3):422-425.

169. Goring HH *et al. (*2007) Discovery of expression QTLs using large-scale transcriptional profiling in human lymphocytes. *Nat.Genet.* 39 (10):1208-1216.

170. Ruiz-Llorente S *et al. (*2007) Association study of 69 genes in the ret pathway identifies low-penetrance loci in sporadic medullary thyroid carcinoma. *Cancer Res.* 67 (19):9561-9567.

171. Yan H *et al. (*2002) Allelic variation in human gene expression. *Science* 297 (5584):1143.

172. Handoko HY *et al. (*2005) Separate and interacting effects within the catechol-O-methyltransferase (COMT) are associated with schizophrenia. *Mol.Psychiatry* 10 (6):589-597.

173. Craddock N, Owen MJ, and O'Donovan MC (2006) The catechol-O-methyl transferase (COMT) gene as a candidate for psychiatric phenotypes: evidence and lessons. *Mol.Psychiatry* 11 (5):446-458.

174. Sawa A and Snyder SH (2002) Schizophrenia: diverse approaches to a complex disease. *Science* 296 (5568):692-695.

175. Dudbridge F. Pedigree disequilibrium test for multilocus haplotypes. *Genet. Epidemiol.* 25:115-121 (2003).

176. Stranger BE *et al. (*2005) Genome-wide associations of gene expression variation in humans. *PLoS.Genet.* 1 (6):e78.

177. Philibert RA *et al. (*2007) Transcriptional profiling of lymphoblast lines from subjects with panic disorder. *Am.J.Med.Genet.B Neuropsychiatr.Genet.* 144 (5):674-682.

178. Bland M (2000) **An Introduction to Medical Statistics**. 3rd ed. Oxford University Press: New York.

179. Bolstad BM *et al. (*2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics.* 19 (2):185-193.

180. Cheung VG *et al. (*2005) Mapping determinants of human gene expression by regional and genome-wide association. *Nature* 437 (7063):1365-1369.

181. Spielman RS *et al. (*2007) Common genetic variants account for differences in gene expression among ethnic groups. *Nat.Genet.* 39 (2):226-231.

182. Morley M *et al. (*2004) Genetic analysis of genome-wide variation in human gene expression. *Nature* 430 (7001):743-747.

183. Haller F *et al. (*2004) Equivalence test in quantitative reverse transcription polymerase chain reaction: confirmation of reference genes suitable for normalization. *Anal.Biochem.* 335 (1):1-9.

184. Radonic A *et al. (*2004) Guideline to reference gene selection for quantitative real-time PCR. *Biochem.Biophys.Res.Commun.* 313 (4):856-862.

185. de Jonge HJ *et al. (*2007) Evidence based selection of housekeeping genes. *PLoS.ONE.* 2 (9):e898.

186. Vandesompele J *et al. (*2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3 (7):3-7.

187. Dixon AL *et al. (*2007) A genome-wide association study of global gene expression. *Nat.Genet.* 39 (10):1202-1207.

188. Davidson S *et al. (*2006) A remote and highly conserved enhancer supports amygdala specific expression of the gene encoding the anxiogenic neuropeptide substance-P. *Mol.Psychiatry* 11 (4):323, 410-323, 421.

189. Williams NM *et al. (*2008) Strong evidence that GNB1L is associated with schizophrenia. Hum.Mol.Genet. 17 (4):555-566.

190. Miller W *et al. (*2007) 28-way vertebrate alignment and conservation track in the UCSC Genome Browser. *Genome Res.* 17 (12):1797-1808.

191. Corneliussen B *et al. (*1991) Helix-loop-helix transcriptional activators bind to a sequence in glucocorticoid response elements of retrovirus enhancers. *J Virol.* 65 (11):6084-6093.

192. Chang HR *et al. (*2006) Genetic and cellular characterizations of human TCF4 with microsatellite instability in colon cancer and leukemia cell lines. Cancer Lett. 233 (1):165-171.

193. Yao J *et al. (*2003) Catechol estrogen 4-hydroxyequilenin is a substrate and an inhibitor of catechol-O-methyltransferase. *Chem.Res.Toxicol.* 16 (5):668-675.

194. James SJ *et al. (*2006) Metabolic endophenotype and related genotypes are associated with oxidative stress in children with autism. *Am.J.Med.Genet. Neuropsychiatric Genet*. 141B (8):947-956.

195. Raftogianis R *et al. (*2000) Estrogen metabolism by conjugation. *J.Natl.Cancer Inst.Monogr* (27):113-124.

196. Worda C *et al. (*2003) Influence of the catechol-O-methyltransferase (COMT) codon 158 polymorphism on estrogen levels in women. *Hum.Reprod.* 18 (2):262-266.

197. D'Andrea G *et al. (*2007) Biochemistry of neuromodulation in primary headaches: focus on anomalies of tyrosine metabolism. *Neurol.Sci.* 28 Suppl 2:S94-S96.

198. Yamamoto N, Pierce RC, and Soghomonian JJ (2006) Subchronic administration of L-DOPA to adult rats with a unilateral 6-hydroxydopamine lesion of dopamine neurons results in a sensitization of enhanced GABA release in the substantia nigra, pars reticulata. *Brain Res.* 1123 (1):196-200.

199. Eisenhofer G *et al. (*1998) Plasma metanephrines are markers of pheochromocytoma produced by catechol-O-methyltransferase within tors. *J.Clin.Endocrinol.Metab* 83 (6):2175-2185.

200. Helkamaa T *et al. (*2007) Increased catechol-O-methyltransferase activity and protein expression in OX-42-positive cells in the substantia nigra after lipopolysaccharide microinfusion. *Neurochem.Int.* 51 (6-7):412-423.

201. Williams RB *et al. (*2007) The influence of genetic variation on gene expression. *Genome Res.* 17 (12):1707-1716.

202. Nagarajan R *et al. (*2001) EGR2 mutations in inherited neuropathies dominant-negatively inhibit myelin gene expression. *Neuron* 30 (2):355-368.

203. Szigeti K *et al. (*2007) Functional, histopathologic and natural history study of neuropathy associated with EGR2 mutations. *Neurogenetics.* 8 (4):257-262.

204. Ding YQ *et al. (*2003) Lmx1b is essential for the development of serotonergic neurons. *Nat.Neurosci.* 6 (9):933-938.

205. Soosaar A *et al. (*1994) Expression of basic-helix-loop-helix transcription factor ME2 during brain development and in the regions of neuronal plasticity in the adult brain. *Brain Res.Mol.Brain Res.* 25 (1-2):176-180.

206. Del-Favero J *et al. (*2002) European combined analysis of the CTG18.1 and the ERDA1 CAG/CTG repeats in bipolar disorder. *Eur. J. Hum. Genet.* 10 (4):276-280.

207. Zweier C *et al. (*2007) Haploinsufficiency of TCF4 causes syndromal mental retardation with intermittent hyperventilation (Pitt-Hopkins syndrome). *Am.J Hum.Genet.* 80 (5):994-1001.

208. Pscherer A *et al. (*1996) The helix-loop-helix transcription factor SEF-2 regulates the activity of a novel initiator element in the promoter of the human somatostatin receptor II gene. *EMBO J* 15 (23):6680-6690.

209. Dorflinger U *et al. (*1999) Activation of somatostatin receptor II expression by transcription factors MIBP1 and SEF-2 in the murine brain. *Mol.Cell Biol.* 19 (5):3736-3747.

210. Abelson JL, Nesse RM, and Vinik A (1990) Treatment of panic-like attacks with a long-acting analogue of somatostatin. *J.Clin.Psychopharmacol.* 10 (2):128-132.

211. Yamada Y *et al. (*1992) Somatostatin receptors, an expanding gene family: cloning and functional characterization of human SSTR3, a protein coupled to adenylyl cyclase. Mol.Endocrinol. 6 (12):2136-2142.

212. Small EM and Krieg PA (2003) Transgenic analysis of the atrialnatriuretic factor (ANF) promoter: Nkx2-5 and GATA-4 binding sites are required for atrial specific expression of ANF. *Dev.Biol.* 261 (1):116-131.

213. Saban R *et al. (*2007) Bladder inflammatory transcriptome in response to tachykinins: neurokinin 1 receptor-dependent genes and transcription regulatory elements. *BMC.Urol.* 7:7.

214. Zhang W *et al. (*2007) Gender-specific differences in expression in human lymphoblastoid cell lines. *Pharmacogenet.Genomics* 17 (6):447-450.

4.9. Figures and tables

Figure 4.1. Coefficient of variance of expressed transcripts in LCLs

The distribution of the average percent standard deviation (ave%SD, or %CV) for detectable transcripts in ninety lymphoblastoid cell lines (LCLs) using the Illumina BeadChip assay.

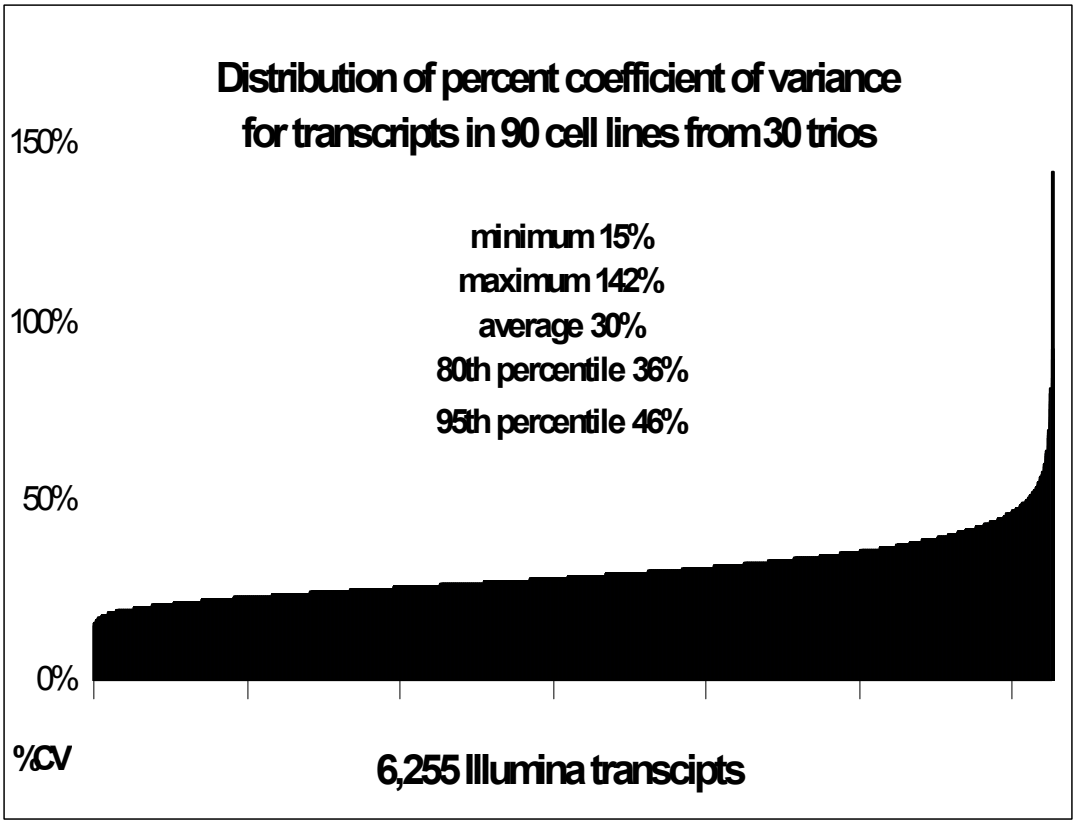

Figure 4.2. Mean transcript intensities in LCLs

The distribution of the average transcript level, measured as arbitrary intensity values, in ninety lymphoblastoid cell lines (LCLs).

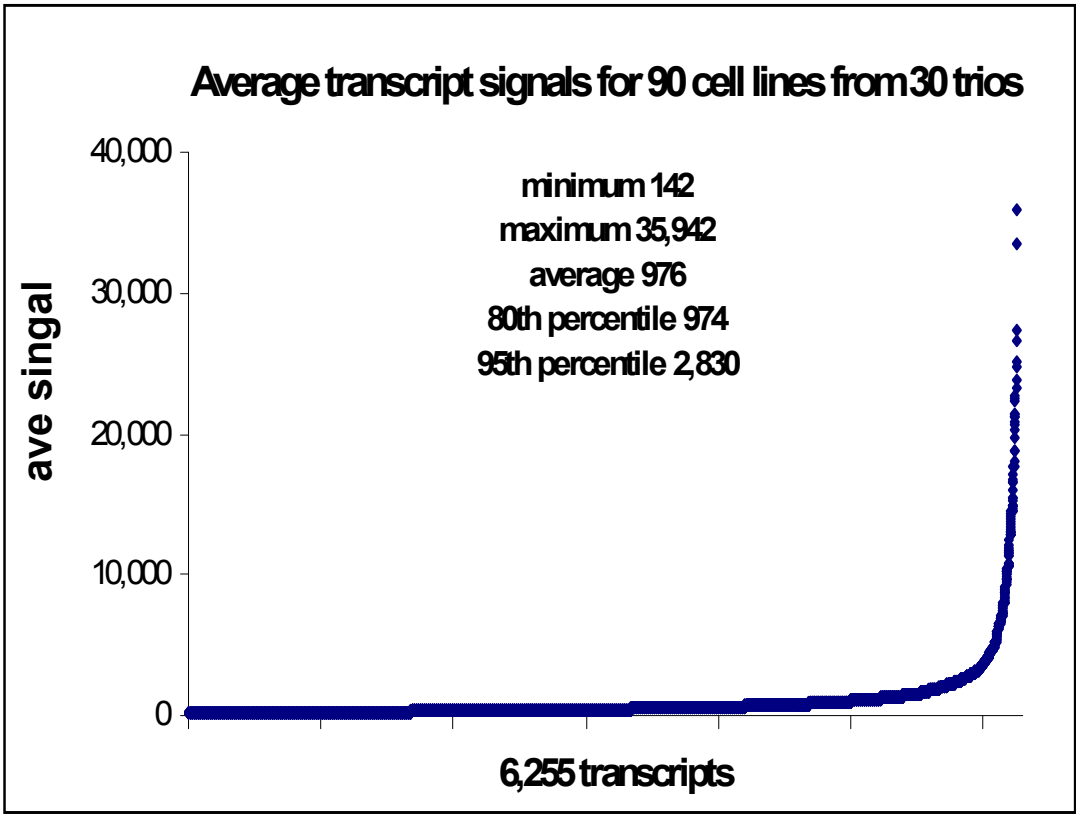

Figure 4.3. Mean fold change in transcripts expressed in LCLs

The distribution of the mean fold change (maximum:minimum expression ratio in the sample set) for ninety lymphoblastoid cell lines (LCLs).

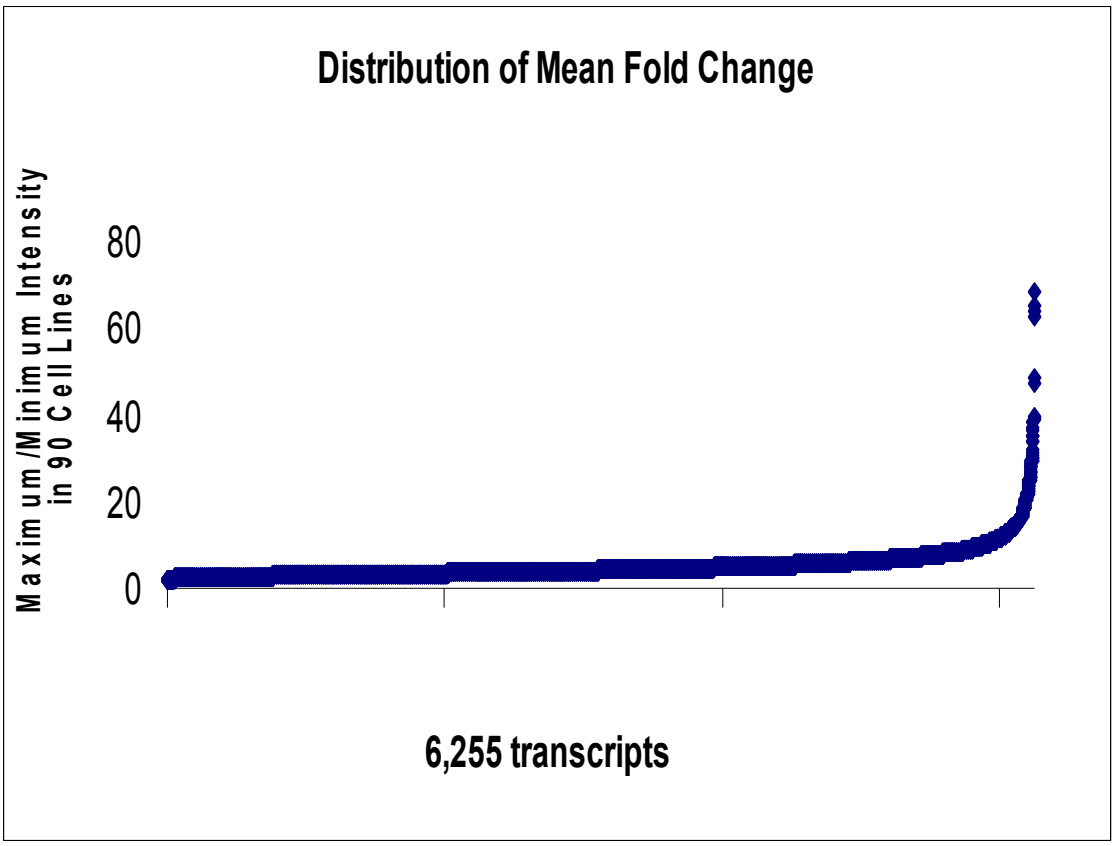

Figure 4.4. Association of SNPs with COMT expression in Caucasian trios

The significance values for the association of global COMT transcript levels in LCLs from thirty trios with 303 SNP genotypes are plotted as the negative log (base 10) of the *p*-value on the y-axis against the position of the interrogated SNP on the x-axis. Chr = chromosome, Mb = megabase. Genes and microsatellite markers are denoted at the top of the graph.

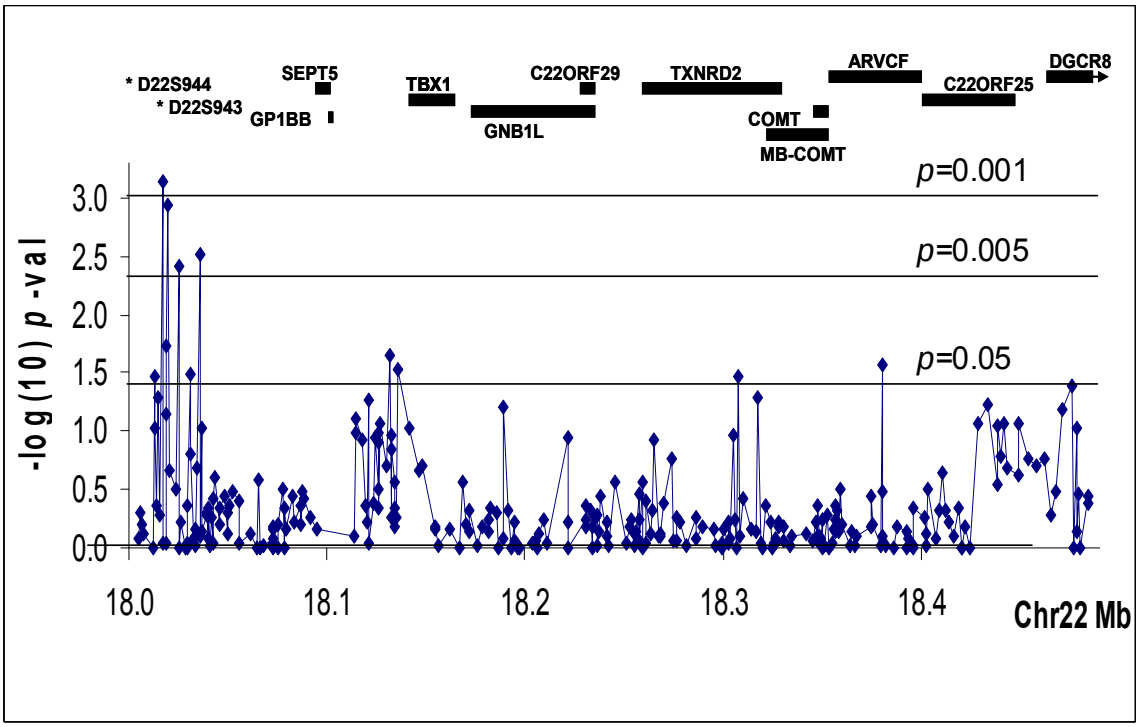

Figure 4.5. Association of SNPs with COMT expression in unrelated Caucasians

The significance values for the association of global COMT transcript levels in LCLs from sixty unrelated parents with the 303 SNP genotypes are plotted as the negative log (base 10) of the p-value on the y-axis against the position of the interrogated SNP on the x-axis. Chr = chromosome, Mb = megabase. Genes and microsatellite markers are denoted at the top of the graph.

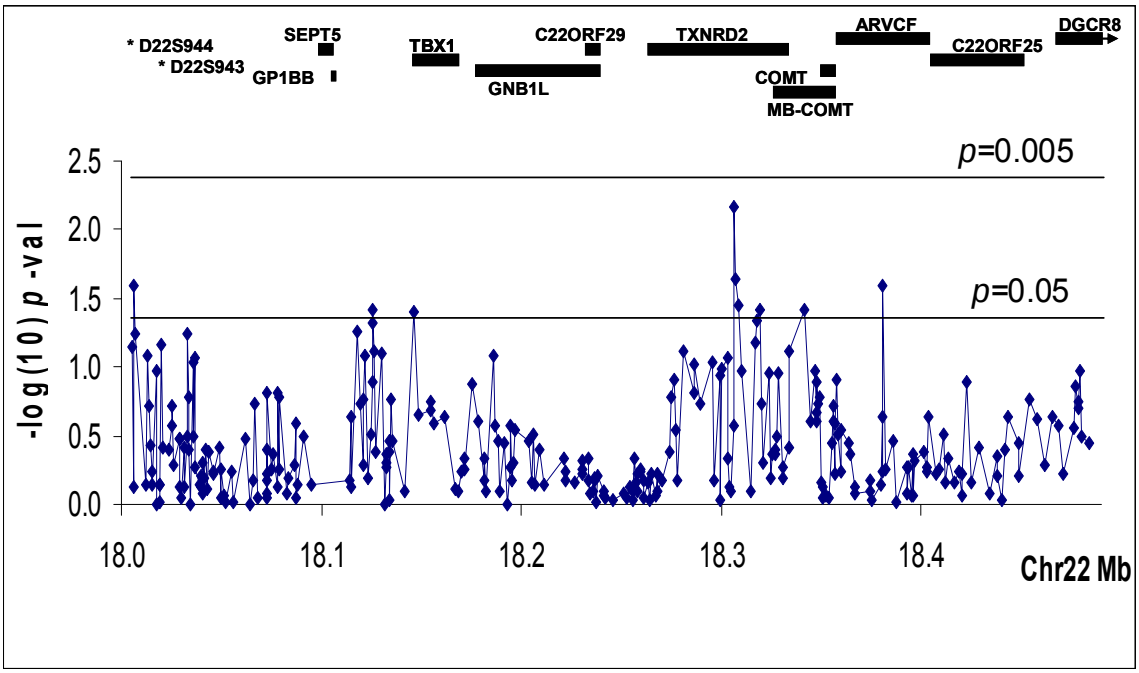

Figure 4.6. Association of SNPs with MB-COMT expression in Caucasian trios

The significance values for the association of MB-COMT transcript levels in LCLs from twenty-eight trios with 302 SNP genotypes are plotted as the negative log (base 10) of the p-value on the y-axis against the position of the interrogated SNP on the x-axis. Chr = chromosome, Mb = megabase. Genes and microsatellite markers are denoted at the top of the graph.

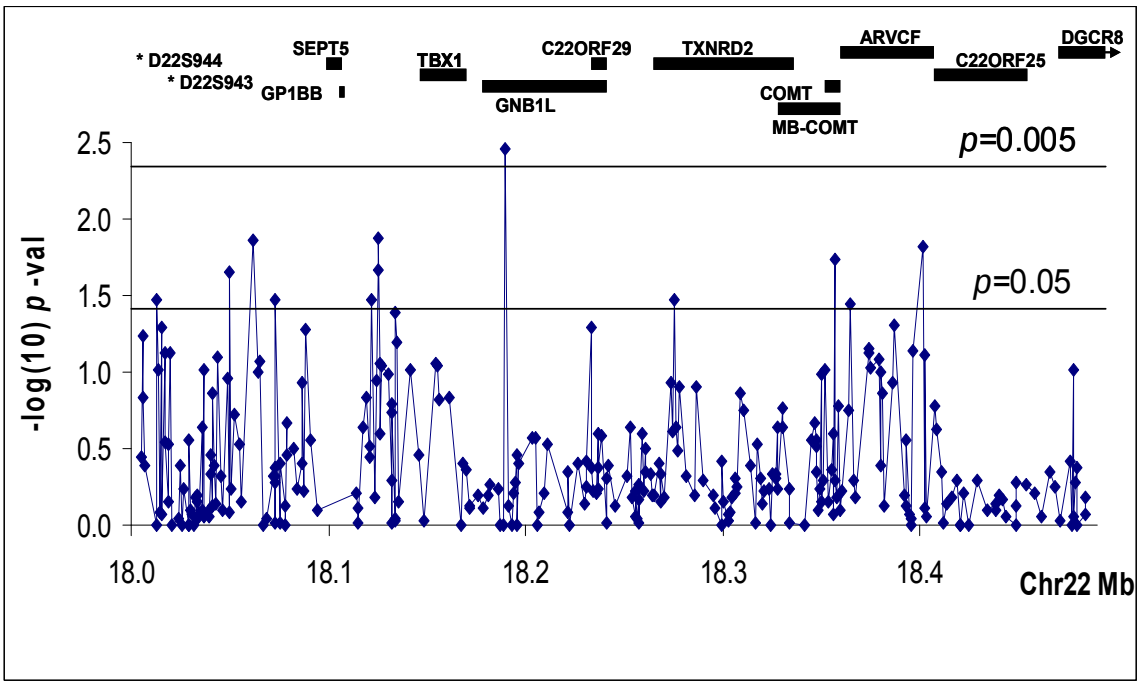

Figure 4.7. Association of SNPs with MB-COMT expression in unrelated Caucasians

The significance values for the association of MB-COMT transcript levels in LCLs from fifty-nine unrelated parents with 302 SNP genotypes are plotted as the negative log (base 10) of the p-value on the y-axis against the position of the interrogated SNP on the x-axis. Chr = chromosome, Mb = megabase. Genes and microsatellite markers are denoted at the top of the graph.

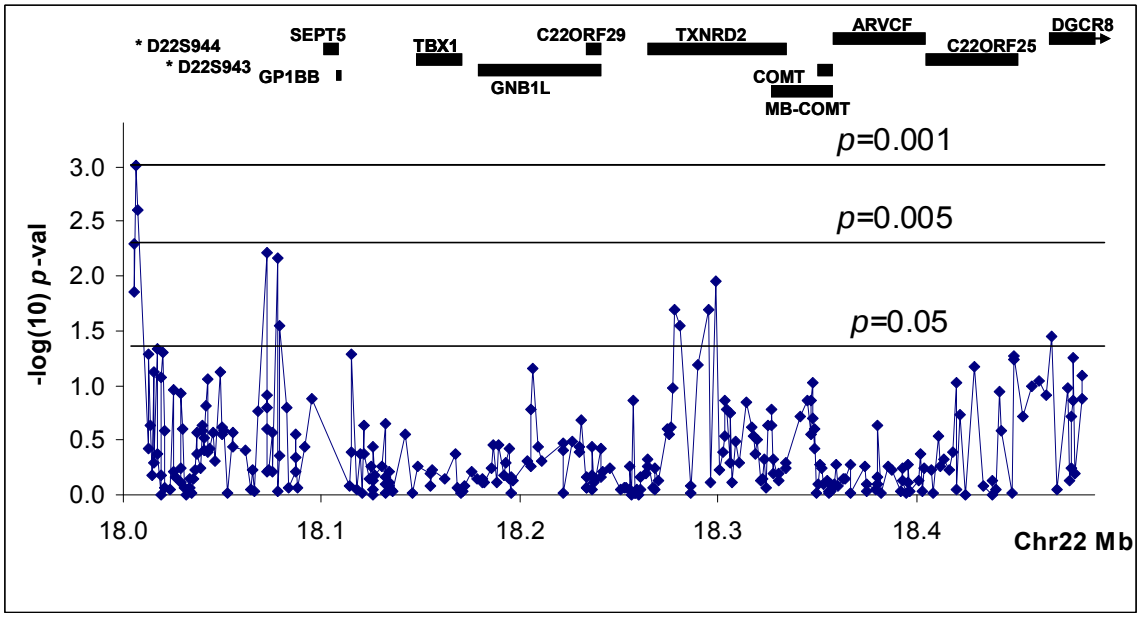

Figure 4.8. Sequence alignment of MB-COMT hybridization probe

Alignment of the probe sequence for the isoform-specific MB-COMT hybridization probe (GI_6466451_I) to the reference sequence of the May 2004 NCBI Genome Browser, showing one basepair mismatch and two rare SNPs within the probe sequence.

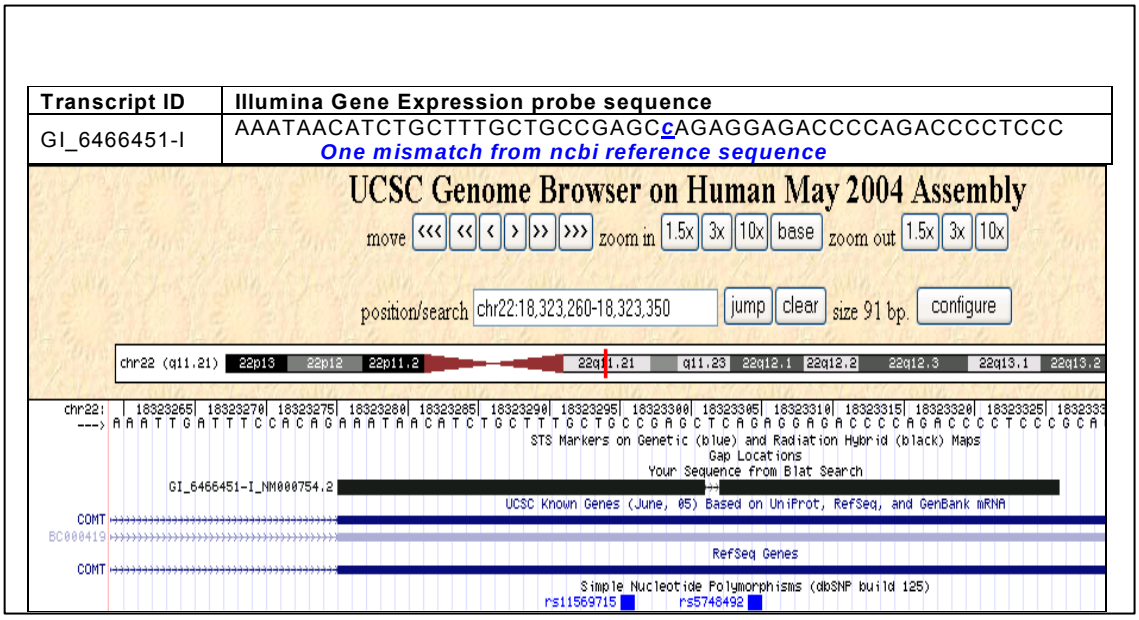

Table 4.1. Association of 7 SNPs ($p \leq 0.05$) with global COMT expression in Caucasian trios given 10,000 permutations

TFBS = transcription factor binding site, LD = r^2>0.8 linkage disequilibrium, sequence positions per May 2004 NCBI build 35.

SNP	$-\log_{10} p$ Permuted 10,000	Comments
rs11912807	4.0	~930bp proximal to D22S943; TCF4 TFBS; ~4,900bp LD block_ chr22:17,993,087-17,998,020_spans D22S943 and NKX22 and NKX3A TFBSs
rs2078747	4.0	~3,700bp distal to D22S943
rs2871047	4.0	TXNRD2 first intron; high LD with rs10483103 at ESR1 TFBS
rs737935	4.0	DGCR8 intron
rs8139968 (rs5748374)	1.9	~19kb distal to D22S943
rs11089328	1.7	DGCR8 intron; SRF, PAX6, and MEIS1AHOXA9 TFBSs
rs739374	1.5	~8,800bp proximal to TBX1; NRSF TFBS

Table 4.2. Association of 9 SNPs ($p \leq 0.05$) with global COMT expression in unrelated Caucasians given 10,000 permutations

TFBS = transcription factor binding site, LD = r^2>0.8 linkage disequilibrium, sequence positions per May 2004 NCBI build 35.

SNP	$-\log_{10} p$ Permuted 10,000	Comments
rs1012157	2.3	TXNRD2 intron
rs11089325	1.7	ARVCF intron
rs9617813	1.6	~500bp proximal to D22S944 within a gene desert of >182kb; ~2,000bp LD block_chr22:17,983,211-17,985,236_spans D22S944; two SOX9 and one TST1 TFBS
rs2157732	1.6	no genes within >20kb; between SEPT5 and TBX1
rs9306229	1.5	TXNRD2 intron
rs740603	1.5	MB-COMT intron
rs11089305	1.4	no genes for >20kb; between SEPT5 and TBX1
rs1978060	1.3	TBX1 intron; ISRE TFBS
rs12483887	1.3	~10kb distal to SETP5

Table 4.3. Association of 8 SNPs ($p \leq 0.05$) with MB-COMT expression in Caucasian trios given 10,000 permutations

TFBS = transcription factor binding site, LD = r^2>0.8 linkage disequilibrium, sequence positions per May 2004 NCBI build 35.

SNP	$-\log_{10} p$ Permuted 10,000	Comments
rs11912807	4.0	~4,900bp LD block_chr22:17,993,087-17,998,020_spans D22S943; NKX22 and NKX3A TFBSs
rs4141526	4.0	~2,900bp distal to D22S943
rs11705143	4.0	GNB1L intron
rs13055873	4.0	GNB1L intron
rs2871047	4.0	TXNRD2 first intron
rs2531716	1.9	~390bp distal to ARVCF; in predicted sequence for C22ORF25; CpG island; LUN1 TFBS; ~46kb LD block _chr22:18,333,223-18,379,258_spans most of ARVCF
rs2157732	1.7	middle of ~33kb region between SEPT5 and TBX1
rs5993834	1.5	GNB1L intron/exon; ~4400bp LD block_chr22:18,162,901-18,167,266_PAX4, CHX10, and CEBPA TFBSs

Table 4.4. Association of 15 SNPs ($p \leq 0.05$) with MB-COMT expression in unrelated Caucasians given 10,000 permutations

TFBS = transcription factor binding site, LD = r^2>0.8 linkage disequilibrium, sequence positions per May 2004 NCBI build 35.

SNP	$-\log_{10} p$ Permuted 10,000	Comments
rs9617813	3.2	~700bp proximal to D22S944; ~680bp LD block_chr22:17,984,426-17,985,109_in D22S944; high LD with rs110854553 in D22S944
rs10854554	2.6	~100bp distal to D22S944; high LD with rs9617857 (rs12168204) and rs13054962
rs13054962	2.5	~1,900bp proximal to D22S944; high LD with rs10854553; high conservation; LUN1 and TST1 TFBSs
rs5748403	2.0	no genes within ~40kb; high regulatory sequence conservation; ~3,900bp LD block _chr22:18,051,567-18,055,430
rs4819811	2.0	~1,300bp proximal to D22S944
rs4597638	2.0	TXNRD2 intron
rs1123657	1.9	no genes within ~60kb; high regulatory sequence conservation
rs3788309	1.9	TXNRD2 intron; ~1300bp LD block_chr22:18,252,916-18,254,191_ EGR2, RP58, and GATA1 TFBSs
rs5992493	1.8	TXNRD2 intron
rs9606176	1.7	TXNRD2 intron
rs732262	1.6	TXNRD2 intron; ~4,300bp LD block _chr22:18,254,894-18,259,211 spanning TXNRD2 exon
rs5992465	1.5	~7,500bp proximal to D22S1624; ~19kb distal to SEPT5; no TFBSs within 15kb; no genes within >37kb
rs1558496	1.4	DGCR8 intron; RFX TFBS
rs7291726	1.4	~1,300bp distal to D22S943; NKX22 and NKX3A TFBSs
rs2078747	1.3	~3,600bp distal to D22S943; ~3,200bp LD block chr22:17,997,914-18,001,083_~1,800bp distal to D22S943; high regulatory sequence conservation

5. Chapter Five. Integrative hypothesis for PD

PD is a complex, multigenic disorder, thought to arise from more than one gene. The challenge is therefore to detect the contribution of different genes which may explain only a portion of the overall genetic liability for PD. Phenotypic heterogeneity can be observed in gender-specific prevalence rates, population-specific propensities for different symptoms and comorbidities, as well as differences in drug response, potentially owed to the fractional genetic contributions of different genes. This may in part explain the difficulty in replicating correlations that have been observed for other candidate genes in different populations (see section 1.1.10.). Ethnicity explains some of the discordance even in the two replicated candidate genes for PD (COMT and ADORA2A)[1,2]. To our knowledge, the 120 multiplex PD pedigrees used in this study constitute the largest PD sample available. Further gains may therefore benefit from an integrative approach to identifying the polygenic origins of PD.

5.1. The potential role of transcription factors

Data from various sources, including gene expression profiles, animal studies, and basic research have uncovered gene-gene interactions that would otherwise not be predicted to play a role in phenotypic outcomes. Our data point to transcription factors involved of with our candidate genes. Two transcription factor binding sites (TFBSs) are altered in our GABRB3 mutation screen of PD probands (section 3.5., table 3.6.), and several TFBSs lie near variants that are associated with heritable COMT and MB-COMT expression patterns in an

unphenotyped population (sections 4.5.4. and 4.5.5., and tables 4.1.-4.4.). The transcription factor SP1, is an interesting example, since its expression is altered in a gender-specific way in individuals with PD[3]. A mutation in the SP1 TFBS in the CRHR1 gene is associated with alcohol abuse[4]. The SP1 TFBS is also present in the S-COMT promoter[5], where it can also serve as a site for estrogenic effects via the ESR1 transcription factor[6]. ESR1 is known to downregulate COMT expression, and has several predicted TFBSs in GABRA5[7]. The TCF4 transcription factor (also named ITF-2, SEF2, E2-2, and ME2), which is implicated in the heritable regulation of S- and MB-COMT expression in HapMap CEU samples, is predicted to contain TFBSs in TAC1, TACR1, CRHR1, GRP, GRPR, and GABRB3. A known target gene for the TCF4 transcription factor is the glucocorticoid receptor (GR) via glucocorticoid response elements (GREs)[8], which are also present in COMT, TAC1, and TACR1.

Glucocorticoids (GCs) that are released during the stress response, are blunted in CRHR1 knockouts[9], and altered in patients with PD[10,11]. Normalization of GC levels by BZ treatment in PD probands implicates the $GABR_A$ genes[11]. Chronic GC exposure causes GABRB3 upregulation in the hippocampus[12], related to the development of long-term contextual fear learning[13] (see Chapter Three). Congenital effects of high GC levels on offspring, for example, due to maternal stress with pregnancy, are correlated with increased CRH mRNA and neuroendocrine dysregulation in the progeny through adulthood[14]. Finally, work by Weiss *et al*. showed that GC levels show sexually dimorphic linkage, with significant linkage to variants in females that are not linked in males[15]. Thus,

gender-specific genetics are correlated with phenotypic differences in SP1, TCF4, and glucocorticoid transcription factor systems.

Other overlaps include a 3'UTR SNP discovered in a mutation screen of GABRB3, which lies in a GATA1 TFBS. The GATA1 TFBS also lies near a SNP implicated in the heritable expression of COMT. Binding sites for the more ubiquitous NKX family members of transcription factors, which are implicated in long-range COMT expression, are also observed in TAC1, TACR1, GABRB3, GRP, GRPR, and COMT. Interestingly, inflammatory bladder effects are mediated by NKX25 via TACR1. This is intriguing because the pathogenesis of PD is thought to derive from complex, oligogenic origins with putative pleiotropic effects. Finally, the NRSF TFBS, associated with COMT expression, also regulates CRH expression[16]. These are a few examples of the multiple lines of evidence for the phenotypic and genetic role of transcription factors in our candidate genes for PD.

5.2. Gene-gene interactions

Exploiting known interactions among pathway genes may provide more power to detect culprit alleles for PD. The interaction of genetic variables necessary to produce a clinically relevant phenotype could effectively increase the genetic load for PD. For example, a candidate gene association study in Korean alcoholics demonstrated that by combining the alleles of missense mutations from two alcohol-metabolizing genes, investigators were able to derive a synergistic 91-fold increased risk of alcoholism (OR=91.4), which explained approximately 86.5% of the population risk in Koreans[17]. Presumably, genetic

factors with small effect size by themselves could be combined with other postively associated genetic factors to determine possible synergistic effects in PD.

The literature is replete with known interactions between our candidate genes. Some gene-gene relationships are borne out the expression studies, and others come from positional data. For example, somatostatin has overlapping distribution with COMT, and plays a role in reducing anxiety[18], as well as mediating autonomic signals, nociception, and learning and memory[19]. As well, significant linkage (LOD=4.1) was found in seventy PD pedigrees with syndromic features at the microsatellite D22S445 (chr22, 33.8 Mb) at the somatostatin receptor gene SSTR3.

Anxiety-modulating drugs that either directly or indirectly target PD candidate genes point to potential gene-gene interactions, for example, between COMT and $GABR_A$s, with different levels of overlapping effects. For example, the ADRA2A blocker, yohimbine, raises COMT metabolites, blood pressure, and anxiety. $GABR_A$-targetting alprazolam, and ADRA2A agonist, clonidine, each lower COMT metabolite levels, blood pressure, and anxiety[32], although the $GABR_A$ agonist, diazepam, lowers anxiety without altering COMT metabolite levels or autonomic endpoints[20].

Other potentially important gene-gene interactions to be pursued are the transcription factors that impinge on our candidate genes. Clues as to the role of the transcription factors which harbor TFBSs in our candidate genes, and at regions associated with heritable expression, come from the literature. Gender-

related differences in transcription factors could explain sexually dimorphic phenomena. For example, SP1 transcription factor is differentially regulated in females with PD[3]. Hormonal differences, acting at the same genetic locus could explain gender differences. However there is also evidence of gender-specific linkage to quantitative traits related to neurotransmission and anxiety. Work by Weiss *et al*., demonstrated that normal variation in stress system effectors showed gender-specific genetic correlations, for example for serotonin levels, which showed large sex-specific differences in heritability and linkage peaks, and cortisol levels, which revealed an additional linkage peak for females only[15]. At the level of our low-resolution genome scans, little evidence of sex-specific loci exists, and segregation studies suggest that gender differences occur by phenomena acting at the same genetic loci in males and females. However epistatic gender differences could be tested by parsing the affected population into narrow categories reflecting observed phenotypic differences. Subcategories of probands could be categorized by differential drug response, symptomology, syndromic features, and comorbidity, as well as gender, to test for significant variables that may resolve related genetic and/or phenotypic heterogeneity. For example, respiratory symptoms are more prevalent in females, correlated with greater refractory response to drug treatment in females. Females are prone to hormonal supression of respiration via progesterone associated with increased incidence of PD in females, and certain drugs are more successful at treating these respiratory symptoms, presumable via specific molecular mechanisms related to respiration. Overlaps in transcriptional

regulation of our candidate genes by hormones are common (e.g. COMT, TACR1, GABRB3, and GABRA5)[21], and the preponderance of hormone-sensitive TFBSs (including ER and GR) in the genes points to yet undiscovered hormonal interactions. Therefore, hormone status, drug responsiveness, SP1 levels (dysregulated in PD)[3], and hormone-responsive TFBS genotypes could be used to further distinguish subcategories for comparison.

5.3. Physiological themes to identify gene interactions

A unifying theme related to a physiological overlap in the effects of our candidate genes is highly coincidental with processes responsible for oxidative stress, hypoxia, and pH balance. In addition to the important role for the $GABR_A$s, COMT, and the neuropeptidergic genes in neurotransmission and autonomic signaling cascades, our candidate genes have important roles for oxidative homeostasis, including cicurmstances of hypoxia, as well as oxidative stress. This commonality provides another source of putative variables that may influcence the disposition toward PD, which may be exploited to find further candidates genes of PD. Support for this less-cited role has been in the literature for a long time, related to the dubbed “false suffocation alarm” hypothesis for PD. It is based on the premise that the PD subjects exhibit altered oxidative homeostasis, both in the physiological triggering of responses to oxygen shortage, and reduced ability to buffer homeostatic control of vagal tone and autonomic responses (see sections 1.1.2., 3.2.6., and 3.2.8.). COMT variation is also implicated with reduced ability to mediate oxidative stress (see sections 4.2.2.2. and 4.2.3.2.). Subjects with PD are hypothesized to be hypersensitive to

signals of oxygen shortage (or an increase in CO_2, lactate, and metabolite, bicarbonate), causing inappropriate activation of neuroendocrine and autonomic signaling cascades (section 3.2.6.).

Mechanisms for the normal physiological regulation of intracellular pH includes acid/base transport across the cell membrane, include transmembrane bicarbonate influx by $GABR_As$[22], and metabolic responses acting to normalize the pH[23]. Thus, metabolic or respiratory alkalosis prompts the generation of lactic acid in response. Exaggerated elevation in blood lactate levels (and intracellular alkalosis) in response to challenge agents, such as CO_2 inhalation and sodium lactate infusion, is characteristic of subjects with PD. Trait dysregulation of blood gases in PD are also measured as lower urinary pH[24]. Hypoxic and ischemic conditions also cause transcriptional downregulation of GABRAs[25], and upregulation of COMT as well as related neuronal genes (e.g. GAD1, CCKAR, NRG1, RELN, GABRB1, MECP2)[26]. Haploinsufficiency of methyl-CpG-binding protein 2, MECP2, is associated with decreased GABRB3 expression in Rett syndrome (RS) patients, having phenotypic overlaps with PD, such as hyperventilation, EEG abnormalities, and urinary pH differences (section 3.6.). Interestingly, the transcription factors SOX9 and EGR2[28] are also downregulated with hypoxia. SOX9 and EGR2 lie in LD blocks that are associated with COMT expression in our data (see section 4.5.4., and tables 4.2. and 4.4.). Other oxidative state-responsive transcription factors that were noted in the heritable expression of COMT were TST1 (also named POU3F1) and PAX4[28]. POU3F1 and GABRA5 are differentially expressed in hippocampal tissue in response to

oxidative stress[29]. Finally, estrogen via ESR1 has protective effects against oxidative stress[31].

$GABR_A$ genes directly mediate transmembrane bicarbonate efflux, dependent on the biocarbonate transmembrane gradient, having an excitatory GABAergic effect that is associated with long-term potentiation of habituation responses, short-term neuroplasticity, and paired pulse depression[22]. This less-cited $GABR_A$ function has implications on the development of maladaptive behavior, and long-term molecular effects of PD. COMT, as well as other genes known to be regulated by brain ischemia/hypoxia in the adult brain (e.g. BDNF and CCKAR) are operant in protecting the developing brain against endogenous stressors, such as maternal starvation during pregnancy associated with non-affective and affective pychosis[26], or perinatal hypoxia associated with the development of SCZ[27]. But besides such severe effects of chromosomal aberrations, as seen with Rett Syndrome, or trauma due to complications at birth, studies in animals show that early adverse experience and separation anxiety can alter trait hypoxic ventilatory responses (section 3.2.9.).

Similar to genetic study of PD, genes responsible for the risk of SCZ have also been elusive, despite the greater heritability of SCZ compared to PD (0.80 vs. 0.48). It is likely that the polygenic nature of PD makes the contribution of any given susceptibility gene difficult to detect. Positional data at chromosome 15q12 helped to pinpoint identify the $GABR_A$ genes as candidates for PD. However, COMT and ADORA2A, which are more established candidate genes for PD, had no positional support for their involvement among the genome scans

conducted to date. Of benefit, PD is characterized by far more easily discernable symptoms than depression, for example. Therefore the lion's share of further exploration may depend on empirical data generated from clinicians, drug studies, and expression profiles to inform necessarily hypothesis-based research in the genetic analysis of PD.

5.4. References

1. Domschke K *et al. (*2007) Meta-analysis of COMT val158met in panic disorder: ethnic heterogeneity and gender specificity. *Am.J.Med.Genet.B Neuropsychiatr.Genet.* 144 (5):667-673.

2. Lam P, Hong CJ, and Tsai SJ (2005) Association study of A2a adenosine receptor genetic polymorphism in panic disorder. *Neurosci.Lett.* 378 (2):98-101.

3. Philibert RA *et al. (*2007) Transcriptional profiling of lymphoblast lines from subjects with panic disorder. *Am.J.Med.Genet.B Neuropsychiatr.Genet.* 144 (5):674-682.

4. Treutlein J *et al. (*2006) Genetic association of the human corticotropin releasing hormone receptor 1 (CRHR1) with binge drinking and alcohol intake patterns in two independent samples. Mol.Psychiatry 11 (6):594-602.

5. Tenhunen J *et al. (*1994) Genomic organization of the human catechol O-methyltransferase gene and its expression from two distinct promoters. Eur.J.Biochem. 223 (3):1049-1059.

6. Klinge CM (2001) Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res.* 29 (14):2905-2919.

7. Kim Y *et al. (*1997) Human y-aminobutyric acid-type A receptor alpha 5 subunit gene (GABRA5): Characterization and structural organization of the 5' flanking region. *Genomics*. 42:378-387.

8. Corneliussen B *et al. (*1991) Helix-loop-helix transcriptional activators bind to a sequence in glucocorticoid response elements of retrovirus enhancers. *J Virol.* 65 (11):6084-6093.

9. Smith GW *et al. (*1998) Corticotropin Releasing Factor Receptor 1-Deficient Mice Display Decreased Anxiety, Impaired Stress Response, and Aberrant Neuroendocrine Development. *Neuron* 20 (6):1093-1102.

10. Brambilla F *et al. (*1992) Psychoimmunoendocrine aspects of panic disorder. *Neuropsychobiology* 26 (1-2):12-22.

11. Curtis GC, Abelson JL, and Gold PW (1997) Adrenocorticotropic hormone and cortisol responses to corticotropin-releasing hormone: changes in panic disorder and effects of alprazolam treatment. *Biol.Psychiatry* 41 (1):76-85.

12. Bremner, J. D. *et al.* SPECT [I-123]iomazenil measurement of the benzodiazepine receptor in panic disorder. *Biol. Psychiatry* **47**, 96-106 (2000).

13. Sanchez, M. M., Ladd, C. O. & Plotsky, P. M. Early adverse experience as a developmental risk factor for later psychopathology: evidence from rodent and primate models. *Dev. Psychopathol.* **13**, 419-449 (2001).

14. Welberg LA, Seckl JR, and Holmes MC (2000) Inhibition of 11beta-hydroxysteroid dehydrogenase, the foeto-placental barrier to maternal glucocorticoids, permanently programs amygdala GR mRNA expression and anxiety-like behaviour in the offspring. *Eur.J.Neurosci.* 12 (3):1047-1054.

15. Weiss LA *et al. (*2006) The sex-specific genetic architecture of quantitative traits in humans. *Nat.Genet.* 38 (2):218-222.

16. Seth KA and Majzoub JA (2001) Repressor element silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) can act as an enhancer as well as a repressor of corticotropin-releasing hormone gene transcription. *J.Biol.Chem.* 276 (17):13917-13923.

17. Kim DJ *et al. (*2008) Major genetic components underlying alcoholism in Korean population. *Hum. Mol. Genet*. 17 (6):854-858.

18. Meis S *et al. (*2005) Mechanisms of somatostatin-evoked responses in neurons of the rat lateral amygdala. *Eur.J.Neurosci.* 21 (3):755-762.

19. Schulteis G and Martinez JL, Jr. (1992) Peripheral modulation of learning and memory: enkephalins as a model system. *Psychopharmacology (Berl)* 109 (3):347-364.

20. Charney DS, Heninger GR, and Redmond DE, Jr. (1983) Yohimbine induced anxiety and increased noradrenergic function in humans: effects of diazepam and clonidine. Life Sci. 33 (1):19-29.

21. Pierson RC, Lyons AM, and Greenfield LJ, Jr. (2005) Gonadal steroids regulate GABAA receptor subunit mRNA expression in NT2-N neurons. Brain Res.Mol.Brain Res. 138 (2):105-115.

22. White AM and Platt B (2001) Ionic mechanisms of GABA-induced long-term potentiation in the rat superior colliculus. Exp.Brain Res. 140 (4):486-494.

23. Maddock RJ (2001) The lactic acid response to alkalosis in panic disorder: an integrative review. J.Neuropsychiatry Clin.Neurosci. 13 (1):22-34.

24. Lacerda AL, Caetano D, and Keshavan MS (2005) Urinary pH in panic disorder. Psychiatry Res. 134 (2):199-203.

25. Li H, Siegel RE, and Schwartz RD (1993) Rapid decline of GABAA receptor subunit mRNA expression in hippocampus following transient cerebral ischemia in the gerbil. Hippocampus 3 (4):527-537.

26. Schmidt-Kastner R *et al. (*2006) Gene regulation by hypoxia and the neurodevelopmental origin of schizophrenia. Schizophr.Res. 84 (2-3):253-271.

27. Murray RM *et al. (*2004) A developmental model for similarities and dissimilarities between schizophrenia and bipolar disorder. Schizophr.Res. 71 (2-3):405-416.

28. Zhou D *et al. (*2008) Gene expression in mouse brain following chronic hypoxia: role of sarcospan in glial cell death. Physiol Genomics 32 (3):370-379.

29. Shan Y *et al. (*2006) Role of Bach1 and Nrf2 in up-regulation of the heme oxygenase-1 gene by cobalt protoporphyrin. FASEB 20:2651-2653.

30. Wang X *et al. (*2007) Genome-wide transcriptome profiling of region-specific vulnerability to oxidative stress in the hippocampus. Genomics. 90 (2):201-212.

31. Le May C *et al. (*2006) Estrogens protect pancreatic beta-cells from apoptosis and prevent insulin-deficient diabetes mellitus in mice. Proc.Natl.Acad.Sci. U S A. 103 (24):9232-9237.

32. Charney DS *et al. (*1986) Behavioral, biochemical, and blood pressure responses to alprazolam in healthy subjects: interactions with yohimbine. Psychopharmacology (Berl) 88 (2):133-140.

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