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Tese de Doutorado

Aspectos Genéticos no Transtorno do Pânico

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Sumário

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3. Lista de abreviaturas

5-HTT – Gene transportador da serotonina ou *Serotoninergic transporter gene*

5-HTTLPR – Polimorfismo na região promotora do gene transportador da serotonina ou *Serotonin Transporter Promoter Polymorphism*

AMPc – Adenosina monofosfato cíclico

BDNF – Fator neurotrófico neuronal

CBT – *Cognitive Behavioral Therapy*

CI – Comportamento inibido

CI95% – *Confident Interval of 95%*

CIDI – *Composite International Diagnostic Interview*

COMT – Gene *Catechol O-Methyltransferase*

CREB – AMPc responsive-element binding protein

CREB1 – AMPc responsive-element binding protein gene

CRH – Hormônio liberador de Corticotrofina

CRHR1 – Receptor 1 do Hormônio liberador de Corticotrofina

DL – Desequilíbrio de ligação

DNA – Ácido desoxirribonucléico

DSM – Manual diagnóstico e estatístico de transtorno mental ou *Diagnostic and Statistical Manual*

EFHC2 – EF-hand domain containing 2

GWA – *Genome-wide association*

GxA – Interação Gene-Ambiente

HCPA – Hospital de Clínicas de Porto Alegre

HPA – Eixo Hipotálamo-hipófise-adrenal

HTR1A – Receptor de serotonina 1A

HTR1B – Receptor de serotonina 1B

HTR2A – Receptor de serotonina 2A

HTR2C – Receptor de serotonina 2C

HTR3A – Receptor de serotonina 3A

HWE – Equilíbrio de Hardy-Weinberg ou *Hardy-Weinberg Equilibrium*

ICD – *International Classification of Diseases*

ISRS – Inibidores seletivos de recaptação da serotonina

L – *long*

MAOA – Monoamino oxidase A

Met – Metionina

MGH – *Massachusetts General Hospital*

MINI – *MINI International Neuropsychiatric Interview*

MOOSE – *Meta-analysis of Observational Studies in Epidemiology*

OR – *Odds ratio*

PD – *Panic Disorder*

RFLP – *Restriction fragment length polymorphism*

RGS2 – Regulator of G-protein signaling 2

S – *short*

SLC6A4 – Solute Carrier Family 6 (Neurotransmitter transporter serotonin), member 4

SNC – Sistema Nervoso Central

SNP – *Single nucleotide polymorphism*

SNPs – *Single nucleotide polimorphisms*

SSRIs – *Serotonin Selective Reuptake Inhibitors*

TCLE – Termo de Consentimento livre e esclarecido

TP – Transtorno do Pânico

TPH – Triptofano hidroxilase

UFRGS – Universidade Federal do Rio Grande do Sul

Val – Valina

VNTR – Número variável de repetições em *tandem*

4. Apresentação Parcial dos Resultados

- Blaya C, Paludo A, Torres M, Dornelles M, Seganfredo AC, Heldt E, Manfro GG. Milnaciprano no Transtorno do Pânico: um ensaio aberto. XXIII Jornada de Psiquiatria Psicodinâmica do CELG, Porto Alegre 2006.
- Blaya C, Paludo A, Torres M, Dornelles M, Seganfredo AC, Heldt E, Manfro GG. Milnacipran in Panic Disorder: an open trial. 159th American Psychiatric Association Annual Meeting, Toronto 2006.
- Muños V, Blaya C, Leister-Segal S, Manfro GG. Técnica de digestão com *Msp*I para identificação da variante "l" do 5-HTTLPR. 25ª Semana Científica do HCPA, Porto Alegre 2006.
- Blaya C, Heldt E, Moorjani P, Salum Júnior G, Leistner-Segal S, Smoller J, Manfro GG. Marcadores genéticos como preditores à Terapia Cognitiva Comportamental para Transtorno do Pânico. XXV Congresso Brasileiro de Psiquiatria, Porto Alegre, 2007.
- Blaya C, Moorjani P, Heldt E, Salum Júnior G, Leistner-Segal S, Perlis R, Smoller J, Manfro GG. Genetic markers as a predictor to Cognitive Behavioral Therapy (CBT) for Panic Disorder (PD). XVth World Congress in Psychiatric Genetics, Nova Iorque, 2007.
- Salum Júnior G, Blaya C, Moorjani P, Seganfredo AC, Heldt E, Kipper L, Smoller J, Manfro GG. Interação Gene-Ambiente (GxA): Moderação de um

polimorfismo no receptor da serotonina 1A (5-HTR1A) na relação com Transtorno do Pânico e estressores precoces de vida. XIX Salão de Iniciação Científica da UFRGS, Porto Alegre, 2007. Prêmio destaque.

- Acosta JR, Blaya C, Salum Júnior G, Manfro GG. Polimorfiosmo Val66Met do Fator Neurotrófico Derivado do Cerébro (BDNF) e Transtorno do Pânico: estudo de caso-controle. XIX Salão de Iniciação Científica da UFRGS, Porto Alegre, 2007.

5. Resumo

A herdabilidade estimada do Transtorno do Pânico (TP) é de 28 a 43%, embora ainda permaneça como questão de pesquisa quais os genes envolvidos na sua etiologia. A busca dos genes candidatos para os estudos de associação geralmente é baseada em moléculas envolvidas no tratamento farmacológico, nos agentes que induzem ataques de pânico ou nos estudos de neuroimagem. Genes relacionados ao sistema serotoninérgico, noradrenérgico, eixo hipotálamo-hipófise-adrenal, entre outros, já foram investigados no TP. Os resultados são ainda pouco conclusivos. Para ampliar o conhecimento nessa área, foram realizados quatro estudos nessa tese.

O polimorfismo no promotor do gene transportador da serotonina (*5-HTTLPR*) foi estudado por alguns autores no TP. Realizamos então o estudo 1, que é composto pela revisão sistemática e meta-análise dos estudos de caso-controle que avaliaram esse gene no TP. Foram revisados artigos publicados entre janeiro de 1996 e abril de 2007 nas bases PubMed, PsychInfo, Lilacs e ISI. Identificou-se 19 artigos potenciais, e foram incluídos na análise 10 estudos. Não houve associação do gene *5-HTTLPR* e o TP (OR=0,91, CI95% 0,80 to 1,03, p=0,14). Três subanálises divididas de acordo com a etnia, qualidade do grupo controle e comorbidade com Agorafobia também não encontraram qualquer associação. Entretanto essa análise não possui poder para identificar associações de menor magnitude, e mais estudos são necessários.

O estudo 2 foi realizado para avaliar a associação entre polimorfismos nos genes *5-HTTLPR* e receptores da serotonina 1A (*HTR1A*) e 2A (*HTR2A*) e o TP, bem como a interação com dois fatores ambientais previamente associados ao transtorno: trauma e parentagem na infância. Foram incluídos 107 pacientes e 125 controles. O gene *HTR1A* foi associado ao TP na análise de grupo corrigido para o experimento (p_2=0,027). Três SNPs desse gene foram associados ao TP. Na análise de haplótipo pode-se identificar um haplótipo associado ao TP (p=0,008), e outro protetor para o TP (p=0,004). Na análise de interação, identificou-se que polimorfismos no gene *HTR2A* (rs6311 e rs6313), influenciado por ótima parentagem paterna, conferia proteção ao TP. Essa interação permaneceu significativa após a correção de Bonferroni. Não houve associação do TP com o *5-HTTLPR* mesmo considerando a interação com trauma na infância. Esse estudo replica achados prévios de associação do TP com o *HTR1A* e também reforça a evidência de interação gene-ambiente do *HTR2A* com parentagem, que possivelmente influencia a capacidade dos sujeitos de usarem as experiências familiares como suporte.

O estudo 3 avaliou o TP com o *EF-hand domain containing 2* (*EFHC2*), um gene previamente associado à percepção do medo. Nesse estudo foram incluídos 127 pacientes e 132 controles. Um dos 8 marcadores do *EFHC2* (rs1562875) foi associado ao TP, e também a dois outros fenótipos intermediários associados à ansiedade: comportamento inibido e temperamento evitação de dano. Se for

replicado, esse achado pode colaborar para a elucidação do mecanismo neurobiológico do medo.

O estudo 4 avaliou polimorfismos em 7 genes previamente envolvidos na ansiedade (*BDNF, CREB1, RGS2, CRHR1, 5-HTTLPR, HTR2A, HTR1A)* com a resposta à terapia cognitivo-comportamental (TCC) no TP. Foram incluídos 74 pacientes com TP resistentes a quatro meses de tratamento prévio com antidepressivos que fizeram TCC em grupo. Na análise de grupo, houve associação do *CREB1* com melhora em 1 ano de seguimento (p= 0,016). Dois dos 5 marcadores do *CREB1* estiveram associados a esse desfecho (rs7594560, p=0,003 e rs2253206, p=0,021), mas não permaneceram associados após a correção por permutação. Na análise de haplótipos, um dos dez haplótipos esteve associado à melhor resposta nesse mesmo período. Apesar do limitado tamanho amostral esse estudo promoveu evidências preliminares da associação do *CREB1* na resposta à TCC.

Por fim, essa tese versa sobre alguns aspectos relacionados ao TP, abrangendo revisão sistemática da literatura, estudos de associação, de interação gene-ambiente e de resposta à TCC. Esses artigos estão de acordo com propostas atuais de estudos em genética, incluindo a pesquisa de vários genes e a análise de múltiplos marcadores que buscam aumentar o conhecimento dos fatores genéticos associados ao TP.

6. Summary

The estimated herdability of PD is between 28 to 43%, however which genes are involved in PD etiology still remains to be answered.. The search for candidate genes are usually based on molecules associated to pharmacological treatment, panic-inducing substance or neuroimage studies. Genes related to serotoninergic, noradrenergic, hypothalamic-pituitary-adrenal axis and others have been previously studied with inconclusive results. In order to improve this knowledge, four studies were performed in this thesis.

The polymorphism in the promoter of serotonin transporter gene (*5-HTTLPR*) has been studied in PD. The study 1 was designed in order to perform a systematic review and meta-analysis of case-controls studies. Papers published between January 1996 and April 2007 were eligible for this study. The electronic databases searched included PubMed, PsychInfo, Lilacs and ISI. Nineteen potential articles were identified, and 10 studies were included in this meta-analysis. No statistically significant association between *5-HTTLPR* and PD was found, OR=0.91 (CI95% 0.80 to 1.03, p=0.14). Three sub-analyses divided according to ethnicity, control group and comorbidity with agoraphobia also did not find any association. However, more studies are needed in order to evaluate a possible minor effect.

The study 2 was designed to evaluate the association between *HTR1A, HTR2A* and *5-HTTLPR* in PD patients and controls. Besides this, we ought to evaluate the interaction between these genes and two environmental factors previously associated with PD: childhood trauma and parental bonding. In this case-control candidate gene study, 107 PD patients and 125 controls were included. HTR1A was experiment-wide associated with PD in set-based test (p_2=0.027). Three SNPs of this gene were nominally significantly associated with PD. Additionally, one haplotype was significantly associated with PD (p=0.008), and one haplotype was significantly protective for PD (p=0.004). In the interaction analysis with optimal father parenting, both interaction terms of SNPs of HTR2A (rs6311 and rs6313) were nominally associated with PD and remained significant after Bonferroni's correction. No association was found with *5-HTTLPR* even considering interaction with childhood trauma. This study replicates previous findings regarding the association between PD and *HTR1A*. We also reinforce evidence of gene-environment interaction in *HTR2A* gene with parenting, maybe influencing the capacity of subjects to use familiar experiences as environmental support.

In the study 3 we evaluated the association between PD and EF-hand domain containing 2 (*EFHC2*), a gene previously related to fear recognition. In this study we included 127 patients and 132 controls. One of 8 *EFHC2* markers (rs1562875) was associated with PD and also with two intermediate phenotypes related to anxiety: behavioral inhibition and harm avoidance. If replicated, this finding may contribute to elucidate the neurobiological path of fear.

In the study 4 we evaluated 7 genes previously related to anxiety (*BDNF, CREB1, RGS2, CRHR1, 5-HTTLPR, HTR2A, HTR1A)* with cognitive-behavioral therapy (CBT) response. We included 74 patients resistant to 4 months of antidepressant treatment that received add-on treatment with group CBT. In the set-based test, *CREB1* achieved gene-wide significant association with improvement at 1-year follow-up (empirical p-value = 0.016). Out of the 5 *CREB1* single markers SNPs, two showed nominally significant associations (rs7594560, p-value 0.003 and rs2253206, p-value 0.021), that did not remain significant after 10,000 permutations. In the haplotype analysis, we identified one haplotype significantly associated with 1-year response (p=0.0002). Although limited by small sample size, these results provide preliminary evidence that variations in *CREB1* may be related to long-term CBT response among pharmacological-resistant patients with PD.

Finally, this thesis shows some aspects related to genetics in PD, including a systematic search, association, gene-environment and CBT response studies. Papers included are in accordance with what has been proposed for new studies in genetic, including the analysis of different genes and markers in order to contribute to the knowledge of genetic factors of PD.

7. Introdução

Transtorno do Pânico

O Transtorno do Pânico (TP) é caracterizado pela presença de ataques súbitos de ansiedade, acompanhado de ansiedade antecipatória e evitação de locais ou situações nas quais já ocorreu um ataque de pânico (1). O TP freqüentemente é complicado pela presença de Agorafobia, que é a evitação fóbica de locais nos quais é difícil de sair ou obter ajuda. Estudos recentes estimam que o TP com ou sem Agorafobia afeta em torno de 4,8% da população geral (2-4). O TP é duas ou três vezes mais comum em mulheres e geralmente inicia no final da adolescência ou início da vida adulta (5).

O TP, em geral, tem um curso crônico. Alguns estudos evidenciaram que cerca de 20 a 30% dos pacientes apresentavam sintomatologia igual ou pior após vários anos de tratamento (1, 6). Outros estudos de seguimento mostraram que o TP pode apresentar um curso crônico em até 88% dos pacientes (1, 7). Andersch *et al*. (2003) seguiram pacientes com TP ao longo de 15 anos e evidenciaram que mais de 20% da amostra seguia sintomática (8).

O diagnóstico do TP, como uma entidade nosológica individual, surgiu em 1980 a partir do DSM III (Manual Diagnóstico e Estatístico de Transtorno Mental). No DSM IV (1), o TP caracteriza-se pela presença de ataques espontâneos e recorrentes que atingem o pico máximo em até 10 minutos e são acompanhados por uma sensação de medo iminente ou urgência para escapar. Após um dos

ataques, deve haver preocupação persistente com a possibilidade de ocorrer um novo episódio, com suas implicações e consequências ou uma alteração significativa no comportamento em função dos ataques por um período de pelo menos um mês. No TP, os ataques de pânico não ocorrem como uma conseqüência de efeitos fisiológicos diretos de alguma substância ou condição médica, assim como não são sintomas de outro transtorno mental (1). O paciente deve ter vivenciado ataques espontâneos ao menos uma vez, embora os ataques possam passar a ser situacionais com o desenvolvimento da doença.

Etiologia

Diversos fatores vêm sendo atribuídos à gênese do Transtorno do Pânico, e tanto fatores genéticos quanto psicológicos e ambientais parecem contribuir para esse transtorno (9).

Fatores genéticos

Os Transtornos de Ansiedade geralmente se agregam em famílias (10). Estudos de famílias com TP demonstraram um padrão familiar, no qual o risco de TP em parentes de primeiro grau dos pacientes com esse transtorno é maior que no grupo controle (11). Estudos com gêmeos também corroboram esses achados, mostrando maior concordância do TP em gêmeos monozigóticos em comparação aos dizigóticos (12).

Estudos realizados em gêmeos sugerem que fatores de risco genéticos comuns provavelmente colaboram para as altas taxas de comorbidade presente nos Transtornos de Ansiedade. Togerson (13) encontrou uma concordância de 2:1 em gêmeos monozigóticos:dizigóticos quando considerou todos os Transtornos de Ansiedade, mas não encontrou essa relação quando considerou um mesmo Transtorno de Ansiedade. Estudos mais recentes em uma ampla amostra de gêmeos descreveram que tanto fatores genéticos quanto ambientais colaboram para o desenvolvimento de Fobias, Depressão Maior, Transtorno de Ansiedade Generalizada e Transtorno do Pânico (14-17).

Fatores Psicológicos

Vários estudos psicológicos sobre o TP seguem a teoria cognitivo-comportamental. Segundo Clark (18), os ataques de pânico derivam de interpretações catastróficas disfuncionais de certas manifestações corporais. A suposição é centralizada no processamento inadequado de informações vindas de um estímulo externo (ruído, luminosidade) ou interno (sensação de taquicardia, sudorese, vertigem). A interpretação é de perigo iminente que dispara ou intensifica as sensações corporais confirmando o “perigo”, gerando então mais interpretações catastróficas e ansiedade em uma espiral crescente e rápida. O modelo de Barlow (19) amplia este conceito, no qual o ataque de pânico inicial é um “alarme falso” quando ocorre um aumento momentâneo de estresse da vida em indivíduos com vulnerabilidades biológicas e/ou psicologicas. Após o primeiro ataque, a pessoa torna-se apreensiva em relação a ataques futuros. Para Barlow

(19), o medo primário no TP é o medo das sensações físicas, particularmente as associadas à ativação autonômica. Esta teoria também salienta a influência dos fatores sócio-culturais para o surgimento da Agorafobia, onde o medo estaria associado aos estímulos ambientais.

Alguns autores dedicaram-se ao estudo dos fatores psicodinâmicos associados ao início do TP ou a uma maior vulnerabilidade psicológica ao transtorno (20). Pacientes com TP freqüentemente se descrevem como crianças medrosas, nervosas e tímidas, e também referem desconforto com os sentimentos agressivos, sentimentos crônicos de baixa auto-estima, frustração e ressentimento que precediam o início do TP.

A qualidade da relação parental é um fator de risco para o TP (21). Por exemplo, os pacientes com TP freqüentemente descrevem seus pais como pessoas brabas, críticas, amedrontadoras e controladoras (22). O estudo da relação parental na psicopatologia evoluiu muito com o surgimento de instrumentos que avaliam esse constructo de forma estruturada, como o *Parental Bonding Inventory* (PBI) (23). O PBI é um instrumento auto-aplicável de 25 questões que avalia separadamente os cuidados do pai e da mãe durante os primeiros 16 anos de vida em duas dimensões: a superproteção e o afeto. Uma análise fatorial recentemente sugeriu uma estrutura de três fatores para o PBI, com a dimensão adicional do fator de autoritarismo (24-26). Alguns estudos demostraram uma boa correlação entre os cuidados reais e a medida parental avaliada pelo instrumento (27-30). Além disso, uma coorte de 20 anos demostrou uma boa estabilidade do instrumento em longo prazo (31). O PBI está disponível

na versão traduzida e adaptada para o português brasileiro (32). Estudos que avaliaram pacientes com TP quanto ao vínculo parental com o PBI demontraram uma associação desse transtorno com pais com altos níveis de superproteção e baixos níveis de afeto (21, 33-36).

Fatores ambientais

Estudos prévios têm associado experiências traumáticas na infância (37-39) ao desenvolvimento do TP na idade adulta. Eventos estressantes na vida adulta também estão relacionados ao desenvolvimento de Transtornos de Ansiedade (14) e ao TP (40). Scocco *et al.* (41) descreveram que cerca de 90% dos pacientes com TP experienciaram transição de papéis e 40% tiveram perdas no ano que antecedeu o início do TP.

No entanto a aferição do trauma, principalmente do trauma infantil, é algumas vezes pouco confiável, possivelmente devido ao medo, culpa e vergonha relacionado ao evento. Alguns autores sugerem que a medida de trauma torna-se mais confiável quando realizada através de uma medida contínua (42) e auto-aplicável (43). Um instrumento usado para avaliar trauma na infância é o *Childhood Trauma Questionnaire* (CTQ), que foi traduzido e adaptado para a versão brasileira (42). O CTQ é um instrumento auto-aplicável composto por 28 questões tipo Likert que avalia cinco subescalas: abuso emocional, físico e sexual, e negligência física e emocional. Os ítens são pontuados de 1 a 5, e o resultado de cada subescala varia de 5 a 25. O CTQ fornece também um escore total de maus-tratos obtido através da soma de todas as subescalas, variando de 25 a 125

pontos, sendo que os escores maiores indicam maiores maus-tratos. Estudos prévios indicam boa estabilidade do CTQ avaliado em crianças vítimas de trauma após 6 meses de tratamento. As subescalas do CTQ apresentam boa consistência interna e validade convergente com avaliação clínica (44, 45).

Delineamentos de estudos em Genética Psiquiátrica

Durante muitos anos, os estudos em genética se restringiam aos estudos de famílias (46) através de análise de ligação de marcadores moleculares utilizando um grande número de indivíduos afetados e não afetados dentro de uma mesma família. No entanto, essa abordagem não contribuiu muito para identificar genes de doenças complexas, como a doença coronariana, asma, diabetes e doenças psiquiátricas (46). Os estudos mudaram então o foco do mapeamento genético para estudos de associação.

Os estudos de associação buscam identificar se um padrão de polimorfismos varia sistematicamente em um determinado fenótipo (47). Existem dois desenhos fundamentais nos estudos de associação: os realizados em famílias (trios) e os realizados em indivíduos não-relacionados (caso-controle). Em termos de poder estatístico, os estudos de trios e caso-controle pouco se diferem (46). Já em termos de coleta de dados, os estudos de trios geralmente exigem mais esforços em comparação aos de caso-controle, exceto em crianças, pois o acesso aos pais é mais fácil (46). Por outro lado, o delineamento de trios é mais robusto que o de caso-controle na medida que permite determinar se o alelo de

risco é de origem materna ou paterna (48); além disso, o estudo de trios possibilita a construção de um modelo e teste de múltiplas hipóteses (46) e reduz as chances de associação por estratificação étnica (48, 49).

Os estudos de trios baseiam-se no teste de desequilíbrio de transmissão, que compara o número de alelos transmitidos para os sujeitos afetados com o que seria esperado pela transmissão Mendeliana. Um excesso de determinado alelo na prole afetada indica que aquele fenótipo está em desequilíbrio de ligação com o marcador (46). Já os estudos de caso-controle comparam se um determinado polimorfismo é mais freqüente em um determinado grupo (47).

Classificação dos estudos de associação

Existem mais de 30.000 genes no genoma humano, e a probabilidade *a priori* de que algum gene selecionado ao acaso possa influenciar um determinado fenótipo é muito baixa (50). Dessa forma, ou a seleção de SNPs (*Single Nucleotides Polimorphisms*) deve englobar um número muito grande de marcadores, ou os SNPs devem ser selecionados baseado em algum referencial teórico. Os estudos de associação são então classificados de acordo com método de busca dos SNPs estudados da seguinte forma:

a) Gene candidato, no qual cerca de 5-50 polimorfismos, ao redor de um determinado gene, são avaliados. O gene pode ser determinado pela sua posição, por ter estudos de ligação prévios, ou mesmo por ser um candidato funcional, ou seja, ter a sua função já demonstrada em outras espécies;

b) Mapa fino, que são estudos conduzidos numa região candidata de cerca de 1-10Mb e pode envolver centenas de genes;

c) *Genome-wide Association* (GWA), que busca identificar variações ao longo do genoma, com cerca de 300.000 SNPs.

As vantagens dos estudos de gene candidato é que o custo é infinitamente menor; no entanto, a escolha do gene deve ser feita de forma bastante criteriosa. Já os estudos que envolvem múltiplos SNPs, como o GWA, apresentam um risco elevado de encontrar associações espúrias devido às múltiplas associações, portanto o valor p deve ser adequadamente corrigido (47).

Seleção de SNPs

Nos estudos de gene candidato, o processo de seleção dos SNPs no gene deve ser feito de forma que os SNPs que retém maior variação genética sejam escolhidos, e a esse processo se denomina *Single Nucleotide Polymorphism (SNP) Tagging*. Dessa forma, dentre dois SNPs disponíveis que estão em forte desequilíbrio de ligação (DL), será escolhido apenas um, preferencialmente aquele com maior freqüência alélica (47, 51). Existem programas que desempenham essa função, como por exemplo, o *Tagger program* (51) (http://www.broad.mit.edu/mpg/tagger/).

Peculiaridades da análise estatística

Um dos problemas da análise estatística de estudos na área da genética é que o grande número de polimorfismos possibilita encontrar associações

estatisticamente significativas ao acaso. Para evitar isso, algumas abordagens são feitas no tratamento estatístico.

A primeira etapa é avaliar a qualidade do material genotipado. Indivíduos que não foram genotipados na maioria dos polimorfismos devem-se, provavelmente, ao comprometimento da qualidade do ácido desoxirribonucléico (DNA), devendo então ser excluídos. Da mesma forma, SNPs que não forem genotipados na maioria dos indivíduos provavelmente também não são de boa qualidade (50). Testar para o equilíbrio de Hardy-Weinberg (HWE) também pode ser útil, uma vez que desvio de HWE freqüentemente se deve a consangüinidade, estratificação populacional, seleção (47) ou erro no experimento (52). Portanto, alguns autores preconizam que HWE deva também ser usado como controle de qualidade, e descartar aqueles SNPs que desviarem em controles, por exemplo, com um nível de significância alfa de 10^{-3} ou 10^{-4}.

O objetivo final dos estudos de associação é encontrar associação de determinado gene com o fenótipo avaliado. No entanto, analisar os SNPs individualmente pode negligenciar a informação conjunta (47). Uma das formas de abordar a associação conjunta do gene é analisar através do teste baseado no grupo (*set-based test*). Dessa forma, além do teste de associação individual de cada SNP com o fenótipo, obtém-se também um teste de associação para o efeito aditivo dos SNPs. Outra estratégia para avaliar um conjunto de SNPs é a análise de haplótipos, que busca a correlação de SNPs em regiões de pouca recombinação (47). Os achados da análise de haplótipo são mais consistentes quando são incluídos SNPs com forte DL entre eles. No entanto essa abordagem

oferece pouca ou nenhuma vantagem sobre a análise de grupo, particularmente quando se utiliza a estratégia de busca de SNPs através do método *Tagging*, pois esse método não inclui SNPs que estão em forte DL (47).

Esses recursos não evitam, todavia, o grande problema dos estudos genéticos: múltiplos testes. O genoma é muito grande, então qualquer SNP (ou grupo de SNP) tem uma baixa probabilidade de estar realmente associado ao fenótipo estudado. Uma estratégia para evitar falso-positivos é adotar a correção de Bonferroni, mas para SNPs com forte DL essa correção é bastante conservadora. Outra estratégia é adotar o procedimento de permutação. Dessa forma os dados do genótipo são mantidos preservando a estrutura de DL, mas os fenótipos são randomizados n vezes entre os indivíduos para gerar dados que satisfaçam a hipótese nula de ausência de associação com o fenótipo (47). Nenhuma associação biológica deve ser encontrada nos dados permutados, pois o fenótipo foi modificado. Compara-se então o melhor resultado permutado, que representa um achado ao acaso, aos dados reais obtidos (48). Como a permutação preserva o esquema de correlação entre os SNPs, torna-se um método de correção menos restrito que a correção de Bonferroni, que assume que todos os testes são independentes (53).

Meta-análise

Existem três problemas freqüentes no delineamento de estudos com grande poder em genética. O primeiro deles é a freqüência do fenótipo estudado, que torna compreensível a razão pela qual existem estudos de maior poder na

Depressão Maior que no Transtorno do Pânico. A segunda grande limitação é a freqüência do polimorfismo estudado. Por exemplo, para identificar uma associação com uma razão de chances (RC) de 1,2 de um SNP cuja freqüência nos controles é de 5%, seriam necessários mais de 12 mil sujeitos (50). E a terceira limitação são os custos da genotipagem de genes em tantos indivíduos (48). Dessa forma, os estudos são geralmente pequenos, incluindo de 100 a 300 sujeitos (54, 55). Os estudos pequenos possuem um papel fundamental ao gerar hipóteses, mas devem ser replicados em uma segunda amostra, preferencialmente maior (50).

De acordo com Hirschhorn *et al*. (56), de um total de 166 estudos de associação de variações genéticas com doenças, apenas 6 replicaram achados prévios. Sabe-se que a RC de associação de um polimorfismo com uma doença é geralmente baixa. Dentre os transtornos psiquiátricos, apenas a Doença de Alzheimer foi associada a um gene com RC maior que 2 (50), enquanto que as demais associações geralmente se situam numa RC entre 1,1 à 1,5 (57). Assim, a estratégia estatística final é compilar os resultados de vários pequenos estudos para averiguar o real impacto de determinada variação genética em um fenótipo, através do recurso da meta-análise.

Estratificação Étnica

Outro problema freqüente nos estudos de associação de caso-controle são os potenciais confundidores, como por exemplo, estratificação étnica. Como a freqüência de muitos SNPs difere de acordo com a origem étnica, é possível que

uma determinada associação se deva a uma diferença de representação étnica dos grupos. Uma estratégia pode ser restringir as análises a um determinado grupo étnico, tornando a população mais homogênea possível (58). A desvantagem desse método é que ele pode ser influenciado pela a definição cultural de uma determinada ancestralidade (59). Por exemplo, no Brasil a ancestralidade africana é freqüentemente omitida e alguns autores preconizam que a descrição fenotípica não prediz a ancestralidade do brasileiro (60). No entanto, no Rio Grande do Sul o percentual de mistura africana em indivíduos classificados fenotipicamente como caucasianos é de cerca de 6% (61), sugerindo que a avaliação fenotípica é um bom parâmetro de ancestralidade para essa região.

Outro recurso é utilizar alguns marcadores para avaliar o impacto da ancestralidade da população na amostra. Uma forma descrita para controlar essa variável é o controle genômico (62), no qual se utiliza de variação de marcadores genéticos da amostra para inferir a subestrutura da população e corrigir a amostra para a estratificação. Também pode ser usado o método de associação estruturada, que parte do princípio de que uma população heterogênea é composta por subpopulações homogêneas, então se utiliza de múltiplos lócus para inferir a estrutura da população de forma detalhada (63). Os testes de associação levam então em conta a estrutura inferida da população (59)

A busca de genes candidatos

O TP é considerado um transtorno psiquiátrico de herança complexa, pois não foi identificado nenhum padrão de herança Mendeliana (64). Provavelmente vários genes contribuam com um pequeno efeito para a suscetibilidade ao TP. Desta forma, a busca de genes candidatos tem sido feita baseada no conhecimento de moléculas envolvidas no tratamento farmacológico, nos agentes que induzem ataques de pânico (65) ou nos estudos de neuroimagens. Por exemplo, a resposta clínica que os pacientes com TP apresentam quando tratados com Inibidores Seletivos de Recaptação da Serotonina (ISRS) e a piora quando usam agentes serotoninérgicos colabora para a hipótese de disfunção serotoninérgica no TP (12, 66). Outros autores buscam genes candidatos baseado em modelos animais e alterações neuroendócrinas encontradas nesses pacientes.

Sistema serotoninérgico

A disfunção do sistema serotoninérgico tem sido implicada em diversas condições, como nos Transtornos de Ansiedade, enxaqueca, epilepsia e Transtornos de Humor (12). No TP, tem sido descrito que tanto o excesso, quanto a falta desse neurotransmissor em algumas regiões cerebrais estão envolvidos na sua etiopatogenia (66). Existem duas hipóteses opostas que tentam explicar a disfunção serotoninérgica no TP: excesso ou superatividade de serotonina (67, 68), e déficit ou subatividade da serotonina (69), e ambas as interpretações encontram respaldo ao serem confrontadas com a resposta farmacológica. A

teoria do excesso de serotonina sugere que os pacientes com TP apresentam liberação exagerada de serotonina na fenda sináptica ou hipersensibilidade dos receptores pós-sinápticos. A piora clínica que os pacientes com TP apresentam quando iniciam tratamento com ISRS corrobora a teoria de excesso, indicando que a hipersensibilidade dos receptores pós-sinápticos piora as crises. Por outro lado, a teoria do déficit propõe que em determinadas regiões cerebrais, como na substância cinzenta periarquedutal, a serotonina previne o pânico, e o déficit desse neurotransmissor propicia o ataque de pânico. A administração do precursor da serotonina (5-hidroxi-triptofano) (70) diminui os ataques de pânico e após um tempo os ISRS são efetivos em diminuir as crises de ansiedade. Não se espera que toda a complexidade do TP seja explicada de forma simplista por uma disfunção em um sistema monoaminérgico. Entretanto, os modelos biológicos de interpretação partem de bases simples com o intuito de explicar uma parte do transtorno que pode estar relacionada à neurotransmissão específica de uma determinada monoamina.

Existem vários genes envolvidos no sistema serotoninérgico, e um candidato freqüentemente estudados nos transtornos ansiosos e de humor (71) é o gene transportador da serotonina (*5-HTT*). O *5-HTT* está localizado no cromossomo 17q11.1-q12 (72) e codifica uma proteína de membrana integral que tem um papel na recaptação deste neurotransmissor na fenda sináptica. Existem dois polimorfismos neste gene que têm sido pesquisados: um VNTR que corresponde a um número variável de repetições em *tandem* no intron 2 (72) e o polimorfismo de tamanho de repetição (RFLP – *restriction fragment length polymorphism*) que

corresponde a uma inserção/ deleção de 44 pares de bases na região promotora deste gene (*5 - HTTLPR*), que origina 2 alelos (l- *long* e s-*short*). O alelo l*,* constituído de 528 pares de base, vem sendo relacionado a uma transcrição do gene transportador da serotonina duas a três vezes mais eficiente quando comparado ao alelo s de 484 pares de base (73, 74). Isso significa que o alelo s seria menos ativo, resultando numa captação em níveis menores da serotonina na fenda sináptica.

Lesch *et al.* (73) relataram que o polimorfismo *5-HTTLPR* é responsável por 3-4% da variabilidade de neuroticismo. Indivíduos com genótipos s/s ou s/l teriam mais características associadas ao neuroticismo do que pessoas com o genótipo l/l. Osher *et al.* (75) conseguiram reproduzir os achados de Lesch *et al.*, encontrando relação entre o alelo curto do *5-HTTLPR* e medidas de personalidade relacionadas à ansiedade. Entretanto, estudos subseqüentes não replicaram esses achados (76). A associação do *5-HTTLPR* com neuroticismo torna esse gene um bom candidato a ser avaliado no TP. No entanto, alguns estudos não encontraram qualquer relação entre esse gene e o TP (77, 78), enquanto outro grupo encontrou associação com o alelo l (79). Já os demais polimorfismos existentes nesse gene não foram associados ao TP (tabela 1).

Alguns estudos pré-clínicos e clínicos apontam para a participação do receptor de serotonina 1A (*HTR1A*) como envolvido no TP. Ratos modificados para esse gene apresentam diversos comportamentos relacionados à ansiedade em testes de conflito (80). O receptor *HTR1A* é expresso em duas populações de neurônios: como auto-receptor pré-sináptico nos núcleos da Rafe e como

heteroreceptor pós-sináptico no córtex frontal, predominantemente no hipocampo, septum e córtex (81). A ativação do receptor pré-sináptico do receptor *HTR1A* por um agonista seletivo resulta numa supressão da síntese, *turn-over* e liberação de serotonina nas áreas projetivas (82), resultando num efeito ansiolítico. Por outro lado, a ativação dos receptores pós-sinápticos resulta num efeito ansiogênico (83). Nos pacientes com TP existe uma redução da responsividade do receptor *HTR1A* (84), sugerindo que uma variação alélica no gene desse receptor possa predispor o desenvolvimento desse transtorno (9). Recentemente foi descrito que um polimorfismo (C1019G) no promotor do *HTR1A* possa contribuir para a patogênese de Agorafobia no TP (9). O alelo G libera a repressão da expressão dos auto-receptores *HTR1A* ao alterar o sítio de ligação de um fator inibitório da transcrição, resultando então na redução da neurotransmissão serotonérgica (85). Um estudo de neuroimagem (86) descreveu uma associação entre o alelo G e a diminuição da ativação do córtex cerebral quando expostos a estímulo ansiogênico em pacientes com TP. O alelo G também foi associado à ansiedade e temperamento de evitação de dano em voluntários saudáveis (87) e ao TP com Agorafobia (88). No entanto esse achado não foi replicado num estudo de duas amostras independentes que avaliou o neuroticismo, um fenótipo intermediário da ansiedade (89).

Drogas com ação no receptor de serotonina 2A (*HTR2A*) apresentam ação ansiolítica (90). O gene do *HTR2A* consiste de dois introns e três éxons e está localizado no cromossomo 13q14-q21. Alguns polimorfismos foram descritos nesse gene (91), sendo que o polimorfismo T102C tem sido bastante estudado. O

alelo C expressa uma proteína do HTR2A menos ativo que alelo T, indicando um papel funcional para esse polimorfismo (92). Alguns estudos demonstraram associação entre variações no gene do *HTR2A* com comportamento suicida no transtorno depressivo (93, 94). No TP foi descrita associação de um polimorfismo (T102C) em duas amostras independentes (12, 95). No entanto esse achado não foi replicado em duas outras amostras (88, 96). Unschuld *et al.* (97) recentemente descreveram associação do *HTR2A* com temperamento dependente de gratificação em pacientes com TP.

A serotonina é metabolizada pele enzima monoamino oxidadese A (*MAOA*), que é codificada pelo gene localizado no cromossomo X (Xp11.23-11.4) (98). Foi descrito um polimorfismo na região promotora do gene que influencia a atividade da enzima: os alelos longos possuem maior atividade enzimática em comparação com os alelos curtos (65). Três estudos (65, 79, 99) encontraram associação do alelo longo com TP em mulheres, enquanto que um estudo não replicou esse achado (100).

A triptofano hidroxilase 1 (*TPH1*) é uma enzima que modula a síntese de serotonina. Três polimorfismos não funcionais foram avaliados no TP, mas nenhum dos quatro estudos (96, 101-104) encontrou associação com o TP. Recentemente descobriu-se que a isoforma triptofano hidroxilase 2 (*TPH2*) é preferencialmente expressa no tecido neuronal e é então responsável pela modulação da síntese da serotonina no cérebro. Dois estudos avaliaram o *TPH2* no TP e não encontraram qualquer associação (105, 106).

Os polimorfismos estudados nos demais receptores da serotonina não mostraram resultados consistentes e replicáveis. A tabela 1 descreve os principais achados encontrados nos genes da serotonina e o TP.

Tabela 1. Estudos que avaliaram genes da serotonina no Transtorno do Pânico

Gene Candidato	Polimorfismo	Referência	Amostra	Resultado principal
5-HTT	5-HTTLPR	Hamilton *et al*. 1999(107)	45 famílias, 74 trios	**Sem associação**
		Deckert *et al*. 1997 (77)	158 pacientes 169 controles	**Sem associação**
		Ishiguro *et al*. 1997 (78)	66 pacientes 150 controles	**Sem associação**
		Maron *et al*. 2005 (79)	158 pacientes 215 controles	**Associação com alelo l**
		Matsushita *et al*. 1997 (108)	86 pacientes 213 controles	**Sem associação**
		Kim *et al*. (104)	244 pacientes 227 controles	**Sem associação**
	VNTR	Maron *et al*. 2004 (102)	158 pacientes 215 controles	**Sem associação**
	18784A-C 10647G-A 167G-C	Maron *et al*. 2004b (103)	127 pacientes 146 controles	**Sem associação**
MAO-A	VNTR	Deckert *et al*. 1999 (65)	209 pacientes 190 controles	**Associação com o alelo longo em mulheres com TP**
		Hamilton *et al*. 2000 (100)	70 famílias, 81 trios	**Sem associação**
		Maron *et al*. 2005 (79)	158 pacientes 215 controles	**Associação alelo longo em mulheres com TP e Ago**
		Samochowiec *et al*. 2004 (99)	101 pacientes 202 controles	**Associação alelo longo em mulheres com Ago**
	Fnu4HI	Tadic *et al*. 2003(109)	38 pacientes 276 controles	**Sem associação**
TPH 1	1095T-C	Han *et al*. 1999(101)	45 pacientes 142 controles	**Sem associação**

	218A-C	Fehr *et al*. 2001(96)	35 pacientes 87 controles	**Sem associação**
		Maron *et al*. 2004 (102)	158 pacientes 215 controles	**Sem associação**
		Kim *et al*. 2006 (104)	244 pacientes 227 controles	**Sem associação**
	779A-C	Maron *et al*. 2004b(103)	127 pacientes 146 controles	**Sem associação**
TPH2	19776C-T 79727C-T	Maron *et al.*(2007)(105)	213 pacientes 303 controles	**Sem associação**
	844G-T 4002C-T	Mössner *et at.*(2006)(106)	134 pacientes 134 controles	**Sem associação**
HTR1A	-1019C-G	Rothe *et al*. 2004a (88)	133 pacientes 134 controles	**Associação do genótipo GG e alelo G em mulheres com Ago**
		Maron *et al*. 2004b (103)	127 pacientes 146 controles	**Associação do alelo C em TP com TH**
		Huang *et al*. 2004(110)	87 pacientes 107 controles	**Associação do genótipo GG e alelo G no ataque de pânico**
	294G-A	Inada *et al*. 2003(12)	63 pacientes 100 controles	**Sem associação**
	-480A-del	Maron *et al*. 2004b(103)	127 pacientes 146 controles	**Sem associação**
HTR1B	861G-C	Fehr *et al*. 2000a (111)	32 pacientes 74 controles	**Sem associação**
		Maron *et al*. 2004(102)	158 pacientes 215 controles	**Sem associação**
	-1089T-C, -700C-A, -511G-T, -161A-T, 129 C-T, 276G-A, 371T-G, 705C-T, 1180G-A	Maron *et al*. 2004b(103)	127 pacientes 146 controles	**Sem associação**
HTR2A	102T-C	Fehr *et al*. 2001(96)	35 pacientes 87 controles	**Sem associação**
		Inada *et al*. 2003 (12)	63 pacientes 100 controles	**Associação do alelo C no TP com Ago**

		Rothe *et al*. 2004b (9)	94/86 pacientes 94/86 controles	**Sem associação**
		Maron *et al*. 2004b(103)	127 pacientes 146 controles	**Associação do alelo C no TP puro**
	-143A-G, 73C-A, 1354 C-T	Maron *et al*. 2004b (103)	127 pacientes 146 controles	**Sem associação**
HTR2C	Cys23Ser	Fehr *et al*. 2000b(112)	35 pacientes 89 controles	**Sem associação**
		Inada *et al*. 2003 (12)	63 pacientes 100 controles	**Sem associação**
		Maron *et al*. 2004b(103)	127 pacientes 146 controles	**Associação com TP com ou sem comorbidades**
	(12-18)G-T (4-5)T-G	Deckert *et al*. 2000 (113)	87/124 pacientes 131/95 controles	**Sem associação**
	2831T-G	Maron *et al*. 2004b(103)	127 pacientes 146 controles	**Sem associação**
HTR3A	1302T-C 1596G-A	Maron *et al*. 2004b(103)	127 pacientes 146 controles	**Sem associação**

Abreviações. TP, Transtorno do Pânico; Ago, Agorafobia; TH, Transtorbo de Humor. *5-HTT*, gene transportador da serotonina. *5-HTTLPR*, polimorfismo no promotor do gene transportador da serotonina; *MAO-A*, enzima monoamino-oxidase A; *TPH 1*, triptofano hidroxilase 1; *HTR1A*, receptor da serotonina 1A; *HTR1B*, receptor da serotonina 1B; *HTR2A*, receptor da serotonina 2A; *HTR2C*, receptor da serotonina 2C; *HTR3A*, receptor da serotonina 3A; *VNTR*, número variável de repetições em tandem.
Nota. Adaptado e atualizado de Maron et al. (114).

Sistema Noradrenérgico

Algumas evidências apontam para o papel da norepinefrina na patogênese do TP: elevação do seu principal metabólito em urina de pacientes (115), uso de agentes com ação noradrenérgica no tratamento farmacológico desse transtorno (66, 116, 117), e indução de ataques de pânico com o aumento da liberação da noradrenalina (118). Além disso, o *locus coeruleus*, que contém a maior

concentração de células noradrenérgicas no cérebro, é conhecido por estar envolvido no medo e na ansiedade (119, 120).

Alguns autores mostraram dados consistentes relacionando o polimorfismo Val158Met do gene *Catechol O-Methyltransferase* (*COMT*) com o TP (95, 121, 122). *COMT* é uma enzima que inativa as catecolaminas, incluindo a noradrenalina, adrenalina e dopamina. O gene que codifica a *COMT* está localizado no cromossomo 22q11.2. Uma substituição de um único nucleotídeo (guanina por adenosina), na primeira posição do códon 158 desse gene leva a uma mudança funcional do aminoácido valina por metionina (rs4680). Essa transição de valina por metionina está associada a um aumento de três a quatro vezes na termolabilidade. O alelo com valina (Val) apresenta uma maior atividade da enzima *COMT* que o alelo com metionina (Met) (121). O alelo Met foi associado ao Transtorno Obsessivo-Compulsivo (123), apesar de não ter sido replicado em outro estudo (124).

Alguns estudos demonstram uma relação entre o alelo de alta atividade (Val) com o TP em caucasianos (121, 122, 125), no entanto em coreanos essa relação foi inversa (126). Recentemente uma meta-análise avaliou seis estudos de associação e não encontrou qualquer associação entre o polimorfismo Val158Met com o TP (127). No entanto, foi identificada uma grande heterogeneidade nos estudos incluídos na meta-análise. Nas análises estratificadas, identificou-se uma associação do alelo valina em amostras caucasianas, enquanto que foi descrita uma associação com o alelo metionina nas amostras asiáticas.

Hormônio liberador de Corticotrofina (CRH)

O CRH é um peptídeo que regula a função da hipófise e adrenal no eixo hipotálamo-hipófise-adrenal (HPA) e é também um hormônio extra-hipotalâmico relacionado a vários comportamentos, incluindo expressões de medo (128). Os corpos celulares dos neurônios que contém CRH são largamente distribuídos no cérebro e, além dos neurônios encontrados no núcleo para-ventricular do hipotálamo, encontram-se no núcleo da estria terminal e núcleo central da amígdala. Com base em uma série de dados proveniente de estudos com animais, sabe-se que a administração central de CRH produz uma série de condutas análogas àquelas vistas em Transtornos de Ansiedade e transtornos afetivos, incluindo: anorexia, insônia, diminuição da libido, aumento da atividade locomotora, aumento da freqüência cardíaca e pressão arterial, distúrbios gastrointestinais, aumento da conduta de autolimpeza, redução da atividade exploratória e aumento da resposta de sobressalto (129). Camundongos transgênicos com alterações no gene do CRH mostraram excesso de produção do hormônio de liberação da corticotrofina. Esses camundongos exibiam também anormalidades endócrinas, incluindo elevação do hormônio adrenocorticotrófico e corticosterona, reforço da responsividade ao novo e uma resposta semelhante à que é vista no labirinto em cruz elevado. Essas manipulações genéticas reforçam a hipótese de que o CRH desempenha um importante papel nas respostas comportamentais a estressores e na psicopatologia humana (128). Há evidências de que o sistema de CRH do Sistema Nervoso Central (SNC) comumente medeia

a associação entre estresse precoce na vida e o desenvolvimento de Transtornos de Humor e Ansiedade na vida adulta (130). Um estudo recente (131) demonstrou que variações no gene do CRH estão associadas ao comportamento inibido em crianças, um temperamento que está relacionado ao desenvolvimento de Transtorno de Ansiedade na vida adulta.

O receptor 1 do CRH (*CRHR1*) está envolvido na ação ansiogênica do CRH (132, 133). Modelos animais mostraram que ratos deficientes de *CRHR1* apresentam menos comportamento ansioso em comparação com ratos selvagens (134). Alguns estudos demonstraram que antidepressivos de diferentes classes suprimem a expressão do gene CRH em roedores (135), e que a atividade do eixo HPA está deprimida em humanos saudáveis (136, 137). Além disso, foi descrito que os ISRS exercem parte do seu efeito através da redução da atividade dos neurônios do CRH (138). Liu *et al.* (139) recentemente encontraram um efeito moderado de polimorfismos do *CRHR1* e resposta em curto prazo à fluoxetina para Depressão Maior.

Neurotrofinas e mensageiros intermediários

O Fator neurotrófico neuronal (*BDNF*) é a mais abundante neurotrofina cerebral (140) e é uma molécula candidata a estar envolvida na fisiopatologia dos Transtornos de Humor (141). O *BDNF* participa da plasticidade cerebral envolvendo mecanismos como aprendizado e memória (142). O nível sérico do *BDNF* foi relacionado à resposta à Terapia Cognitiva Comportamental de pacientes com TP (143).

Um polimorfismo funcional Val66Met do gene *BDNF* foi relacionada à atividade da secreção dessa neurotrofina (144). O modelo animal recentemente mostrou que ratos Met/Met apresentam mais comportamento de ansiedade, acompanhado de uma redução de secreção de *BDNF* atividade-dependente, mas não no nível total (145). O comportamento ansioso dos ratos não foi modificado pelo uso da fluoxetina, sugerindo a importância dessa neurotrofina como mediador do efeito ansiolítico dos antidepressivos. Considerando o papel do *BDNF* na sobrevivência, diferenciação e plasticidade neuronal, o polimorfismo Val66Met passou a ser um grande candidato a ser avaliado nos transtornos psiquiátricos (142). Entretanto a associação desse polimorfismo com ansiedade é bastante errática. Por um lado, Lang *et al*. (146) encontraram associação entre o genótipo Val/Val e traços de ansiedade, enquanto o alelo Met estava associado a níveis mais elevados de temperamento ansioso em outro estudo (147). No TP esse polimorfismo somente foi avaliado em amostras orientais, e não houve associação entre esse SNP e TP em japoneses (148) e chineses (149).

Modelos animais demonstraram que a ansiedade está relacionada ao *AMPc responsive-element binding protein* (*CREB*) (150). O *CREB* é expresso em todas as células cerebrais e é uma proteína que age como fator de transcrição (151). Wallace *et al.* (152) demonstraram em um modelo animal que a expressão do *CREB* na amígdala basolateral influencia os comportamentos de depressão, medo e ansiedade. O aumento da expressão de *CREB* na amígdala de ratos tem um efeito semelhante ao que é visto no treinamento massivo em campo aberto no desenvolvimento do medo condicionado (153). Em humanos, o *CREB* está

associado ao Transtorno de Humor (154), suicídio (155, 156), agressividade (157) e resposta ao antidepressivo (158). O *CREB* também está envolvido no circuito de recompensa e regula então a sensibilidade individual ao estímulo emocional, contribuindo então para neuroadaptação ao estímulo (151). Alguns estudos associaram também o *CREB* à memória em diversos organismos (159). O propranolol, que é um tratamento experimental para o Transtorno do Estresse Pós-Traumático, modifica a consolidação da memória ao atuar como antagonista beta-adrenérgico, que inibe a fosforilação do AMPc (160). O TP está intimamente ligado à interpretação catastrófica dos sintomas corporais e à memória dos ataques de pânico, sendo essa a base da terapia cognitivo-comportamental (161).

O *RGS2* (*regulator of G-protein signaling 2*) é uma proteína que reduz a atividade da proteína G (162, 163). Os neurotransmissores envolvidos na biologia da ansiedade, como a serotonina e a noradrenalina, agem através da proteína G acoplada ao receptor de signalina (164). Ratos *RGS2 knockout* mostraram-se mais ansiosos que os ratos selvagens (165), e um estudo recente (166) mostrou associação entre 4 SNPs do *RGS2* com TP.

Fenótipos intermediários

Os estudos de genética em Transtornos de Ansiedade são bastante erráticos (167), provavelmente devido à grande dificuldade em definir qual a real parte que é herdável desse fenótipo. Alguns autores descreveram que o aspecto genético dos Transtornos de Ansiedade se torna mais aparente quando a definição do

estado “afetado” é expandido, e inclui variações sub-sindrômicas (13, 168). Sabe-se que os Transtornos de Ansiedade são influenciados por fatores genéticos quando considerados como um conjunto; no entanto, os estudos de gêmeos demonstraram pouca concordância para um mesmo Transtorno de Ansiedade (13). Além disso, existe uma grande sobreposição de Ansiedade e Depressão. Estudos em família demonstraram uma maior suscetibilidade para o desenvolvimento de Depressão em familiares de pacientes com TP (169). Parece que a nosologia genética não segue os parâmetros diagnósticos propostos pelo DSM-IV (1).

Dentro desse contexto surge o conceito de fenótipos intermediários. Propõem-se então que os fatores genéticos, modulados por experiências ambientais, influenciam a expressão de “fenótipos mínimos” e o agrupamento desses determina os “fenótipos intermediários”, como o comportamento inibido, um temperamento que pode estar associado aos Transtornos de Ansiedade ou Depressivos. Os fenótipos intermediários, por sua vez, caracterizam as síndromes clínicas, como por exemplo, a síndrome ansiosa ou a síndrome depressiva, configurando o temperamento ou o funcionamento pré-mórbido. As síndromes passam então a ser consideradas transtornos quando levam a um prejuízo funcional do indivíduo. O prejuízo funcional pode ocorrer devido ao tempo de sofrimento, à desadaptação funcional, ao prejuízo na qualidade de vida ou mesmo ao aumento dos custos sociais ou da morbi-mortalidade (Figura 1). Dentro desse paradigma é possível compreender a grande sobreposição da comorbidade

Ansiedade e Depressão, uma vez que essas compartilham de endofenótipos comuns.

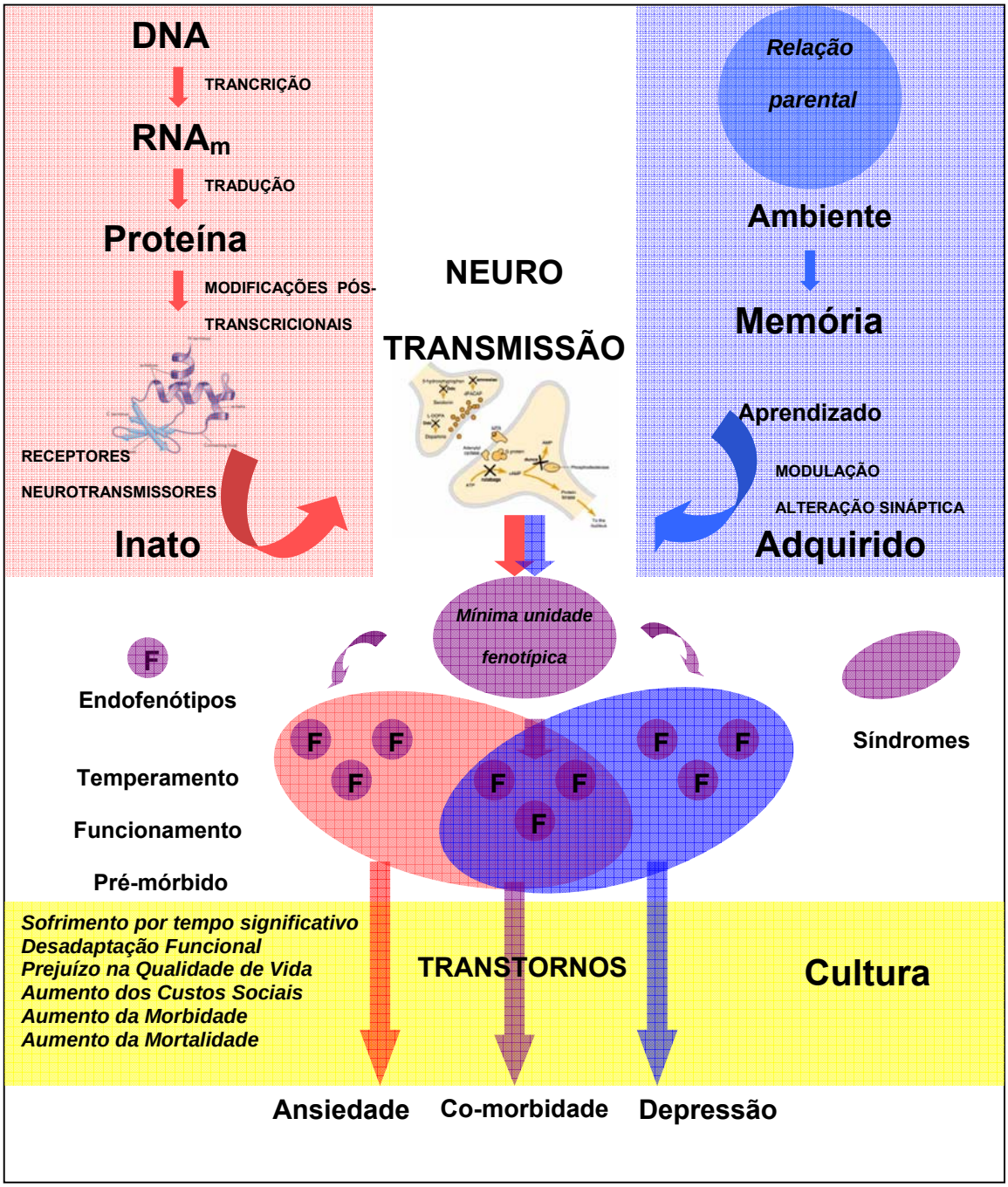

Figura 1. Modelo dimensional da nosologia genética. Autor Salum Ga, 2008.

Alguns autores sugerem que existe um pequeno número de dimensões nas quais os indivíduos diferem, e a psicopatologia representa então extremos de dimensões que compõe a personalidade normal (167). Muitas dessas dimensões ou temperamentos que contribuem para diferenças individuais na personalidade estão diretamente relacionadas com a ansiedade (167).

O temperamento dos indivíduos é um fenótipo intermediário bastante relacionado a variações herdáveis. Cloninger (170) definiu três traços de personalidade que ocorrem em resposta a estímulos específicos: os “procuradores por novidade”, que é um padrão de atividade exploratória freqüente de intensa excitação frente um novo estímulo; os “evitadores de dano”, que é uma tendência herdada a responder de forma extremamente intensa a um estímulo aversivo, aprendendo a evitar novidades; e os “dependentes de gratificação” que é uma tendência a responder de forma intensa a reforços positivos. A integração dessas dimensões neurobiológicas forma padrões de respostas diferentes a punição, recompensa e novidades, e favorece a capacidade discriminatória de situações seguras e perigosas. Existem evidências que a variação em cada dimensão está fortemente associada com alterações específicas nas vias monoaminérgicas; por exemplo, os “evitadores de dano” estão relacionados com alta atividade serotoninérgica. Nos indivíduos com alta evitação de dano, a ansiedade crônica caracteriza-se por preocupações antecipatórias freqüentes (170), um padrão clinicamente semelhante ao encontrado em pacientes com Transtorno do Pânico. Os pacientes com TP são descritos como possuírem predominantemente um temperamento de evitadores de dano (171, 172).

O comportamento inibido (CI) também é um fenótipo intermediário de ansiedade que vem sendo estudado em estudos genéticos (131). CI na infância é caracterizado pela presença de irritabilidade em bebês, timidez e medos excessivos na criança pré-escolar e, na criança em idade escolar, introversão, dificuldade e constrangimento ao enfrentar novas situações (173). O comportamento inibido apresenta tanto componentes comportamentais, tais como o retraimento social e timidez, quanto componentes fisiológicos, como aumento dos níveis de cortisol salivar, dos níveis de catecolaminas urinárias, da freqüência cardíaca e dilatação pupilar (173). Comportamento inibido é mais freqüente em crianças cujos pais têm Agorafobia e Transtorno do Pânico (174), e Transtornos de Ansiedade são mais freqüentes nas famílias de crianças com comportamento inibido (175). Crianças identificadas como tendo comportamento inibido têm taxas mais altas de Transtornos de Ansiedade na infância (176).

A percepção das emoções é outro fenótipo avaliado no TP em estudos de neuroimagem (86). Kessler *et al.* (177) descreveram que o reconhecimento das emoções tende a ser pior nos pacientes com TP, que interpretam outras emoções como raiva. Esse estudo levanta a hipótese de que os pacientes com TP tendem a interpretar os estímulos sociais como sendo perigosos. A percepção do medo tem sido estudada em diferentes transtornos (178), no qual a amígdala parece desempenhar um papel crucial (179). Weiss *et al.* (180) realizaram um mapeamento do reconhecimento do medo em duas amostras independentes de pacientes com Síndrome de Turner e encontraram associação com o *EF-hand domain containing 2* (*EFHC2*), um gene localizado no cromossomo X.

Interação gene-ambiente

As associações entre marcadores genéticos com transtornos psiquiátricos vêm sendo um tanto erráticas (71), provavelmente devido à desconsideração dos fatores ambientais que interagem com fatores genéticos. Os transtornos psiquiátricos, de uma forma geral, desenvolvem-se devido à interação entre uma predisposição familiar e estressores biopsicossociais. Esse modelo é contemplado pela teoria de diátese-estresse que prediz o desenvolvimento de Depressão (71), no qual a sensibilidade a um evento estressor depende da sua genética. Mann *et al.* (181) sugerem a construção de um modelo que pode ser visto como um modelo gatilho-limiar para explicar essa suscetibilidade e compreender esse fenômeno. A diátese (vulnerabilidade) determina como um indivíduo reage a um evento estressor e depende de fatores que moldam a sua personalidade como os fatores genéticos e ambientais, experiências infantis entre outros. Baseado neste modelo, os fatores de risco podem ser categorizados como pertencentes a um desses dois domínios (gatilho ou limiar pessoal).

Essa tem sido a base dos estudos de interação gene-ambiente. Caspi *et al.* (71) ao seguir uma amostra por mais de duas décadas encontraram que a presença do alelo curto no *5-HTTLPR* influenciava a probabilidade de desenvolver um quadro Depressivo Maior frente a uma situação estressora (71). A associação gene-ambiente do *5-HTTLPR* para Depressão foi demonstrada em muitos estudos (71, 182-189), mas não em todos (190, 191). Surtees *et al*. (191), no maior estudo

disponível de interação gene-ambiente para Depressão, não encontrou relação entre o genótipo e experiências na infância ou na vida adulta com o risco de Depressão maior. Por outro lado, foi identificada uma interação somente com os homens, mas na direção oposta àquela descrita por Caspi *et al.* (71).

A interação gene-ambiente do *5-HTTLPR* com maus tratos na infância não foi estudada exclusivamente na Depressão Maior. Kaufman *et al*. (192) descreveram em um estudo de seguimento de crianças vítimas de maus-tratos e controles uma interação gene-ambiente também com o alelo s e uso precoce de álcool.

Nenhum estudo testou a interação gene-ambiente no TP, apenas com fenótipos intermediários da ansiedade. Stein *et al.* (193) avaliaram a interação do *5-HTTLPR* com trauma na infância e encontraram que os sujeitos com o alelo s estavam associados à maior sensibilidade à ansiedade quando expostos a maior abuso emocional, o que não ocorreu com os sujeitos ll. No entanto, não houve interação gene-ambiente com o fenótipo neuroticismo pelo inventário de personalidade NEO.

Outro gene candidato para interação gene-ambiente é o *HTR1A*. Ratos com deleção de *HTR1A*, particularmente no córtex pré-frontal, apresentam um fenótipo de ansiedade somente quando essa inativação ocorre no período pós-natal, uma vez que a inativação do *HTR1A* na vida adulta não afeta a ansiedade (194). Recentemente foi demonstrado que a expressão do *HTR1A* é menor em animais sensíveis ao estresse (195). No entanto, Mandelli *et al.* (182) não

encontrou qualquer associação entre o *HTR1A* e eventos estressantes no início da Depressão Maior.

Os estudos de interação não se restringem ao efeito deletério do ambiente, mas também avaliam o efeito protetor. Jokela *et al.* (196) avaliaram um coorte prospectiva de 21 anos e encontraram que os sujeitos portadores do alelo T do polimorfismo T102C do *HTR2A* eram mais responsivos ao aspecto protetor do cuidado materno para o desenvolvimento de depressão. Esse estudo mostra que variações genéticas podem influenciar na forma como o indivíduo utiliza os suportes ambientais.

Os estudos de interação devem ser vistos com cuidado, pois freqüentemente os achados se restringem a subanálises, o que resulta de um maior número de testes estatísticos e então uma maior probabilidade de recair no erro tipo I (197). Por exemplo, Grabe (184) e Eley (183) encontraram associação gene-ambiente com o *5-HTTLPR* apenas nas mulheres, enquanto que Surtees *et al*. (191) encontraram uma relação em homens com direção inversa (198). Já Stein *et al*. (193) descreveu uma interação do gene transportador da serotonina com uma escala de trauma na infância e sensibilidade à ansiedade, enquanto que não há referência às demais escalas, que refletem fatores de risco mais bem estabelecidos para ansiedade como o abuso sexual. Sjoberg *et al*. (199) descreveram interação entre *5-HTTLPR* e adversidades com Depressão, mas a interação foi observada em direções opostas em homens e mulheres.

Outra limitação desses estudos é que a baixa freqüência de eventos no grupo de comparação diminui o poder do estudo que provavelmente detectará

somente interações de grande efeito. Os estudos de interação geralmente apresentam um poder pequeno, e os achados iniciais freqüentemente estão superestimados, necessitando ser replicado em amostras maiores. Curiosamente, os estudos de tamanho amostral pequeno geralmente descrevem uma associação positiva, enquanto que apenas estudos grandes (190, 191) mostram resultados negativos. Possivelmente, existe um viés de publicação e a interpretação do resultado passa a ser ainda mais problemática (198). Ainda, a presença de interação estatística encontrada nos estudos não significa necessariamente uma interação biológica plausível (198).

Moffitt et al. (200) propuseram sete etapas para conduzir um bom estudo de interação, sendo que primeiramente deve-se buscar os modelos quantitativos de genética comportamental. Os coeficientes de herdabilidade não indicam apenas a contribuição de genes, mas também o efeito desses na interação com o ambiente. No caso do TP, a herdabilidade é estimada em 28%, sendo que os fatores ambientais contribuem em 70% (14). A segunda etapa é identificar o evento ambiental candidato, e ter sua medida otimizada. Posteriormente, devem-se identificar os genes candidatos, e então testar a interação. No passo seguinte, deve-se testar se a interação permanece ao substituir um gene por outro ou mesmo um evento ambiental por outro, para avaliar a especificidade da interação. Porém essa relação somente é firmada quando o achado é replicado e concluído por meta-análise (200).

Interação gene-gene

Diante do possível papel de múltiplos genes no desenvolvimento de patologias psiquiátricas, alguns autores passaram a investigar a interação gene-gene. O sucesso dessas interações depende muito de um tamanho amostral adequado (201).

Freitag *et al*. (202) avaliaram diversas interações possíveis para o desenvolvimento do TP e encontraram uma interação entre o *HTR1A* (-1019C/G) e o *COMT* (Val/Met). Os autores propõem que o alelo G do HTR1A levaria a um aumento da expressão dos auto-receptores no núcleo da rafe, levando então a uma diminuição da transmissão serotoninérgica. Nas células pós-sinápticas, por outro lado, o alelo G levaria à diminuição da expressão dos receptores *HTR1A* pós-sinápticos. Essa proposta está de acordo com estudos de neuroimagem nos quais se identificou diminuição de ligação nos receptores no giro do cíngulo e nos núcleos da rafe (203). A liberação de norepinefrina no *locus coeruleus* é modulada em ratos pela ação inibitória do *HTR1A* nos neurônios glutamatérgicos (204), sugerindo-se, então, que a redução da transmissão serotoninérgica causada pelo alelo G conseqüentemente levaria a um aumento da liberação de norepinefrina. Já o alelo G do *COMT* leva a uma inativação monoaminérigica mais rápida, e a redução local de norepinefrina nos hetero-receptores alfa 1 nos neurônios serotoninérgicos da rafe leva à diminuição da liberação de serotonina (205). Dessa forma, a redução da neurotransmissão serotoninérgica poderia ocorrer pela elevação da atividade da *COMT* ou pelo aumento dos auto-receptores ou

diminuição dos hetero-receptores *HTR1A*, ou mesmo pela interação desses fatores. No entanto, os autores encontraram uma razão de chances (RC) para esse achado de 1,08 em uma amostra de 230 sujeitos. Gauderman (201) sugere que quando a RC esperada em um estudo de interação for de 1,5, necessitar-se-iam mais de 2000 sujeitos, ou seja, resultados vindos de amostras pequenas podem estar recaindo no erro tipo I.

Farmacogenética

A resposta a psicofármacos também parece ser influenciada por variações genéticas. Ela é resultado da interação de múltiplas variações genômicas com influências ambientais e depende da estrutura ou expressão funcional de produtos gênicos (receptores, transportadores, enzimas), os quais são alvos diretos das drogas ou modificam indiretamente o desenvolvimento e a plasticidade de conexões neurais envolvidas em efeitos farmacológicos. Como o mecanismo de ação detalhado dos antidepressivos continua sendo enigmático, os estudos recentes modificaram o foco que vinha sendo depositado nos neurotransmissores, ou seja, sua liberação, recaptura e metabolismo para modulação da expressão gênica, plasticidade sináptica e neurogênese (206, 207).

Os estudos de farmacogenética envolvendo antidepressivos geralmente se concentram em pacientes com Depressão Maior. Serretti *et al*. (208) trataram pacientes com depressão unipolar e bipolar com fluvoxamina e encontraram uma pior resposta ao fármaco para os pacientes bipolares com a variação G do

polimorfismo (-109C/G) no *HTR1A*. Lemonde *et al.* (209) descreveram a resposta à fluoxetina, à nefazodona e ao agonista do *HTR1A* fibanserina e também encontraram uma pior resposta nos pacientes com o alelo G. Evidências indicam uma associação entre o tempo de resposta e a resposta global dos ISRS e o *5-HTTLPR* em pacientes com Depressão (210, 211).

Apenas um estudo examinou a influência de marcadores genéticos no TP. Perna *et al*. testaram a hipótese de que a variação alélica do *5-HTTLPR* poderia estar relacionada com a eficácia da paroxetina no tratamento do TP. A presença do alelo l em homozigose ou heterozigose foi associada com melhor resposta aos ataques de pânico em mulheres (212).

Apesar dos avanços nas pesquisas de farmacogenética, nenhum estudo avaliou a influência dos marcadores gênicos com a psicoterapia em nenhum transtorno psiquiátrico.

8. Justificativa do Estudo

Estudos de caso e controle não têm encontrado qualquer associação entre o polimorfismo do gene transportador da serotonina (*5-HTTLPR*) com o Transtorno do Pânico (TP). No entanto, os estudos geralmente são realizados com amostras que variam de 100 a 300 indivíduos, justificando o uso da meta-análise para aumentar o poder do estudo.

Além disso, nenhum estudo avaliou o *5-HTTLPR* na sua forma trialélica (213), e os resultados de associação dos genes dos receptores de serotonina *HTR1A* e *HTR2A* com o TP são bastante controversos. Não existe nenhum estudo publicado em amostras brasileiras que avalie essas variações gênicas no TP. Nenhum estudo avaliou também a interação dessas variações gênicas com estressores associados ao TP.

O gene *EFHC2* (*EF-hand domain containing 2*) esteve recentemente relacionado à percepção do medo (180) e ainda não foi avaliado no TP nem nos fenótipos intermediários da ansiedade. Como medo é um sintoma central do TP, o *EFCH2* pode ser um gene candidato para o TP.

Por fim, apesar de alguns avanços na farmacogenética, nenhum estudo avaliou a influência de marcadores gênicos à terapia cognitivo-comportamental. Esses estudos possivelmente poderiam contribuir para elucidar o mecanismo biológico envolvido no processo psicoterápico.

9. Hipóteses

Considerando que os estudos dos aspectos genéticos no TP ainda apresentam resultados controversos, as hipóteses desse estudo são:

1. Uma vez que os estudos de associação com o *5-HTTLPR* e o TP são relativamente pequenos, nossa primeira hipótese é de que com o recurso da meta-análise poderíamos encontrar uma associação. Devido à associação do alelo s com traços de ansiedade em sujeitos saudáveis (214), a hipótese é de que o alelo s também estaria associado ao TP.
2. Como estudos prévios associaram o TP com variações nos genes *HTR1A* e *HTR2A* (12, 88), nossa hipótese é de que replicaríamos esse achado na amostra brasileira. Em relação aos estudos de interação gene-ambiente, nossa hipótese é de que encontraríamos uma interação do *5-HTTLPR* com trauma na infância, e do *HTR2A* com vínculo parental no TP.
3. Como o medo é um sintoma central do TP, nossa hipótese é de que o gene *EFHC2* estaria relacionado ao TP e aos seus 2 fenótipos intermediários (comportamento inibido e temperamento de evitação de danos).
4. Alguns estudos sugerem mecanismos semelhantes para a ação da psicoterapia e de psicofármacos (215-217). Nossa hipótese é de que

algum gene previamente relacionado à ansiedade (*BDNF, CREB, CRHR1, RGS2, HTR1A, HTR2A*) esteja associado à resposta à Terapia Cognitivo-Comportamental.

10. Objetivos

Gerais:

- O objetivo desse estudo é pesquisar a influência do polimorfismo nos genes dos receptores de serotonina *HTR1A e HTR2A, 5-HTTLPR, CRHR1, RGS2, BDNF, CREB* e *EFHC2* no TP. Como esse estudo envolve vários genes, aumentando conseqüentemente a chance de erro tipo I, os genes estudados com cada fenótipo foram estabelecidos *a priori*, resultando em quatro objetivos específicos.

Específicos:

1. Revisão sistemática da literatura em busca da associação entre Transtorno do Pânico e o gene transportador da serotonina.
2. Evidenciar associação entre o gene transportador da serotonina e os receptores *HTR1A* e *HTR2A* com o diagnostico de TP na população brasileira e a interação entre gene-ambiente com estressores e vínculo parental na infância.
3. Evidenciar associação entre marcadores do *EFHC2* com o TP e fenótipos intermediários.
4. Evidenciar a influência dos marcadores gênicos do *BDNF, CREB1, CRHR1, RGS2, HTR1A, HTR2A* e transportador da serotonina na resposta à Terapia Cognitivo-Comportamental.

11. Considerações Éticas

Os sujeitos incluídos nos estudos assinaram o Termo de Consentimento livre e esclarecido (TCLE) que está em anexo e um adendo explicando a parceria internacional. O projeto está de acordo com a resolução CNS 347/05 e foi avaliado pelo Comitê de Ética e Pesquisa da HCPA (04-272 e 05-056) e do *Massachusetts General Hospital* (MGH 2007-P-000119). Os TCLE utilizados nos estudos estão no anexo A.

12. Referências Bibliográficas

1. Association AP. Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). 4th edition ed. Washington; 1994.
2. Kessler RC, Chiu WT, Jin R, Ruscio AM, Shear K, Walters EE. The epidemiology of panic attacks, panic disorder, and agoraphobia in the National Comorbidity Survey Replication. Arch Gen Psychiatry. 2006 Apr;63(4):415-24.
3. Kessler RC, McGonagle KA, Zhao S, Nelson CB, Hughes M, Eshleman S, et al. Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. Arch Gen Psychiatry. 1994 Jan;51(1):8-19.
4. Weinstein RS. Panic disorder. Am Fam Physician. 1995 Nov 15;52(7):2055-63, 67-8.
5. Rosenberg R, Ottosson JO, Bech P, Mellergard M, Rosenberg NK. Validation criteria for panic disorder as a nosological entity. Acta Psychiatr Scand Suppl. 1991;365:7-17.
6. Rosenbaum JF, Pollack MH, Pollock RA. Clinical issues in the long-term treatment of panic disorder. J Clin Psychiatry. 1996;57 Suppl 10:44-8; discussion 9-50.
7. Pollack MH, Smoller JW. The longitudinal course and outcome of panic disorder. Psychiatr Clin North Am. 1995 Dec;18(4):785-801.
8. Andersch S, Hetta J. A 15-year follow-up study of patients with panic disorder. Eur Psychiatry. 2003 Dec;18(8):401-8.
9. Rothe C, Koszycki D, Bradwejn J, King N, De Luca V, Shaikh S, et al. Association study of serotonin-2A receptor gene polymorphism and panic disorder in patients from Canada and Germany. Neurosci Lett. 2004 Jun 17;363(3):276-9.
10. Hettema JM, Neale MC, Kendler KS. A review and meta-analysis of the genetic epidemiology of anxiety disorders. Am J Psychiatry. 2001 Oct;158(10):1568-78.
11. Crowe RR, Noyes R, Pauls DL, Slymen D. A family study of panic disorder. Arch Gen Psychiatry. 1983 Oct;40(10):1065-9.
12. Inada Y, Yoneda H, Koh J, Sakai J, Himei A, Kinoshita Y, et al. Positive association between panic disorder and polymorphism of the serotonin 2A receptor gene. Psychiatry Res. 2003 May 1;118(1):25-31.
13. Torgersen S. Genetic factors in anxiety disorders. Arch Gen Psychiatry. 1983 Oct;40(10):1085-9.
14. Hettema JM, Prescott CA, Myers JM, Neale MC, Kendler KS. The structure of genetic and environmental risk factors for anxiety disorders in men and women. Arch Gen Psychiatry. 2005 Feb;62(2):182-9.
15. Kendler KS, Myers JM, Neale MC. A multidimensional twin study of mental health in women. Am J Psychiatry. 2000 Apr;157(4):506-13.
16. Kendler KS, Neale MC, Kessler RC, Heath AC, Eaves LJ. The genetic epidemiology of phobias in women. The interrelationship of agoraphobia, social phobia, situational phobia, and simple phobia. Arch Gen Psychiatry. 1992 Apr;49(4):273-81.

17. Kendler KS, Walters EE, Truett KR, Heath AC, Neale MC, Martin NG, et al. A twin-family study of self-report symptoms of panic-phobia and somatization. Behav Genet. 1995 Nov;25(6):499-515.
18. Clark DM. A cognitive approach to panic. Behav Res Ther. 1986;24(4):461-70.
19. Barlow DH. Anxiety and its disorders: the nature and treatment of anxiety and panic. . New York: Guilford Press; 1988.
20. Kipper L, Blaya C, Teruchkin B, Heldt E, Isolan L, Mezzomo K, et al. Evaluation of defense mechanisms in adult patients with panic disorder: before and after treatment. J Nerv Ment Dis. 2005 Sep;193(9):619-24.
21. Heider D, Matschinger H, Bernert S, Alonso J, Brugha TS, Bruffaerts R, et al. Adverse parenting as a risk factor in the occurrence of anxiety disorders : A study in six European countries. Soc Psychiatry Psychiatr Epidemiol. 2008 Jan 14.
22. Shear MK, Cooper AM, Klerman GL, Busch FN, Shapiro T. A psychodynamic model of panic disorder. Am J Psychiatry. 1993 Jun;150(6):859-66.
23. Parker G, Tupling H, Brown LB. Parental bonding instrument. Br J Med Psychol. 1979;52:1-10.
24. Kendler KS, Sham PC, MacLean CJ. The determinants of parenting: an epidemiological, multi-informant, retrospective study. Psychol Med. 1997 May;27(3):549-63.
25. Sato T, Narita T, Hirano S, Kusunoki K, Sakado K, Uehara T. Confirmatory factor analysis of the Parental Bonding Instrument in a Japanese population. Psychol Med. 1999 Jan;29(1):127-33.
26. Cox BJ, Enns MW, Clara IP. The Parental Bonding Instrument: confirmatory evidence for a three-factor model in a psychiatric clinical sample and in the National Comorbidity Survey. Soc Psychiatry Psychiatr Epidemiol. 2000 Aug;35(8):353-7.
27. Mackinnon AJ, Henderson AS, Scott R, Duncan-Jones P. The Parental Bonding Instrument (PBI): an epidemiological study in a general population sample. Psychol Med. 1989 Nov;19(4):1023-34.
28. Parker G. Parental reports of depressives. An investigation of several explanations. J Affect Disord. 1981 Jun;3(2):131-40.
29. Parker G. The Parental Bonding Instrument: psychometric properties reviewed. Psychiatr Dev. 1989 Winter;7(4):317-35.
30. Plantes MM, Prusoff BA, Brennan J, Parker G. Parental representations of depressed outpatients from a U.S.A. sample. J Affect Disord. 1988 Sep-Oct;15(2):149-55.
31. Wilhelm K, Niven H, Parker G, Hadzi-Pavlovic D. The stability of the Parental Bonding Instrument over a 20-year period. Psychol Med. 2005 Mar;35(3):387-93.
32. Hauck S, Schestatsky S, Terra L, Knijnik L, Sanchez P, Ceitlin LH. Cross-cultural adaptation of Parental Bonding Instrument (PBI) to Brazilian Portuguese Rev Psiquiatr Rio Gd Sul. 2006;28(2):162-8.
33. Faravelli C, Panichi C, Pallanti S, Paterniti S, Grecu LM, Rivelli S. Perception of early parenting in panic and agoraphobia. Acta Psychiatr Scand. 1991 Jul;84(1):6-8.
34. Wiborg IM, Dahl AA. The recollection of parental rearing styles in patients with panic disorder. Acta Psychiatr Scand. 1997 Jul;96(1):58-63.

35. Silove D, Parker G, Hadzi-Pavlovic D, Manicavasagar V, Blaszczynski A. Parental representations of patients with panic disorder and generalised anxiety disorder. Br J Psychiatry. 1991 Dec;159:835-41.
36. Pacchierotti C, Bossini L, Castrogiovanni A, Pieraccini F, Soreca I, Castrogiovanni P. Attachment and panic disorder. Psychopathology. 2002 Nov-Dec;35(6):347-54.
37. Fergusson DM, Lynskey MT, Horwood LJ. Childhood sexual abuse and psychiatric disorder in young adulthood: I. Prevalence of sexual abuse and factors associated with sexual abuse. J Am Acad Child Adolesc Psychiatry. 1996 Oct;35(10):1355-64.
38. Kendler KS, Bulik CM, Silberg J, Hettema JM, Myers J, Prescott CA. Childhood sexual abuse and adult psychiatric and substance use disorders in women: an epidemiological and cotwin control analysis. Arch Gen Psychiatry. 2000 Oct;57(10):953-9.
39. Kendler KS, Neale MC, Kessler RC, Heath AC, Eaves LJ. Childhood parental loss and adult psychopathology in women. A twin study perspective. Arch Gen Psychiatry. 1992 Feb;49(2):109-16.
40. Faravelli C. Life events preceding the onset of panic disorder. J Affect Disord. 1985 Jul;9(1):103-5.
41. Scocco P, Barbieri I, Frank E. Interpersonal problem areas and onset of panic disorder. Psychopathology. 2007;40(1):8-13.
42. Grassi-Oliveira R, Stein LM, Pezzi JC. [Translation and content validation of the Childhood Trauma Questionnaire into Portuguese language]. Rev Saude Publica. 2006 Apr;40(2):249-55.
43. Dill DL, Chu JA, Grob MC, Eisen SV. The reliability of abuse history reports: a comparison of two inquiry formats. Compr Psychiatry. 1991 Mar-Apr;32(2):166-9.
44. Paivio SC. Stability of retrospective self-reports of child abuse and neglect before and after therapy for child abuse issues. Child Abuse Negl. 2001 Aug;25(8):1053-68.
45. Bernstein DP, Stein JA, Newcomb MD, Walker E, Pogge D, Ahluvalia T, et al. Development and validation of a brief screening version of the Childhood Trauma Questionnaire. Child Abuse Negl. 2003 Feb;27(2):169-90.
46. Laird NM, Lange C. Family-based designs in the age of large-scale gene-association studies. Nat Rev Genet. 2006 May;7(5):385-94.
47. Balding DJ. A tutorial on statistical methods for population association studies. Nat Rev Genet. 2006 Oct;7(10):781-91.
48. Hirschhorn JN, Daly MJ. Genome-wide association studies for common diseases and complex traits. Nat Rev Genet. 2005 Feb;6(2):95-108.
49. Burton PR, Tobin MD, Hopper JL. Key concepts in genetic epidemiology. Lancet. 2005 Sep 10-16;366(9489):941-51.
50. Hattersley AT, McCarthy MI. What makes a good genetic association study? Lancet. 2005 Oct 8;366(9493):1315-23.
51. de Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D. Efficiency and power in genetic association studies. Nat Genet. 2005 Nov;37(11):1217-23.
52. Wittke-Thompson JK, Pluzhnikov A, Cox NJ. Rational inferences about departures from Hardy-Weinberg equilibrium. Am J Hum Genet. 2005 Jun;76(6):967-86.
53. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 2007 Sep;81(3):559-75.

54. Ioannidis JP. Molecular bias. Eur J Epidemiol. 2005;20(9):739-45.
55. Salanti G, Amountza G, Ntzani EE, Ioannidis JP. Hardy-Weinberg equilibrium in genetic association studies: an empirical evaluation of reporting, deviations, and power. Eur J Hum Genet. 2005 Jul;13(7):840-8.
56. Hirschhorn JN, Lohmueller K, Byrne E, Hirschhorn K. A comprehensive review of genetic association studies. Genet Med. 2002 Mar-Apr;4(2):45-61.
57. Ioannidis JP. Genetic associations: false or true? Trends Mol Med. 2003 Apr;9(4):135-8.
58. Cordell HJ, Clayton DG. Genetic association studies. Lancet. 2005 Sep 24-30;366(9491):1121-31.
59. Pritchard JK, Donnelly P. Case-control studies of association in structured or admixed populations. Theor Popul Biol. 2001 Nov;60(3):227-37.
60. Parra FC, Amado RC, Lambertucci JR, Rocha J, Antunes CM, Pena SD. Color and genomic ancestry in Brazilians. Proc Natl Acad Sci U S A. 2003 Jan 7;100(1):177-82.
61. Zembrzuski VM, Callegari-Jacques SM, Hutz MH. Application of an African Ancestry Index as a genomic control approach in a Brazilian population. Ann Hum Genet. 2006 Nov;70(Pt 6):822-8.
62. Devlin B, Roeder K. Genomic control for association studies. Biometrics. 1999 Dec;55(4):997-1004.
63. Devlin B, Roeder K, Wasserman L. Genomic control, a new approach to genetic-based association studies. Theor Popul Biol. 2001 Nov;60(3):155-66.
64. Vieland VJ, Goodman DW, Chapman T, Fyer AJ. New segregation analysis of panic disorder. Am J Med Genet. 1996 Apr 9;67(2):147-53.
65. Deckert J, Catalano M, Syagailo YV, Bosi M, Okladnova O, Di Bella D, et al. Excess of high activity monoamine oxidase A gene promoter alleles in female patients with panic disorder. Hum Mol Genet. 1999 Apr;8(4):621-4.
66. Versiani M, Cassano G, Perugi G, Benedetti A, Mastalli L, Nardi A, et al. Reboxetine, a selective norepinephrine reuptake inhibitor, is an effective and well-tolerated treatment for panic disorder. J Clin Psychiatry. 2002 Jan;63(1):31-7.
67. Kahn RS, van Praag HM, Wetzler S, Asnis GM, Barr G. Serotonin and anxiety revisited. Biol Psychiatry. 1988 Jan 15;23(2):189-208.
68. Iversen SD. 5-HT and anxiety. Neuropharmacology. 1984 Dec;23(12B):1553-60.
69. Deakin JFW, Graeff FG. 5-HT and mechanisms of defence. J Psychopharmachol. 1991;5:305-15.
70. den Boer JA, Westenberg HG. Behavioral, neuroendocrine, and biochemical effects of 5-hydroxytryptophan administration in panic disorder. Psychiatry Res. 1990 Mar;31(3):267-78.
71. Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, et al. Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science. 2003 Jul 18;301(5631):386-9.
72. Lesch KP, Balling U, Gross J, Strauss K, Wolozin BL, Murphy DL, et al. Organization of the human serotonin transporter gene. J Neural Transm Gen Sect. 1994;95(2):157-62.

73. Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, et al. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science. 1996 Nov 29;274(5292):1527-31.
74. Heils A, Teufel A, Petri S, Stober G, Riederer P, Bengel D, et al. Allelic variation of human serotonin transporter gene expression. J Neurochem. 1996 Jun;66(6):2621-4.
75. Osher Y, Hamer D, Benjamin J. Association and linkage of anxiety-related traits with a functional polymorphism of the serotonin transporter gene regulatory region in Israeli sibling pairs. Mol Psychiatry. 2000 Mar;5(2):216-9.
76. Flory JD, Manuck SB, Ferrell RE, Dent KM, Peters DG, Muldoon MF. Neuroticism is not associated with the serotonin transporter (5-HTTLPR) polymorphism. Mol Psychiatry. 1999 Jan;4(1):93-6.
77. Deckert J, Catalano M, Heils A, Di Bella D, Friess F, Politi E, et al. Functional promoter polymorphism of the human serotonin transporter: lack of association with panic disorder. Psychiatr Genet. 1997 Spring;7(1):45-7.
78. Ishiguro H, Arinami T, Yamada K, Otsuka Y, Toru M, Shibuya H. An association study between a transcriptional polymorphism in the serotonin transporter gene and panic disorder in a Japanese population. Psychiatry Clin Neurosci. 1997 Oct;51(5):333-5.
79. Maron E, Lang A, Tasa G, Liivlaid L, Toru I, Must A, et al. Associations between serotonin-related gene polymorphisms and panic disorder. Int J Neuropsychopharmacol. 2005 Jun;8(2):261-6.
80. Heisler LK, Chu HM, Brennan TJ, Danao JA, Bajwa P, Parsons LH, et al. Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. Proc Natl Acad Sci U S A. 1998 Dec 8;95(25):15049-54.
81. Kent JM, Mathew SJ, Gorman JM. Molecular targets in the treatment of anxiety. Biol Psychiatry. 2002 Nov 15;52(10):1008-30.
82. Handley SL. 5-Hydroxytryptamine pathways in anxiety and its treatment. Pharmacol Ther. 1995 Apr;66(1):103-48.
83. Lesch KP, Gutknecht L. Focus on The 5-HT1A receptor: emerging role of a gene regulatory variant in psychopathology and pharmacogenetics. Int J Neuropsychopharmacol. 2004 Dec;7(4):381-5.
84. Lanzenberger RR, Mitterhauser M, Spindelegger C, Wadsak W, Klein N, Mien LK, et al. Reduced serotonin-1A receptor binding in social anxiety disorder. Biol Psychiatry. 2007 May 1;61(9):1081-9.
85. Lemonde S, Turecki G, Bakish D, Du L, Hrdina PD, Bown CD, et al. Impaired repression at a 5-hydroxytryptamine 1A receptor gene polymorphism associated with major depression and suicide. J Neurosci. 2003 Sep 24;23(25):8788-99.
86. Domschke K, Braun M, Ohrmann P, Suslow T, Kugel H, Bauer J, et al. 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. 2006 Jun;9(3):349-55.
87. Strobel A, Gutknecht L, Rothe C, Reif A, Mossner R, Zeng Y, et al. Allelic variation in 5-HT1A receptor expression is associated with anxiety- and depression-related personality traits. J Neural Transm. 2003 Dec;110(12):1445-53.

88. Rothe C, Gutknecht L, Freitag C, Tauber R, Mossner R, Franke P, et al. Association of a functional 1019C>G 5-HT1A receptor gene polymorphism with panic disorder with agoraphobia. Int J Neuropsychopharmacol. 2004 Jun;7(2):189-92.
89. Hettema JM, An SS, van den Oord EJ, Neale MC, Kendler KS, Chen X. Association study between the serotonin 1A receptor (HTR1A) gene and neuroticism, major depression, and anxiety disorders. Am J Med Genet B Neuropsychiatr Genet. 2007 Dec 28.
90. de Angelis L. 5-HT2A antagonists in psychiatric disorders. Curr Opin Investig Drugs. 2002 Jan;3(1):106-12.
91. Erdmann J, Shimron-Abarbanell D, Rietschel M, Albus M, Maier W, Korner J, et al. Systematic screening for mutations in the human serotonin-2A (5-HT2A) receptor gene: identification of two naturally occurring receptor variants and association analysis in schizophrenia. Hum Genet. 1996 May;97(5):614-9.
92. Polesskaya OO, Sokolov BP. Differential expression of the "C" and "T" alleles of the 5-HT2A receptor gene in the temporal cortex of normal individuals and schizophrenics. J Neurosci Res. 2002 Mar 15;67(6):812-22.
93. Arias B, Gasto C, Catalan R, Gutierrez B, Pintor L, Fananas L. The 5-HT(2A) receptor gene 102T/C polymorphism is associated with suicidal behavior in depressed patients. Am J Med Genet. 2001 Dec 8;105(8):801-4.
94. Du L, Bakish D, Lapierre YD, Ravindran AV, Hrdina PD. Association of polymorphism of serotonin 2A receptor gene with suicidal ideation in major depressive disorder. Am J Med Genet. 2000 Feb 7;96(1):56-60.
95. Maron E, Nikopensius T, Koks S, Altmae S, Heinaste E, Vabrit K, et al. Association study of 90 candidate gene polymorphisms in panic disorder. Psychiatr Genet. 2005 Mar;15(1):17-24.
96. Fehr C, Schleicher A, Szegedi A, Anghelescu I, Klawe C, Hiemke C, et al. Serotonergic polymorphisms in patients suffering from alcoholism, anxiety disorders and narcolepsy. Prog Neuropsychopharmacol Biol Psychiatry. 2001 Jul;25(5):965-82.
97. Unschuld PG, Ising M, Erhardt A, Lucae S, Kloiber S, Kohli M, et al. Polymorphisms in the serotonin receptor gene HTR2A are associated with quantitative traits in panic disorder. Am J Med Genet B Neuropsychiatr Genet. 2007 Jun 5;144(4):424-9.
98. Levy ER, Powell JF, Buckle VJ, Hsu YP, Breakefield XO, Craig IW. Localization of human monoamine oxidase-A gene to Xp11.23-11.4 by in situ hybridization: implications for Norrie disease. Genomics. 1989 Aug;5(2):368-70.
99. Samochowiec J, Hajduk A, Samochowiec A, Horodnicki J, Stepien G, Grzywacz A, et al. Association studies of MAO-A, COMT, and 5-HTT genes polymorphisms in patients with anxiety disorders of the phobic spectrum. Psychiatry Res. 2004 Aug 30;128(1):21-6.
100. Hamilton SP, Slager SL, Heiman GA, Haghighi F, Klein DF, Hodge SE, et al. No genetic linkage or association between a functional promoter polymorphism in the monoamine oxidase-A gene and panic disorder. Mol Psychiatry. 2000 Sep;5(5):465-6.
101. Han L, Nielsen DA, Rosenthal NE, Jefferson K, Kaye W, Murphy D, et al. No coding variant of the tryptophan hydroxylase gene detected in seasonal affective disorder, obsessive-compulsive disorder, anorexia nervosa, and alcoholism. Biol Psychiatry. 1999 Mar 1;45(5):615-9.

102. Maron E, Kuikka JT, Shlik J, Vasar V, Vanninen E, Tiihonen J. Reduced brain serotonin transporter binding in patients with panic disorder. Psychiatry Res. 2004 Dec 15;132(2):173-81.
103. Maron E, Tasa G, Toru I, Lang A, Vasar V, Shlik J. Association between serotonin-related genetic polymorphisms and CCK-4-induced panic attacks with or without 5-hydroxytryptophan pretreatment in healthy volunteers. World J Biol Psychiatry. 2004 Jul;5(3):149-54.
104. Kim W, Choi YC, Yoon KS, Cho DY, Pae CU, Woo JM. Tryptophan hydroxylase and serotonin transporter gene polymorphism does not affect the diagnosis, clinical features and treatment outcome of panic disorder in the Korean population. Prog Neuropsychopharmacol Biol Psychiatry. 2006;30:1413-8.
105. Maron E, Toru I, Must A, Tasa G, Toover E, Vasar V, et al. Association study of tryptophan hydroxylase 2 gene polymorphisms in panic disorder. Neurosci Lett. 2007 Jan 16;411(3):180-4.
106. Mossner R, Freitag CM, Gutknecht L, Reif A, Tauber R, Franke P, et al. The novel brain-specific tryptophan hydroxylase-2 gene in panic disorder. J Psychopharmacol. 2006 Jul;20(4):547-52.
107. Hamilton SP, Heiman GA, Haghighi F, Mick S, Klein DF, Hodge SE, et al. Lack of genetic linkage or association between a functional serotonin transporter polymorphism and panic disorder. Psychiatr Genet. 1999 Mar;9(1):1-6.
108. Matsushita S, Muramatsu T, Kimura M, Shirakawa O, Mita T, Nakai T, et al. Serotonin transporter gene regulatory region polymorphism and panic disorder. Mol Psychiatry. 1997 Sep;2(5):390-2.
109. Tadic A, Rujescu D, Szegedi A, Giegling I, Singer P, Moller HJ, et al. Association of a MAOA gene variant with generalized anxiety disorder, but not with panic disorder or major depression. Am J Med Genet B Neuropsychiatr Genet. 2003 Feb;117(1):1-6.
110. Huang YY, Battistuzzi C, Oquendo MA, Harkavy-Friedman J, Greenhill L, Zalsman G, et al. Human 5-HT1A receptor C(-1019)G polymorphism and psychopathology. Int J Neuropsychopharmacol. 2004 Dec;7(4):441-51.
111. Fehr C, Grintschuk N, Szegedi A, Anghelescu I, Klawe C, Singer P, et al. The HTR1B 861G>C receptor polymorphism among patients suffering from alcoholism, major depression, anxiety disorders and narcolepsy. Psychiatry Res. 2000 Dec 4;97(1):1-10.
112. Fehr C, Szegedi A, Anghelescu I, Klawe C, Hiemke C, Dahmen N. Sex differences in allelic frequencies of the 5-HT2C Cys23Ser polymorphism in psychiatric patients and healthy volunteers: findings from an association study. Psychiatr Genet. 2000 Jun;10(2):59-65.
113. Deckert J, Meyer J, Catalano M, Bosi M, Sand P, DiBella D, et al. Novel 5'-regulatory region polymorphisms of the 5-HT2C receptor gene: association study with panic disorder. Int J Neuropsychopharmacol. 2000 Dec;3(4):321-5.
114. Maron E, Shlik J. Serotonin function in panic disorder: important, but why? Neuropsychopharmacology. 2006 Jan;31(1):1-11.
115. Bremner JD, Krystal JH, Southwick SM, Charney DS. Noradrenergic mechanisms in stress and anxiety: II. Clinical studies. Synapse. 1996 May;23(1):39-51.

116. Blaya C, Seganfredo AC, Dornelles M, Torres M, Paludo A, Heldt E, et al. The efficacy of milnacipran in panic disorder: an open trial. Int Clin Psychopharmacol. 2007 May;22(3):153-8.
117. Fahy GT, Easty DL, Collum LM, Benedict-Smith A, Hillery M, Parsons DG. Randomised double-masked trial of lodoxamide and sodium cromoglycate in allergic eye disease. A multicentre study. Eur J Ophthalmol. 1992 Jul-Sep;2(3):144-9.
118. Charney DS, Heninger GR, Breier A. Noradrenergic function in panic anxiety. Effects of yohimbine in healthy subjects and patients with agoraphobia and panic disorder. Arch Gen Psychiatry. 1984 Aug;41(8):751-63.
119. Coplan JD, Lydiard RB. Brain circuits in panic disorder. Biol Psychiatry. 1998 Dec 15;44(12):1264-76.
120. Charney DS. Monoamine dysfunction and the pathophysiology and treatment of depression. J Clin Psychiatry. 1998;59 Suppl 14:11-4.
121. Rothe C, Koszycki D, Bradwejn J, King N, Deluca V, Tharmalingam S, et al. Association of the Val158Met Catechol O-Methyltransferase Genetic Polymorphism with Panic Disorder. Neuropsychopharmacology. 2006 Mar 8.
122. Domschke K, Freitag CM, Kuhlenbaumer G, Schirmacher A, Sand P, Nyhuis P, et al. Association of the functional V158M catechol-O-methyl-transferase polymorphism with panic disorder in women. Int J Neuropsychopharmacol. 2004 Jun;7(2):183-8.
123. Karayiorgou M, Altemus M, Galke BL, Goldman D, Murphy DL, Ott J, et al. Genotype determining low catechol-O-methyltransferase activity as a risk factor for obsessive-compulsive disorder. Proc Natl Acad Sci U S A. 1997 Apr 29;94(9):4572-5.
124. Ohara K, Nagai M, Suzuki Y, Ochiai M. No association between anxiety disorders and catechol-O-methyltransferase polymorphism. Psychiatry Res. 1998 Aug 17;80(2):145-8.
125. Hamilton SP, Slager SL, Heiman GA, Deng Z, Haghighi F, Klein DF, et al. Evidence for a susceptibility locus for panic disorder near the catechol-O-methyltransferase gene on chromosome 22. Biol Psychiatry. 2002 Apr 1;51(7):591-601.
126. Woo JM, Yoon KS, Yu BH. Catechol O-methyltransferase genetic polymorphism in panic disorder. Am J Psychiatry. 2002 Oct;159(10):1785-7.
127. Domschke K, Deckert J, O'Donovan M C, Glatt SJ. Meta-analysis of COMT val158met in panic disorder: ethnic heterogeneity and gender specificity. Am J Med Genet B Neuropsychiatr Genet. 2007 Jul 5;144(5):667-73.
128. Koob GF. Corticotropin-releasing factor, norepinephrine, and stress. Biol Psychiatry. 1999 Nov 1;46(9):1167-80.
129. Sullivan GM, Coplan JD, Gorman JM. Psychoneuroendocrinology of anxiety disorders. Psychiatr Clin North Am. 1998 Jun;21(2):397-412.
130. Heim C, Newport DJ, Heit S, Graham YP, Wilcox M, Bonsall R, et al. Pituitary-adrenal and autonomic responses to stress in women after sexual and physical abuse in childhood. Jama. 2000 Aug 2;284(5):592-7.
131. Smoller JW, Rosenbaum JF, Biederman J, Kennedy J, Dai D, Racette SR, et al. Association of a genetic marker at the corticotropin-releasing hormone locus with behavioral inhibition. Biol Psychiatry. 2003 Dec 15;54(12):1376-81.
132. Contarino A, Heinrichs SC, Gold LH. Understanding corticotropin releasing factor neurobiology: contributions from mutant mice. Neuropeptides. 1999 Feb;33(1):1-12.

133. Muller MB, Wurst W. Getting closer to affective disorders: the role of CRH receptor systems. Trends Mol Med. 2004 Aug;10(8):409-15.
134. Timpl P, Spanagel R, Sillaber I, Kresse A, Reul JM, Stalla GK, et al. Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. Nat Genet. 1998 Jun;19(2):162-6.
135. Brady LS, Gold PW, Herkenham M, Lynn AB, Whitfield HJ, Jr. The antidepressants fluoxetine, idazoxan and phenelzine alter corticotropin-releasing hormone and tyrosine hydroxylase mRNA levels in rat brain: therapeutic implications. Brain Res. 1992 Feb 14;572(1-2):117-25.
136. Michelson D, Galliven E, Hill L, Demitrack M, Chrousos G, Gold P. Chronic imipramine is associated with diminished hypothalamic-pituitary-adrenal axis responsivity in healthy humans. J Clin Endocrinol Metab. 1997 Aug;82(8):2601-6.
137. Gold PW, Chrousos GP. Organization of the stress system and its dysregulation in melancholic and atypical depression: high vs low CRH/NE states. Mol Psychiatry. 2002;7(3):254-75.
138. Nemeroff CB, Owens MJ. Pharmacologic differences among the SSRIs: focus on monoamine transporters and the HPA axis. CNS Spectr. 2004 Jun;9(6 Suppl 4):23-31.
139. Liu Z, Zhu F, Wang G, Xiao Z, Tang J, Liu W, et al. Association study of corticotropin-releasing hormone receptor1 gene polymorphisms and antidepressant response in major depressive disorders. Neurosci Lett. 2007 Mar 6;414(2):155-8.
140. Lindsay RM, Wiegand SJ, Altar CA, DiStefano PS. Neurotrophic factors: from molecule to man. Trends Neurosci. 1994 May;17(5):182-90.
141. Duman RS, Heninger GR, Nestler EJ. A molecular and cellular theory of depression. Arch Gen Psychiatry. 1997 Jul;54(7):597-606.
142. Hashimoto K. BDNF variant linked to anxiety-related behaviors. Bioessays. 2007 Feb;29(2):116-9.
143. Kobayashi K, Shimizu E, Hashimoto K, Mitsumori M, Koike K, Okamura N, et al. Serum brain-derived neurotrophic factor (BDNF) levels in patients with panic disorder: as a biological predictor of response to group cognitive behavioral therapy. Prog Neuropsychopharmacol Biol Psychiatry. 2005 Jun;29(5):658-63.
144. Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, et al. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell. 2003 Jan 24;112(2):257-69.
145. Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, et al. Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. Science. 2006 Oct 6;314(5796):140-3.
146. Lang UE, Hellweg R, Kalus P, Bajbouj M, Lenzen KP, Sander T, et al. Association of a functional BDNF polymorphism and anxiety-related personality traits. Psychopharmacology (Berl). 2005 Jun;180(1):95-9.
147. Jiang X, Xu K, Hoberman J, Tian F, Marko AJ, Waheed JF, et al. BDNF variation and mood disorders: a novel functional promoter polymorphism and Val66Met are associated with anxiety but have opposing effects. Neuropsychopharmacology. 2005 Jul;30(7):1353-61.

148. Shimizu E, Hashimoto K, Koizumi H, Kobayashi K, Itoh K, Mitsumori M, et al. No association of the brain-derived neurotrophic factor (BDNF) gene polymorphisms with panic disorder. Prog Neuropsychopharmacol Biol Psychiatry. 2005 Jun;29(5):708-12.
149. Lam P, Cheng CY, Hong CJ, Tsai SJ. Association study of a brain-derived neurotrophic factor (Val66Met) genetic polymorphism and panic disorder. Neuropsychobiology. 2004;49(4):178-81.
150. Blendy JA. The role of CREB in depression and antidepressant treatment. Biol Psychiatry. 2006 Jun 15;59(12):1144-50.
151. Carlezon WA, Jr., Duman RS, Nestler EJ. The many faces of CREB. Trends Neurosci. 2005 Aug;28(8):436-45.
152. Wallace TL, Stellitano KE, Neve RL, Duman RS. Effects of cyclic adenosine monophosphate response element binding protein overexpression in the basolateral amygdala on behavioral models of depression and anxiety. Biol Psychiatry. 2004 Aug 1;56(3):151-60.
153. Josselyn SA, Shi C, Carlezon WA, Jr., Neve RL, Nestler EJ, Davis M. Long-term memory is facilitated by cAMP response element-binding protein overexpression in the amygdala. J Neurosci. 2001 Apr 1;21(7):2404-12.
154. Zubenko GS, Hughes HB, 3rd, Stiffler JS, Brechbiel A, Zubenko WN, Maher BS, et al. Sequence variations in CREB1 cosegregate with depressive disorders in women. Mol Psychiatry. 2003 Jun;8(6):611-8.
155. Odagaki Y, Garcia-Sevilla JA, Huguelet P, La Harpe R, Koyama T, Guimon J. Cyclic AMP-mediated signaling components are upregulated in the prefrontal cortex of depressed suicide victims. Brain Res. 2001 Apr 20;898(2):224-31.
156. Perlis RH, Beasley CM, Jr., Wines JD, Jr., Tamura RN, Cusin C, Shear D, et al. Treatment-associated suicidal ideation and adverse effects in an open, multicenter trial of fluoxetine for major depressive episodes. Psychother Psychosom. 2007;76(1):40-6.
157. Perlis RH, Purcell S, Fagerness J, Cusin C, Yamaki L, Fava M, et al. Clinical and genetic dissection of anger expression and CREB1 polymorphisms in major depressive disorder. Biol Psychiatry. 2007 Sep 1;62(5):536-40.
158. Koch JM, Kell S, Hinze-Selch D, Aldenhoff JB. Changes in CREB-phosphorylation during recovery from major depression. J Psychiatr Res. 2002 Nov-Dec;36(6):369-75.
159. Garakani A, Mathew SJ, Charney DS. Neurobiology of anxiety disorders and implications for treatment. Mt Sinai J Med. 2006 Nov;73(7):941-9.
160. McGaugh JL. The amygdala modulates the consolidation of memories of emotionally arousing experiences. Annu Rev Neurosci. 2004;27:1-28.
161. Rapee RM, Ancis JR, Barlow DH. Emotional reactions to physiological sensations: panic disorder patients and non-clinical Ss. Behav Res Ther. 1988;26(3):265-9.
162. Watson N, Linder ME, Druey KM, Kehrl JH, Blumer KJ. RGS family members: GTPase-activating proteins for heterotrimeric G-protein alpha-subunits. Nature. 1996 Sep 12;383(6596):172-5.
163. Huang LJ, Durick K, Weiner JA, Chun J, Taylor SS. D-AKAP2, a novel protein kinase A anchoring protein with a putative RGS domain. Proc Natl Acad Sci U S A. 1997 Oct 14;94(21):11184-9.
164. Neubig RR. Regulators of G protein signaling (RGS proteins): novel central nervous system drug targets. J Pept Res. 2002 Dec;60(6):312-6.

165. Oliveira-Dos-Santos AJ, Matsumoto G, Snow BE, Bai D, Houston FP, Whishaw IQ, et al. Regulation of T cell activation, anxiety, and male aggression by RGS2. Proc Natl Acad Sci U S A. 2000 Oct 24;97(22):12272-7.
166. Leygraf A, Hohoff C, Freitag C, Willis-Owen SA, Krakowitzky P, Fritze J, et al. Rgs 2 gene polymorphisms as modulators of anxiety in humans? J Neural Transm. 2006 Dec;113(12):1921-5.
167. Smoller JW, Tsuang MT. Panic and phobic anxiety: defining phenotypes for genetic studies. Am J Psychiatry. 1998 Sep;155(9):1152-62.
168. Kendler KS, Neale MC, Kessler RC, Heath AC, Eaves LJ. Panic disorder in women: a population-based twin study. Psychol Med. 1993 May;23(2):397-406.
169. Maier W, Minges J, Lichtermann D. The familial relationship between panic disorder and unipolar depression. J Psychiatr Res. 1995 Sep-Oct;29(5):375-88.
170. Cloninger CR. A unified biosocial theory of personality and its role in the development of anxiety states. Psychiatr Dev. 1986 Autumn;4(3):167-226.
171. Cowley DS, Roy-Byrne PP, Greenblatt DJ, Hommer DW. Personality and benzodiazepine sensitivity in anxious patients and control subjects. Psychiatry Res. 1993 May;47(2):151-62.
172. Mulder RT, Joyce PR, Cloninger CR. Temperament and early environment influence comorbidity and personality disorders in major depression. Compr Psychiatry. 1994 May-Jun;35(3):225-33.
173. Kagan J, Reznick JS, Snidman N, Gibbons J, Johnson MO. Childhood derivatives of inhibition and lack of inhibition to the unfamiliar. Child Dev. 1988 Dec;59(6):1580-9.
174. Rosenbaum JF, Biederman J, Gersten M, Hirshfeld DR, Meminger SR, Herman JB, et al. Behavioral inhibition in children of parents with panic disorder and agoraphobia. A controlled study. Arch Gen Psychiatry. 1988 May;45(5):463-70.
175. Rosenbaum JF, Biederman J, Hirshfeld DR, Bolduc EA, Chaloff J. Behavioral inhibition in children: a possible precursor to panic disorder or social phobia. J Clin Psychiatry. 1991 Nov;52 Suppl:5-9.
176. Biederman J, Rosenbaum JF, Bolduc-Murphy EA, Faraone SV, Chaloff J, Hirshfeld DR, et al. A 3-year follow-up of children with and without behavioral inhibition. J Am Acad Child Adolesc Psychiatry. 1993 Jul;32(4):814-21.
177. Kessler H, Roth J, von Wietersheim J, Deighton RM, Traue HC. Emotion recognition patterns in patients with panic disorder. Depress Anxiety. 2007;24(3):223-6.
178. Good CD, Lawrence K, Thomas NS, Price CJ, Ashburner J, Friston KJ, et al. Dosage-sensitive X-linked locus influences the development of amygdala and orbitofrontal cortex, and fear recognition in humans. Brain. 2003 Nov;126(Pt 11):2431-46.
179. Adolphs R, Spezio M. Role of the amygdala in processing visual social stimuli. Prog Brain Res. 2006;156:363-78.
180. Weiss LA, Purcell S, Waggoner S, Lawrence K, Spektor D, Daly MJ, et al. Identification of EFHC2 as a quantitative trait locus for fear recognition in Turner syndrome. Hum Mol Genet. 2007 Jan 1;16(1):107-13.
181. Mann JJ, Oquendo M, Underwood MD, Arango V. The neurobiology of suicide risk: a review for the clinician. J Clin Psychiatry. 1999;60 Suppl 2:7-11; discussion 8-20, 113-6.

182. Mandelli L, Serretti A, Marino E, Pirovano A, Calati R, Colombo C. Interaction between serotonin transporter gene, catechol-O-methyltransferase gene and stressful life events in mood disorders. Int J Neuropsychopharmacol. 2007 Aug;10(4):437-47.
183. Eley TC, Sugden K, Corsico A, Gregory AM, Sham P, McGuffin P, et al. Gene-environment interaction analysis of serotonin system markers with adolescent depression. Mol Psychiatry. 2004 Oct;9(10):908-15.
184. Grabe HJ, Lange M, Wolff B, Volzke H, Lucht M, Freyberger HJ, et al. Mental and physical distress is modulated by a polymorphism in the 5-HT transporter gene interacting with social stressors and chronic disease burden. Mol Psychiatry. 2005 Feb;10(2):220-4.
185. Jacobs N, Kenis G, Peeters F, Derom C, Vlietinck R, van Os J. Stress-related negative affectivity and genetically altered serotonin transporter function: evidence of synergism in shaping risk of depression. Arch Gen Psychiatry. 2006 Sep;63(9):989-96.
186. Kaufman J, Yang BZ, Douglas-Palumberi H, Houshyar S, Lipschitz D, Krystal JH, et al. Social supports and serotonin transporter gene moderate depression in maltreated children. Proc Natl Acad Sci U S A. 2004 Dec 7;101(49):17316-21.
187. Kendler KS, Kuhn JW, Vittum J, Prescott CA, Riley B. The interaction of stressful life events and a serotonin transporter polymorphism in the prediction of episodes of major depression: a replication. Arch Gen Psychiatry. 2005 May;62(5):529-35.
188. Wilhelm K, Mitchell PB, Niven H, Finch A, Wedgwood L, Scimone A, et al. Life events, first depression onset and the serotonin transporter gene. Br J Psychiatry. 2006 Mar;188:210-5.
189. Zalsman G, Huang YY, Oquendo MA, Burke AK, Hu XZ, Brent DA, et al. Association of a triallelic serotonin transporter gene promoter region (5-HTTLPR) polymorphism with stressful life events and severity of depression. Am J Psychiatry. 2006 Sep;163(9):1588-93.
190. Gillespie NA, Whitfield JB, Williams B, Heath AC, Martin NG. The relationship between stressful life events, the serotonin transporter (5-HTTLPR) genotype and major depression. Psychol Med. 2005 Jan;35(1):101-11.
191. Surtees PG, Wainwright NW, Willis-Owen SA, Luben R, Day NE, Flint J. Social adversity, the serotonin transporter (5-HTTLPR) polymorphism and major depressive disorder. Biol Psychiatry. 2006 Feb 1;59(3):224-9.
192. Kaufman J, Yang BZ, Douglas-Palumberi H, Grasso D, Lipschitz D, Houshyar S, et al. Brain-derived neurotrophic factor-5-HTTLPR gene interactions and environmental modifiers of depression in children. Biol Psychiatry. 2006 Apr 15;59(8):673-80.
193. Stein MB, Schork NJ, Gelernter J. Gene-by-environment (serotonin transporter and childhood maltreatment) interaction for anxiety sensitivity, an intermediate phenotype for anxiety disorders. Neuropsychopharmacology. 2008 Jan;33(2):312-9.
194. Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, et al. Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. Nature. 2002 Mar 28;416(6879):396-400.
195. Bethea CL, Pau FK, Fox S, Hess DL, Berga SL, Cameron JL. Sensitivity to stress-induced reproductive dysfunction linked to activity of the serotonin system. Fertil Steril. 2005 Jan;83(1):148-55.

196. Jokela M, Keltikangas-Jarvinen L, Kivimaki M, Puttonen S, Elovainio M, Rontu R, et al. Serotonin receptor 2A gene and the influence of childhood maternal nurturance on adulthood depressive symptoms. Arch Gen Psychiatry. 2007 Mar;64(3):356-60.
197. Sullivan PF. Spurious genetic associations. Biol Psychiatry. 2007 May 15;61(10):1121-6.
198. Zammit S, Owen MJ. Stressful life events, 5-HTT genotype and risk of depression. Br J Psychiatry. 2006 Mar;188:199-201.
199. Sjoberg RL, Nilsson KW, Nordquist N, Ohrvik J, Leppert J, Lindstrom L, et al. Development of depression: sex and the interaction between environment and a promoter polymorphism of the serotonin transporter gene. Int J Neuropsychopharmacol. 2006 Aug;9(4):443-9.
200. Moffitt TE, Caspi A, Rutter M. Strategy for investigating interactions between measured genes and measured environments. Arch Gen Psychiatry. 2005 May;62(5):473-81.
201. Gauderman WJ. Sample size requirements for association studies of gene-gene interaction. Am J Epidemiol. 2002 Mar 1;155(5):478-84.
202. Freitag CM, Domschke K, Rothe C, Lee YJ, Hohoff C, Gutknecht L, et al. Interaction of serotonergic and noradrenergic gene variants in panic disorder. Psychiatr Genet. 2006 Apr;16(2):59-65.
203. Neumeister A, Bain E, Nugent AC, Carson RE, Bonne O, Luckenbaugh DA, et al. Reduced serotonin type 1A receptor binding in panic disorder. J Neurosci. 2004 Jan 21;24(3):589-91.
204. Szabo ST, Blier P. Serotonin (1A) receptor ligands act on norepinephrine neuron firing through excitatory amino acid and GABA(A) receptors: a microiontophoretic study in the rat locus coeruleus. Synapse. 2001 Dec 15;42(4):203-12.
205. Baraban JM, Aghajanian GK. Suppression of serotonergic neuronal firing by alpha-adrenoceptor antagonists: evidence against GABA mediation. Eur J Pharmacol. 1980 Sep 5;66(4):287-94.
206. Lesch KP. Variation of serotonergic gene expression: neurodevelopment and the complexity of response to psychopharmacologic drugs. Eur Neuropsychopharmacol. 2001 Dec;11(6):457-74.
207. Santarelli L, Saxe MD. Substance P antagonists: meet the new drugs, same as the old drugs? Insights from transgenic animal models. CNS Spectr. 2003 Aug;8(8):589-96.
208. Serretti A, Artioli P, Lorenzi C, Pirovano A, Tubazio V, Zanardi R. The C(-1019)G polymorphism of the 5-HT1A gene promoter and antidepressant response in mood disorders: preliminary findings. Int J Neuropsychopharmacol. 2004 Dec;7(4):453-60.
209. Lemonde S, Du L, Bakish D, Hrdina P, Albert PR. Association of the C(-1019)G 5-HT1A functional promoter polymorphism with antidepressant response. Int J Neuropsychopharmacol. 2004 Dec;7(4):501-6.
210. Pollock BG, Ferrell RE, Mulsant BH, Mazumdar S, Miller M, Sweet RA, et al. Allelic variation in the serotonin transporter promoter affects onset of paroxetine treatment response in late-life depression. Neuropsychopharmacology. 2000 Nov;23(5):587-90.
211. Weizman A, Weizman R. Serotonin transporter polymorphism and response to SSRIs in major depression and relevance to anxiety disorders and substance abuse. Pharmacogenomics. 2000 Aug;1(3):335-41.

212. Perna G, Favaron E, Di Bela D, Bussi R, Bellodi L. Antipanic efficacy of paroxetine and polymorphism within the Promoter of the Serotonin Transporter Gene. Neuropsychopharmacology. 2005;30(12):2230-5.
213. Hu XZ, Lipsky RH, Zhu G, Akhtar LA, Taubman J, Greenberg BD, et al. Serotonin transporter promoter gain-of-function genotypes are linked to obsessive-compulsive disorder. Am J Hum Genet. 2006 May;78(5):815-26.
214. Sen S, Burmeister M, Ghosh D. Meta-analysis of the association between a serotonin transporter promoter polymorphism (5-HTTLPR) and anxiety-related personality traits. Am J Med Genet. 2004 May 15;127B(1):85-9.
215. Linden DE. How psychotherapy changes the brain--the contribution of functional neuroimaging. Mol Psychiatry. 2006 Jun;11(6):528-38.
216. Brody AL, Saxena S, Stoessel P, Gillies LA, Fairbanks LA, Alborzian S, et al. Regional brain metabolic changes in patients with major depression treated with either paroxetine or interpersonal therapy: preliminary findings. Arch Gen Psychiatry. 2001 Jul;58(7):631-40.
217. Martin SD, Martin E, Rai SS, Richardson MA, Royall R. Brain blood flow changes in depressed patients treated with interpersonal psychotherapy or venlafaxine hydrochloride: preliminary findings. Arch Gen Psychiatry. 2001 Jul;58(7):641-8.
218. Wittchen HU, Kessler RC, Pfister H, Lieb M. Why do people with anxiety disorders become depressed? A prospective-longitudinal community study. Acta Psychiatr Scand Suppl. 2000(406):14-23.
219. Munafo MR. Candidate gene studies in the 21st century: meta-analysis, mediation, moderation. Genes Brain Behav. 2006;5 Suppl 1:3-8.

13. Artigos

Artigo 1 - ***Lack of association between the Serotonin Transporter Promoter Polymorphism (5-HTTLPR) and Panic Disorder: a systematic review and meta-analysis***

Artigo 2 ***- Panic disorder and serotoninergic genes (5-HTTLPR, HTR1A and HTR2A): association and interaction with childhood trauma and parental bonding***

Artigo 3 - ***Association between EFHC2 with Panic Disorder, Harm Avoidance and Behavioral Inhibition***

Artigo 4 - ***CREB1 as a predictor of long term response to cognitive-behavior therapy for pharmacotherapy-resistant panic disorder patients***

Artigo 1 – *Lack of association between the Serotonin Transporter Promoter Polymorphism (5-HTTLPR) and Panic Disorder: a systematic review and meta-analysis*

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Lack of association between the Serotonin Transporter Promoter Polymorphism (5-HTTLPR) and Panic Disorder: a systematic review and meta-analysis

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Abstract

Background: The aim of this study is to assess the association between the Serotonin Transporter Promoter Polymorphism (5-HTTLPR) and Panic Disorder (PD).

Methods: This is a systematic review and meta-analysis of case-control studies with unrelated individuals of any ethnic origin examining the role of the 5-HTTLPR in PD according to standard diagnostic criteria (DSM or ICD). Articles published in any language between January 1996 and April 2007 were eligible. The electronic databases searched included PubMed, PsychInfo, Lilacs and ISI. Two separate analyses were performed: an analysis by alleles and a stratified analysis separating studies by the quality of control groups. Asymptotic DerSimonian and Laird's Q test were used to assess heterogeneity. Results of individual studies were combined using the fixed effect model with respective 95% confidence intervals.

Results: Nineteen potential articles were identified, and 10 studies were included in this meta-analysis. No statistically significant association between 5-HTTLPR and PD was found, OR = 0.91 (CI95% 0.80 to 1.03, p = 0.14). Three sub-analyses divided by ethnicity, control group quality and Agoraphobia comorbidity also failed to find any significant association. No evidence of heterogeneity was found between studies in the analyses.

Conclusion: Results from this systematic review do not provide evidence to support an association between 5-HTTLPR and PD. However, more studies are needed in different ethnic populations in order to evaluate a possible minor effect.

Background

Studies in families have shown that Panic Disorder (PD) has a familiar pattern: its prevalence is higher in first degree family members than in control groups [1]. Studies involving siblings show that PD concordance is higher in monozygotic than dizygotic twins [2]. These findings pro-

pose that genetic factors contribute to the pathogenesis of PD with an estimated heritability of 30–40% [3], whereas a recent meta-analysis suggests a higher heritability of 48% [4].

Studies on candidate genes for association have been selected on the basis of the molecular therapeutic drugs and panic-provoking agents [5]. For instance, the response shown by panic patients when treated with Serotonin Selective Reuptake Inhibitors (SSRIs) and the worsening when using a serotoninergic agonist suggest a possible serotoninergic dysfunction in this disorder [2,6].

The serotoninergic transporter gene (5-HTT) is located in chromosome 17q11.1-q12 [7] and it codes for a membrane protein that reuptakes serotonin from the synaptic cleft. A size repetition polymorphism has been related to functionality of the serotoninergic transporter protein. The polymorphism is a 44 bp insertion or deletion on the promoter gene region (5 – HTTLPR) resulting in two alleles (l-*long* and s-*short*). The l allele transcription is two or three times more efficient than the s allele [8,9]. The s allele is less active, therefore, resulting in lower serotonin reuptake and, consequently, in increased serotonin in the synaptic cleft [10].

Previous studies found a significant association between s allele and anxiety traits in healthy volunteers [11]. Regarding PD, studies have systematically failed to find any association between this disorder and 5-HTTLPR. This lack of association could be related to the small sample size of studies. However, a recent study has raised the possibility that the l allele could be involved in panic disorder [12].

Although the controversies between 5-HTTLPR in PD may be related to methodological differences between studies, such as ethnicity, another limitation is lack of statistical power [13]. According to Hirschhorn et al. [14], out of 166 studies on gene-disease associations, only six replicated previous findings. Possible causes for this inconsistency include studies with small sample sizes [15,16], as the most realistic genetic association between a polymorphic locus and a disease has been claimed to yield an odds ratio between 1.1 and 1.5 [17]. Thus at least 1000 subjects should be required to detect this association, depending on the prevalence of polymorphism. However, studies typically report sample sizes from 100 to 300 and rarely above 1000 subjects [16,18], justifying the use of meta-analysis to increase power.

The aim of this study is to attempt to answer whether there is an association between the 5-HTTLPR and PD. As the s allele of 5-HTTLPR is significantly involved in anxiety traits, the assumption is that this polymorphism should be involved in PD.

Methods

The search

- Electronic databases: studies were identified through PubMed (which encompasses Medline, Premedline, and HelthSTAR), PsychINFO, Lilacs and ISI. The PubMed search was run using the Mesh terms: ("Serotonin Plasma Membrane Transport Proteins" [MeSH] OR "5-HTTLPR" OR "5-HTT" OR "SLC6A4") AND "Panic Disorder" [MeSH]. In PsychINFO, Lilacs and ISI the following words were used: "Panic" AND ("serotonin transporter" OR "serotonergic transporter" OR "5-HTT" OR "5-HTTLPR" OR "SLC6A4"). This search strategy was run in June and rerun in April 2007, and included only human studies, with no language restrictions, and a time scope from January 1996 to April 2007, i.e. since the 5-HTTLPR was described by Heils [9] in 1996.

- Reference cross-checking: the list of references of included studies was searched looking for additional studies.

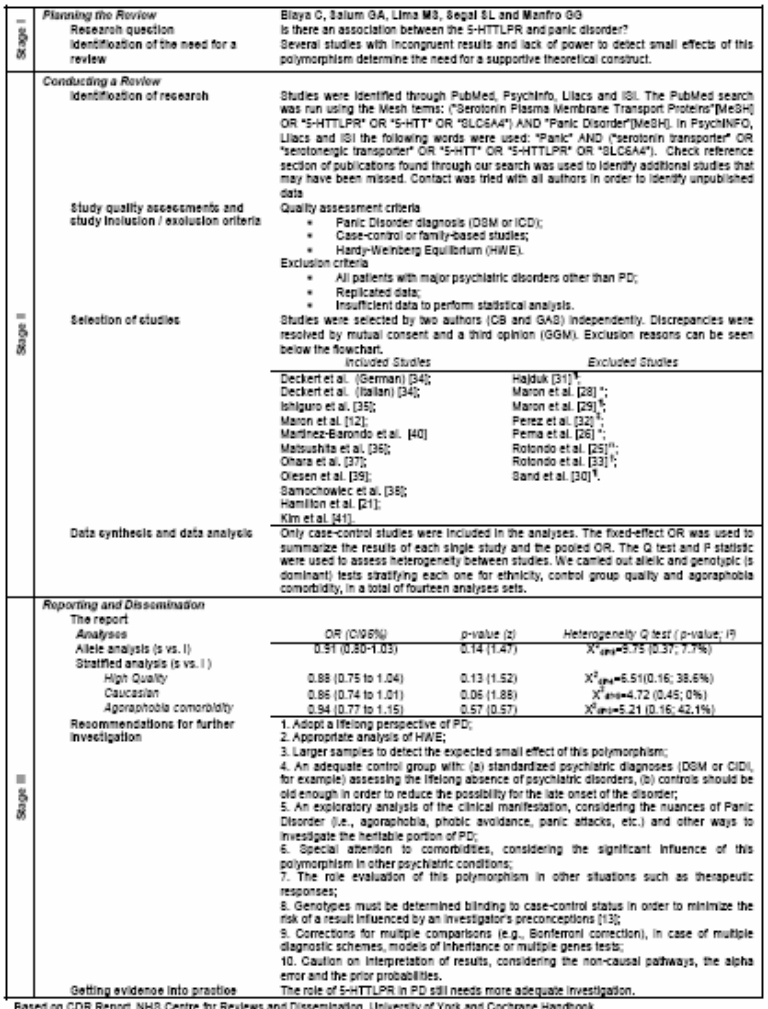
Contact with authors: efforts were made to contact all research groups of studies included in the analysis to identify unpublished data. Three authors replied and no additional study was identified.

Inclusion/Exclusion criteria

Studies reporting the 5-HTTLPR in PD patients of any ethnic origin were evaluated by the authors. The inclusion criteria for this systematic review were: (1) type of studies: case-control and family-based studies; (2) type of participants: diagnoses of PD according to standard diagnostic criteria (DSM or ICD); (3) Hardy-Weinberg Equilibrium (HWE). Testing for HWE is commonly used for quality control of large-scale genotyping and is one of the few ways to identify systematic genotyping errors in unrelated individuals [19], and its assumption is required for the allele analysis [20].

Exclusion criteria included (1) studies in which the main disorders were other than PD; (2) replicated data (a part of sample used for more than one publication); (3) insufficient data to perform statistical analysis (unable even after contacting authors). Regarding replicated data, the included study was selected based on the sample size and the availability of information studied in this meta-analysis. Family studies were not included in the analyses because there is only one study published [21], so no analysis could be performed.

This meta-analysis methodology was performed according to MOOSE (*Meta-analysis of Observational Studies in Epidemiology*) group guidelines [22] and stages of this methodology are presented in a flowchart here in (Figure 1).



Stage I	*Planning the Review*	Biaya C, Salum GA, Lima MS, Segal SL and Manfro GG
	Research question	Is there an association between the 5-HTTLPR and panic disorder?
	Identification of the need for a review	Several studies with incongruent results and lack of power to detect small effects of this polymorphism determine the need for a supportive theoretical construct.
Stage II	*Conducting a Review*	
	Identification of research	Studies were identified through PubMed, PsychInfo, Lilacs and ISI. The PubMed search was run using the Mesh terms: ("Serotonin Plasma Membrane Transport Proteins"[MeSH] OR "5-HTTLPR" OR "5-HTT" OR "SLC6A4") AND ("Panic Disorder"[MeSH]. In PsychINFO, Lilacs and ISI the following words were used: "Panic" AND ("serotonin transporter" OR "serotonergic transporter" OR "5-HTT" OR "5-HTTLPR" OR "SLC6A4"). Check reference section of publications found through our search was used to identify additional studies that may have been missed. Contact was tried with all authors in order to identify unpublished data
	Study quality assessments and study inclusion / exclusion criteria	Quality assessment criteria • Panic Disorder diagnosis (DSM or ICD); • Case-control or family-based studies; • Hardy-Weinberg Equilibrium (HWE). Exclusion criteria • All patients with major psychiatric disorders other than PD; • Replicated data; • Insufficient data to perform statistical analysis.
	Selection of studies	Studies were selected by two authors (CB and GAS) independently. Discrepancies were resolved by mutual consent and a third opinion (GGM). Exclusion reasons can be seen below the flowchart.
		Included Studies: Deckert et al. (German) [34]; Deckert et al. (Italian) [34]; Ishiguro et al. [35]; Maron et al. [12]; Martínez-Barondo et al. [40]; Matsushita et al. [36]; Ohara et al. [37]; Olesen et al. [39]; Samochowiec et al. [38]; Hamilton et al. [21]; Kim et al. [41]. *Excluded Studies*: Hajduk [31] ¶; Maron et al. [28] *; Maron et al. [29] ¶; Perez et al. [32] *; Perna et al. [26] *; Rotondo et al. [25] ||; Rotondo et al. [33] ||; Sand et al. [30] ¶.
	Data synthesis and data analysis	Only case-control studies were included in the analyses. The fixed-effect OR was used to summarize the results of each single study and the pooled OR. The Q test and I² statistic were used to assess heterogeneity between studies. We carried out allelic and genotypic (s dominant) tests stratifying each one for ethnicity, control group quality and agoraphobia comorbidity, in a total of fourteen analyses sets.
Stage III	*Reporting and Dissemination*	
	The report	
	Analyses	*OR (CI95%)* / *p-value (z)* / *Heterogeneity Q test (p-value; I²)*
	Allele analysis (s vs. l)	0.91 (0.80-1.03) / 0.14 (1.47) / $X^2_{df=8}$=8.75 (0.37; 7.7%)
	Stratified analysis (s vs. l)	
	High Quality	0.88 (0.75 to 1.04) / 0.13 (1.52) / $X^2_{df=4}$=6.51(0.16; 38.6%)
	Caucasian	0.86 (0.74 to 1.01) / 0.06 (1.88) / $X^2_{df=5}$=4.72 (0.45; 0%)
	Agoraphobia comorbidity	0.94 (0.77 to 1.15) / 0.57 (0.57) / $X^2_{df=3}$=5.21 (0.16; 42.1%)
	Recommendations for further investigation	1. Adopt a lifelong perspective of PD; 2. Appropriate analysis of HWE; 3. Larger samples to detect the expected small effect of this polymorphism; 4. An adequate control group with: (a) standardized psychiatric diagnoses (DSM or CIDI, for example) assessing the lifelong absence of psychiatric disorders, (b) controls should be old enough in order to reduce the possibility for the late onset of the disorder; 5. An exploratory analysis of the clinical manifestation, considering the nuances of Panic Disorder (i.e., agoraphobia, phobic avoidance, panic attacks, etc.) and other ways to investigate the heritable portion of PD; 6. Special attention to comorbidities, considering the significant influence of this polymorphism in other psychiatric conditions; 7. The role evaluation of this polymorphism in other situations such as therapeutic responses; 8. Genotypes must be determined blinding to case-control status in order to minimize the risk of a result influenced by an investigator's preconceptions [13]; 9. Corrections for multiple comparisons (e.g., Bonferroni correction), in case of multiple diagnostic schemes, models of inheritance or multiple genes tests; 10. Caution on interpretation of results, considering the non-causal pathways, the alpha error and the prior probabilities.
	Getting evidence into practice	The role of 5-HTTLPR in PD still needs more adequate investigation.

Based on CDR Report, NHS Centre for Reviews and Dissemination, University of York and Cochrane Handbook
Exclusion reasons: *Without control group; ¶Replicated data; || All patients with major Psychiatric comorbidity other than PD; ‡ Hardy-Weinberg departure; †Insufficient data;

Figure 1
Flowchart – Stages of Systematic Review with Meta-analysis.

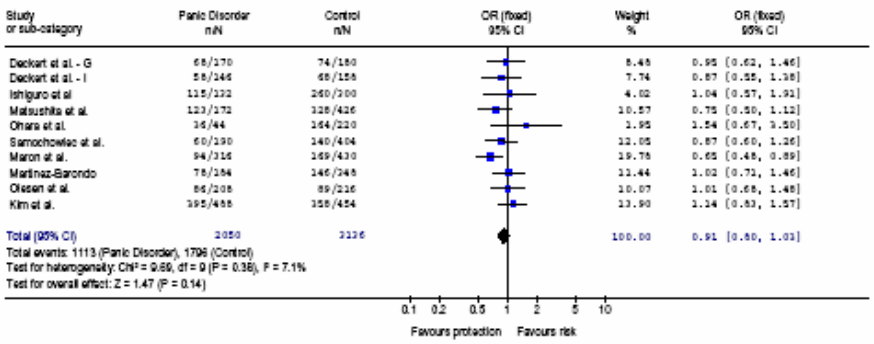


Figure 2
Forest plot with odds ratio of each study and pooled odds ratio for the association between the s allele and risk of Panic Disorder.

Data extraction

Data was independently extracted by two investigators (CB and GAS) using a standardized data extraction form. Discrepancies were resolved by discussion and if consensus was not achieved the decision was made by another reviewer (GGM). Data extracted included number of PD patients and controls for each of the three genotype groups (ll, ls, ss) in each eligible study. Only data regarding 5-HTTLPR and panic disorder were extracted regardless of other polymorphisms, disorders or outcomes reported in the studies. The male/female ratio, mean age, predominant ethnicity of the sample and psychiatric comorbidity were also extracted. Genotype frequencies were used to calculate whether they deviate significantly from HWE. In cases where data was not available in the published reports, authors were contacted directly. The information was classified as not reported when authors did not return to at least three contact trials or did not have this data available.

Statistical analysis

First, an analysis by alleles was performed because this analysis increases the power to detect differences and is the most used analysis in the current literature. Secondly, we analyze the most used model of inheritance (s dominant) to express directly the ll genotype risk [16]. In order to investigate interaction or confusion between studies, a stratified analysis was run after separating studies by ethnicity, quality of control groups and comorbidity with Agoraphobia, as recommended by Munafo and Flint [15]. It was considered as high quality control groups those with appropriate assessment diagnoses. As we performed fourteen different analyses sets of the same genetic data we also present correct p-values according to Finner's modification of the Bonferroni's procedure for multiple comparisons. Despite being an important analysis, we could not perform a stratified analysis for comorbidity with mood disorders and another anxiety disorders because this data was poorly described in studies included in this meta-analysis. We choose to perform an exploratory analysis because the heritable portion of PD that could be related to this polymorphism is not yet defined.

Asymptotic DerSimonian and Laird's Q test was used to assess heterogeneity. Because tests of heterogeneity may be underpowered to detect heterogeneity between studies when the number of studies is small [23], we also explore heterogeneity quantitatively using the I^2 statistic [24].

The results of individual studies, as well as the pooled odds ratio (OR), were synthesized by the fixed effect model with 95% confidence intervals. The significance of the pooled OR was determined by the z test. Publication bias was assessed with a funnel plot (allele analyses). The analyses were done with Review Manager 4.2.8, software developed by the Cochrane Collaboration. The Power Calculator for Genetic Studies software was used in order to estimate the minimum OR that could be detected in this meta-analysis.

Results

In total, 19 potential publications were identified, and 10 studies were included in this meta-analysis. No analysis was performed with family-based studies because only one was found in our search [21]. One study was excluded because all patients had Bipolar Disorder [25], three studies were excluded because there were no control groups [26-28], three studies were excluded because data were replicated [29-31], one study was excluded due to significant deviation of Hardy-Weinberg equilibrium [32] and one study [33] was excluded due to insufficient data. As one study [34] had two different sub samples clinically assessed with different methodologies, it was considered as two separate studies (Deckert German and Deckert Italian), resulting in ten studies included in our analysis (figure 1). The funnel plot suggests a couple of missing studies with OR higher than 1, but the small number of included trials does not allow drawing conclusions about publication bias.

Across all 10 case-control studies investigating 5-HTTLPR and panic disorder [12,34-41], a total of 1,025 patients and 1,568 controls were included, totaling 2,050 alleles among patients and 3,136 alleles between controls. Table 1 depicts data extracted from the included studies. Regarding the allele and stratified analyses, no evidence of heterogeneity was found. The statistics of heterogeneity Q test and I^2 are shown in the figure 2. We found very similar results between the OR calculated by the random-effects model and Peto OR.

In the first analysis, including 5,186 alleles, we compared the 5-HTTLPR allelic distribution between patients with PD and controls of all ten studies included. The pooled OR = 0.91 was not significant (CI95% 0.80 to 1.03; z = 1.47; p = 0.14). The results of this analysis are shown in the forest plot (figure 2). Regarding family studies, we only identified one in this search [21]. The study had evaluated 340 individuals in 45 families with at least three affected people. No linkage between the 5-HTTLPR and PD was observed (p = 0.40).

As the included studies have subjects with different ethnicity and control groups with different quality, we performed stratified analyses. Only five studies – Maron et al. [12], Deckert Italian [34], Samochowiec et al. [38], Olesen et al. [39] and Kim et al. [41] – were classified as having high quality controls. Results showed no significant association between the s allele and PD in both analyses (high and low quality). Regarding the ethnic stratified analysis, no significant association was found in Caucasian or Asian separated analyses. Some studies evaluated the comorbidity with Agoraphobia in their exploratory analysis [12,35,38,41] and no significant association was found in this stratified analysis. Table 2 depicts all these stratified analyses for the allelic analysis and genotype analysis.

We estimated the minimum OR that could be detected in this meta-analysis by allelic analysis. Considering a power of 90%, a PD prevalence of 1.8% in Caucasoid [42], a mean frequency of s allele of 0.45 in a joint analysis with the Power Calculator for genetic studies, the minimum OR that we are capable to found is 1.14.

Discussion

This is the first systematic review between the 5-HTTLPR and PD and no statistically significant association was found. In the overall analysis, there was no evidence for heterogeneity among the studies. This indicates no greater variation among the studies than could be expected by chance and provides the validity to the meta-analysis by suggesting that studies included in this analysis are comparable. However, no association was found between this polymorphism and PD even in all stratified analyses.

The main problem in genetic studies and psychiatric disorders is probably the lack of a phenotype definition. Clinical diagnoses according to DSM-IV may be heterogeneous constructs that combine elements with distinct genetic influences [43]. Additionally, psychiatric disorders usually overlap, and comorbidity might be a bias that impairs some associations. Therefore, some authors are interested in defining "intermediate" phenotypes that might have more direct expression of genes influencing a complex disorder and may have a simpler genetic architecture [43].

Also, a recently detected genetic variation (16A allele) [44] in the long allele of 5-HTTLPR has been linked to a lower expression of the serotonin transporter [45,46]. No study has evaluated this polymorphism neither in panic disorder nor in anxiety traits. Hence, studies should combine the evaluation of different polymorphisms that influence the protein expression.

Limitations of this study

The publication bias could not be adequately assessed in our meta-analysis by the funnel plot due to the small number of studies available. However, we believe that our search strategy was comprehensive enough. Moreover, additional sources of trials like contacting authors of included trials yield no additional study.

The sample size found in our meta-analysis is below that which is required to identify associations with minor effects [17]. In the sub-group analysis the sample size became even smaller. Thus, further investigation using larger sample sizes, in which the control group is properly diagnosed and ethnicity is evaluated, is needed. Such

Table 1: Included Case-control Studies of 5-HTTLPR and Panic Disorder

							Genotype Frequency/ Total (%)			Alelle frequency/ Total (%)		HWE reported (df = 1)		
Studies	Groups (n)	Male (%)	Diagnosis	Comorbidity	Mean Age	Origin	ss	sl	ll	s	l		X^2	p
Ishiguro et al. [35]	*Panic* (66)	30 (45.5)	DSM-IV	NR	40	Japanese	51/66 (77.3)	13/66 (19.7)	2/66 (3)	115/132 (87.1)	17/132 (12.9)	YES	0.986	0.321
	Control (150)	70 (46.7)	Not reported		41		114/150 (76)	32/150 (21.3)	4/150 (2.7)	260/300 (86.7)	40/300 (13.3)	YES	0.887	0.346
Matsushita et al. [36]	*Panic* (86)	54 (62.7)	DSM-III-R	NR	37.0	Japanese	44/86 (51.2)	35/86 (40.7)	7/86 (8.1)	123/172 (71.5)	49/172 (28.5)	YES	<0.001	0.992
	Control (213)	96 (45.1)	Not reported		37.7		125/213 (58.7)	78/213 (36.6)	10/213 (4.7)	328/426 (77)	98/426 (23)	YES	0.242	0.623
Deckert et al. German [34]	*Panic* (85)	NR	DSM-III-R and CIDI	Yes (Mood); Anxiety NR	NR	German	12/66 (14.1)	44/66 (51.8)	29/66 (34.1)	68/170 (40)	102/170 (60)	NO	0.523	0.470
	Control (90)	NR	None		NR		16/90 (17.8)	42/90 (46.7)	32/90 (35.6)	74/180 (41.1)	106/180 (58.9)	YES	0.118	0.731
Deckert et al. Italian [34]	*Panic* (73)	NR	DSM-III-R and SADS-LA	Mood absent; Anxiety NR	NR	Italian	13/73 (17.8)	32/73 (43.8)	28/73 (38.4)	58/146 (39.7)	88/146 (60.3)	NO	0.523	0.470
	Control (79)	NR	DSM-III-R and DIS†		NR		12/79 (15.2)	44/79 (55.7)	23/79 (29.1)	68/158 (43)	90/158 (57)	YES	1.460	0.227
Ohara et al. [37]	*Panic* (22)	11 (50)	DSM-IV	Yes (Anxiety)	35.1	Japanese	14/22 (63.6)	8/22 (36.4)	0/22 (0)	36/44 (81.8)	8/44 (18.2)	NO	1.086	0.769
	Control (110)	NR	Not reported		NR		62/110 (56.4)	40/110 (36.4)	8/110 (7.3)	164/220 (74.5)	56/220 (25.5)	NO	0.192	0.661
Samochowiec et al. [38]	*Panic* (95)	24 (23.8)	CIDI	Yes (Anxiety)	38.7	Polonaise	10/95 (10.5)	40/95 (42.1)	45/95 (47.4)	60/190 (31.6)	130/190 (68.4)	NO	0.062	0.803
	Control (202)	54 (26.7)	ICD-10		35.9		22/202 (10.9)	96/202 (47.5)	84/202 (41.6)	140/404 (34.7)	264/404 (65.3)	NO	0.492	0.483
Maron et al. [12]	*Panic* (158)	32 (20.2)	DSM-IV MINI	Yes (Anxiety and Mood)	38.0	Estonian	11/158 (7)	72/158 (45.6)	75/158 (47.5)	94/316 (29.7)	222/316 (70.3)	YES	1.288	0.256
	Control (215)	56 (26)	MINI†		39.8		34/215 (15.8)	101/215 (47)	80/215 (37.2)	169/430 (39.3)	261/430 (60.7)	YES	0.051	0.822
Olesen et al. [39]	*Panic* (104)	28 (26.9)	DSM-IV	Yes (Anxiety)	NR	Danish	15/104 (14.4)	56/104 (53.8)	33/104 (31.7)	86/208 (41.3)	122/208 (58.7)	NO	1.263	0.261
	Control (108)	30 (27.7)	Clinical Interview		NR		18/108 (16.7)	53/108 (49.1)	37/108 (34.3)	89/216 (41.2)	127/216 (58.8)	NO	0.018	0.894

Table 1: Included Case-control Studies of 5-HTTLPR and Panic Disorder *(Continued)*

Martin et-Barondo et al. [40]	*Panic* (92)	28 (30.4)	DSM-IV	NR	35.8	Spanish	18/92 (19.6)	42/92 (45.7)	32/92 (34.8)	78/184 (42.4)	106/184 (57.6)	NO	0.392	0.531
	Control (174)	67 (38.5)	Not reported		38.4		36/174 (20.7)	74/174 (42.5)	64/174 (36.8)	146/348 (42)	202/348 (58)	NO	2.798	0.094
Kim et al. [41]	*Panic* (244)	143 (58.6)	DSM-IV	Yes (Mood)	36.1	Korean	159/244 (65.2)	77/244 (31.6)	8/244 (3.3)	395/488 (80.9)	93/488 (19.1)	YES	0.1278	0.721
	Control (227)	102 (44.9)	Clinical Interview		33.1		141/227 (62.1)	76/227 (33.5)	10/227 (4.4)	358/454 (78.9)	96/454 (21.1)	YES	0.0035	0.953

Note: NR, Not reported; HWE, Hardy Weinberg Equilibrium; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders IV; ICD-10, International Classification of Diseases; CIDI, Composite International Diagnostic Interview; MINI, MINI International Neuropsychiatric Interview. †Controls with first degree relatives without psychiatric disorders.

studies should focus on exploratory analyses in order to identify the heritable features that might be related to this polymorphism. Additionally, we could not perform a subgroup analysis divided by gender, because only one study reported this data. Neither did we find any stratified analyses regarding co-morbidity (with alcohol, mood and anxiety disorders), even though associations between the 5-HTTLPR and these psychiatric disorders have been reported [30,47,48].

This meta-analysis was carried out with case-control studies. However, some authors [13] suggest that the results from this design finding a positive association between the genome and a disorder could be false. Additionally, a positive result from a case-control study might be due to population stratification rather than linkage disequilibrium [49] affected by the different disease prevalence and different marker frequencies in the subpopulation thereby producing spurious associations [50,51]. The use of Genomic Control, although with limitations [52], may be an alternative in analyzing data of case-control studies. Unfortunately, we could not perform an additional analysis with family studies because we identified only one study with this design. Family-based studies have a unique design in which population stratification is controlled.

Conclusion

In summary, results from this systematic review do not support the hypothesis of a significant association between 5-HTTLPR and PD. All the sub-analyses performed failed to find an association between PD and this polymorphism. However, more studies are needed in different ethnic populations in order to evaluate a possible minor effect. Finally, the 5-HTTLPR does not seem to play a major role in the genetics of panic disorder and, therefore, other polymorphisms should be investigated.

Abbreviations

PD, Panic Disorder

SSRIs, Serotonin Selective Reuptake Inhibitors

5-HTTLPR, Serotonin Transporter Promoter Polymorphism

DSM, Diagnostic and Statistical Manual

ICD, International Classification of Diseases

CIDI, Composite International Diagnostic Interview

MINI, MINI International Neuropsychiatric Interview.

5-HTT, Serotoninergic transporter gene

SLC6A4, Solute Carrier Family 6 (Neurotransmitter transporter serotonin), member 4

HWE, Hardy-Weinberg Equilibrium

MOOSE, Meta-analysis of Observational Studies in Epidemiology

OR, Odds ratio

CI95%, Confident Interval of 95%

Competing interests

Mauricio S. Lima is medical director of Eli Lilly do Brazil.

Carolina Blaya and Gisele G. Manfro are speakers of Eli Lilly Brazil.

Giovanni A. Salum and Sandra Leistner-Segal have no competing interests.

Table 2: Results of the overall studies and sub-group studies stratified by ethnicity, quality of control group and comorbidity with Agoraphobia

	n[a]	OR (95% CI)	Significance			Heterogeneity		
			Z	p-value	Finner's p	χ^{2c}	p-value	I^2
Allelic analyses (s/l)[b]	**10**	**0.91 (0.80–1.03)**	**1.47**	**0.14**	**0.58**	**9.75**	**0.37**	**7.7%**
Ethnicity								
Caucasians	6	0.86 (0.74 – 1.01)	1.88	0.06	0.58	4.72	0.45	0%
Asians	4	1.02 (0.81 – 1.27)	0.14	0.89	0.92	3.67	0.30	18.3%
Quality of Control Group								
High quality control group	5	0.88 (0.75 – 1.04)	1.52	0.13	0.58	6.51	0.16	38.6%
Low quality control group	5	0.96 (0.78 – 1.17)	0.43	0.67	0.76	2.87	0.58	0%
Agoraphobia comorbidity								
With Agoraphobia	4	0.94 (0.77 – 1.15)	0.57	0.57	0.69	5.21	0.16	42.4%
Without Agoraphobia	3	0.80 (0.60 – 1.08)	1.48	0.14	0.58	2.30	0.32	12.9%
Genotype (ls+ss) vs. ll	**10**	**0.87 (0.71 – 1.06)**	**1.39**	**0.17**	**0.58**	**7**	**0.64**	**0%**
Ethnicity								
Caucasians	6	0.85 (0.68 – 1.05)	1.48	0.14	0.58	4.36	0.50	0%
Asians	4	1.01 (0.55 – 1.86)	0.03	0.98	0.98	2.56	0.46	0%
Quality of Control Group								
High quality control group	5	0.80 (0.63 – 1.03)	1.76	0.08	0.58	3.72	0.45	0%
Low quality control group	5	1.02 (0.71 – 1.46)	0.11	0.91	0.93	2.30	0.68	0%
Agoraphobia comorbidity								
With Agoraphobia	4	0.80 (0.57 – 1.11)	1.32	0.19	0.58	2.90	0.41	0%
Without Agoraphobia	3	0.85 (0.51 – 1.40)	0.65	0.51	0.67	1.18	0.55	0%

Abbreviations: OR, odds ratio (fixed effect); Q, Cochran's χ^2-based Q statistic test used to assess the heterogeneity; z test used to determine the significance of the overall OR; F, inconsistency; Finner's p-value, adjusted p-value for multiple comparison

[a] The number of studies included are indicated

[b] The first allele is the risk allele

[c] Degrees of freedom = number of studies - 1.

Authors' contributions

CB conceived of the study, extracted the data, participated in the design and drafted the manuscript. GAS extracted the data, participated in the design, drafted the manuscript and performed the statistical analysis. MSL helped in the statistical analysis and helped to draft the manuscript. SLS helped to draft the manuscript. GGM, participated in its design, coordination and drafted the manuscript. All authors read and approved the final manuscript.

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References

1. Crowe RR, Noyes R, Pauls DL, Slymen D: A family study of panic disorder. *Arch Gen Psychiatry* 1983, 40:1065-1069.
2. Inada Y, Yoneda H, Koh J, Sakai J, Himei A, Kinoshita Y, Akabame K, Hiraoka Y, Sakai T: Positive association between panic disorder and polymorphism of the serotonin 2A receptor gene. *Psychiatry Res* 2003, 118:25-31.
3. Kendler KS, Neale MC, Kessler RC, Heath AC, Eaves LJ: Panic disorder in women: a population-based twin study. *Psychol Med* 1993, 23:397-406.
4. Hettema JM, Neale MC, Kendler KS: A review and meta-analysis of the genetic epidemiology of anxiety disorders. *Am J Psychiatry* 2001, 158:1568-1578.
5. Deckert J, Catalano M, Syagailo YV, Bosi M, Okladnova O, Di Bella D, Nothen MM, Maffei P, Franke P, Fritze J, Maier W, Propping P, Beckmann H, Bellodi L, Lesch KP: Excess of high activity monoamine oxidase A gene promoter alleles in female patients with panic disorder. *Hum Mol Genet* 1999, 8:621-624.
6. Versiani M, Cassano G, Perugi G, Benedetti A, Mastalli L, Nardi A, Savino M: Reboxetine, a selective norepinephrine reuptake inhibitor, is an effective and well-tolerated treatment for panic disorder. *J Clin Psychiatry* 2002, 63:31-37.
7. Lesch KP, Balling U, Gross J, Strauss K, Wolozin BL, Murphy DL, Riederer P: Organization of the human serotonin transporter gene. *J Neural Transm Gen Sect* 1994, 95:157-162.
8. Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, Benjamin J, Muller CR, Hamer DH, Murphy DL: Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science* 1996, 274:1527-1531.
9. Heils A, Teufel A, Petri S, Stober G, Riederer P, Bengel D, Lesch KP: Allelic variation of human serotonin transporter gene expression. *J Neurochem* 1996, 66:2621-2624.
10. Brown JS, Joiner TE: The serotonin transporter gene's association with mental disorder: a meta analysis. In *Department of Psychology* Miami, The Florida State University; 2003:33.
11. Sen S, Burmeister M, Ghosh D: Meta-analysis of the association between a serotonin transporter promoter polymorphism (5-HTTLPR) and anxiety-related personality traits. *Am J Med Genet* 2004, 127B:85-89.
12. Maron E, Lang A, Tasa G, Litvaid L, Toru I, Must A, Vasar V, Shlik J: Associations between serotonin-related gene polymorphisms and panic disorder. *Int J Neuropsychopharmacol* 2005, 8:261-266.
13. Sullivan PF, Eaves LJ, Kendler KS, Neale MC: Genetic case-control association studies in neuropsychiatry. *Arch Gen Psychiatry* 2001, 58:1015-1024.
14. Hirschhorn JN, Lohmueller K, Byrne E, Hirschhorn K: A comprehensive review of genetic association studies. *Genet Med* 2002, 4:45-61.
15. Munafo MR, Flint J: Meta-analysis of genetic association studies. *Trends Genet* 2004, 20:439-444.
16. Salanti G, Amountza G, Ntzani EE, Ioannidis JP: Hardy-Weinberg equilibrium in genetic association studies: an empirical evaluation of reporting, deviations, and power. *Eur J Hum Genet* 2005, 13:840-848.
17. Ioannidis JP: Genetic associations: false or true? *Trends Mol Med* 2003, 9:135-138.
18. Ioannidis JP: Molecular bias. *Eur J Epidemiol* 2005, 20:739-745.
19. Wittke-Thompson JK, Pluzhnikov A, Cox NJ: Rational inferences about departures from Hardy-Weinberg equilibrium. *Am J Hum Genet* 2005, 76:967-986.
20. Sasieni PD: From genotypes to genes: doubling the sample size. *Biometrics* 1997, 53:1253-1261.
21. Hamilton SP, Heiman GA, Haghighi F, Mick S, Klein DF, Hodge SE, Weissman MM, Fyer AJ, Knowles JA: Lack of genetic linkage or association between a functional serotonin transporter polymorphism and panic disorder. *Psychiatr Genet* 1999, 9:1-6.
22. Stroup DF, Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, Moher D, Becker BJ, Sipe TA, Thacker SB: Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. *Jama* 2000, 283:2008-2012.
23. Hardy RJ, Thompson SG: Detecting and describing heterogeneity in meta-analysis. *Stat Med* 1998, 17:841-856.
24. Takkouche B, Cadarso-Suarez C, Spiegelman D: Evaluation of old and new tests of heterogeneity in epidemiologic meta-analysis. *Am J Epidemiol* 1999, 150:206-215.
25. Rotondo A, Mazzanti C, Dell'Osso L, Rucci P, Sullivan P, Bouanani S, Gonnelli C, Goldman D, Cassano GB: Catechol o-methyltransferase, serotonin transporter, and tryptophan hydroxylase gene polymorphisms in bipolar disorder patients with and without comorbid panic disorder. *Am J Psychiatry* 2002, 159:23-29.
26. Perna G, Favaron E, Di Bala D, Bussi R, Bellodi L: Antipanic efficacy of paroxetine and polymorphism within the Promoter of the Serotonin Transporter Gene. *Neuropsychopharmacology* 2005.
27. Perna G, di Bella D, Favaron E, Cucchi M, Liperi L, Bellodi L: Lack of relationship between CO2 reactivity and serotonin transporter gene regulatory region polymorphism in panic disorder. *Am J Med Genet B Neuropsychiatr Genet* 2004, 129:41-43.
28. Maron E, Tasa G, Toru I, Lang A, Vasar V, Shlik J: Association between serotonin-related genetic polymorphisms and CCK-4-induced panic attacks with or without 5-hydroxytryptophan pretreatment in healthy volunteers. *World J Biol Psychiatry* 2004, 5:149-154.
29. Maron E, Nikopensius T, Koks S, Altmae S, Heinaste E, Vabrit K, Tammekivi V, Hallast P, Koido K, Kurg A, Matspalu A, Vasar E, Vasar V, Shlik J: Association study of 90 candidate gene polymorphisms in panic disorder. *Psychiatr Genet* 2005, 15:17-24.
30. Sand P, Lesch KP, Catalano M, Bosi M, Syagailo YV, Okladnova O, Di Bella D, Maffei P, Heils A, Friess F, Politi E, Nothen MM, Franke P, Stober G, Fritze J, Maier W, Propping P, Beckmann H, Bellodi L, Riederer P, Deckert J: Polymorphic MAO-A and 5-HT-transporter genes: analysis of interactions in panic disorder. *World J Biol Psychiatry* 2000, 1:147-150.
31. Hajduk A: [A search for psychobiological determinants of anxiety disorders]. *Ann Acad Med Stetin* 2004, 50:65-75.
32. Perez M, Brown JS, Vrshek-Schallhorn S, Johnson F, Joiner TE Jr.: Differentiation of obsessive-compulsive-, panic-, obsessive-compulsive personality-, and non-disordered individuals by variation in the promoter region of the serotonin transporter gene. *J Anxiety Disord* 2005.
33. Rotondo A, Mazzanti C, Michelini S, Gemignani A, Lazza A, Capovani B, Bouanani S, Coli E, Miniati M, Pfanner C, Presta S, Milanfranchi A, Ramacciotti C, Dell'Osso L, Mauri M, Cassano G, Goldman D: Association study of panic, obsessive-compulsive and eating disorders with a functional polymorphism of the serotonin transporter promoter. *European Neuropsychopharmacology* 1997, 7:236.
34. Deckert J, Catalano M, Heils A, Di Bella D, Friess F, Politi E, Franke P, Nothen MM, Maier W, Bellodi L, Lesch KP: Functional promoter polymorphism of the human serotonin transporter: lack of association with panic disorder. *Psychiatr Genet* 1997, 7:45-47.
35. Ishiguro H, Arinami T, Yamada K, Otsuka Y, Toru M, Shibuya H: An association study between a transcriptional polymorphism in the serotonin transporter gene and panic disorder in a Japanese population. *Psychiatry Clin Neurosci* 1997, 51:333-335.

36. Matsushita S, Muramatsu T, Kimura M, Shirakawa O, Mita T, Nakai T, Higuchi S: Serotonin transporter gene regulatory region polymorphism and panic disorder. *Mol Psychiatry* 1997, 2:390-392.
37. Ohara K, Nagai M, Suzuki Y, Ochiai M: Association between anxiety disorders and a functional polymorphism in the serotonin transporter gene. *Psychiatry Res* 1998, 81:277-279.
38. Samochowiec J, Hajduk A, Samochowiec A, Horodnicki J, Stapien G, Grzywacz A, Kucharska-Mazur J: Association studies of MAO-A, COMT, and 5-HTT genes polymorphisms in patients with anxiety disorders of the phobic spectrum. *Psychiatry Res* 2004, 128:21-26.
39. Olesen OF, Bennike B, Hansen ES, Koefoed P, Woldbye DP, Bolwig TG, Mellerup E: The short/long polymorphism in the serotonin transporter gene promoter is not associated with panic disorder in a Scandinavian sample. *Psychiatr Genet* 2005, 15:159.
40. Martinez-Barrondo S, Saiz PA, Morales B, Garcia-Portilla MP, Coto E, Alvarez V, Bobes J: Serotonin gene polymorphisms in patients with panic disorder. *Acta Esp Psiquiatr* 2005, 33:210-215.
41. Kim W, Choi YC, Yoon KS, Cho DY, Pae CU, Woo JM: Tryptophan hydroxylase and serotonin transporter gene polymorphism does not affect the diagnosis, clinical features and treatment outcome of panic disorder in the Korean population. *Prog Neuropsychopharmacol Biol Psychiatry* 2006, 30:1413-1418.
42. Goodwin RD, Faravelli C, Rosi S, Cosci F, Truglia E, de Graaf R, Wittchen HU: The epidemiology of panic disorder and agoraphobia in Europe. *Eur Neuropsychopharmacol* 2005, 15:435-443.
43. Smoller JW, Rosenbaum JF, Biederman J, Kennedy J, Dai D, Racette SR, Laird NM, Kagan J, Snidman N, Hirshfeld-Becker D, Tsuang MT, Sklar PB, Slaugenhaupt SA: Association of a genetic marker at the corticotropin-releasing hormone locus with behavioral inhibition. *Biol Psychiatry* 2003, 54:1376-1381.
44. Ozaki N, Goldman D, Kaye WH, Plotnicov K, Greenberg BD, Lappalainen J, Rudnick G, Murphy DL: Serotonin transporter missense mutation associated with a complex neuropsychiatric phenotype. *Molecular Psychiatry* 2003, 8:933-936.
45. Kilic F, Murphy DL, Rudnick G: A human serotonin transporter mutation causes constitutive activation of transport activity. *Molecular Pharmacology* 2003, 64:440-446.
46. Hu XZ, Lipsky RH, Zhu G, Akhtar LA, Taubman J, Greenberg BD, Xu K, Arnold PD, Richter MA, Kennedy JL, Murphy DL, Goldman D: Serotonin transporter promoter gain-of-function genotypes are linked to obsessive-compulsive disorder. *Am J Hum Genet* 2006, 78:815-826.
47. Feinn R, Nellissery M, Kranzler HR: Meta-analysis of the association of a functional serotonin transporter promoter polymorphism with alcohol dependence. *Am J Med Genet B Neuropsychiatr Genet* 2005, 133:79-84.
48. Lasky-Su JA, Faraone SV, Glatt SJ, Tsuang MT: Meta-analysis of the association between two polymorphisms in the serotonin transporter gene and affective disorders. *Am J Med Genet B Neuropsychiatr Genet* 2005, 133:110-115.
49. Li CC: Population subdivision with respect to multiple alleles. *Ann Hum Genet* 1969, 33:23-29.
50. Shmulewitz D, Zhang J, Greenberg DA: Case-control association studies in mixed populations: correcting using genomic control. *Hum Hered* 2004, 58:145-153.
51. Bacanu SA, Devlin B, Roeder K: The power of genomic control. *Am J Hum Genet* 2000, 66:1933-1944.
52. Devlin B, Roeder K, Wasserman L: Genomic control, a new approach to genetic-based association studies. *Theor Popul Biol* 2001, 60:155-166.

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Artigo 2 – *Panic disorder and serotoninergic genes (5-HTTLPR, HTR1A and HTR2A): association and interaction with childhood trauma and parental bonding*

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Abstract

Background – Panic disorder (PD) has been related to both genetic and environmental risk factors. However, no study has evaluated a gene-environment interaction for this disorder. The aim of this study is to evaluate the association between HTR1A, HTR2A and 5-HTTLPR in PD patients and controls. We also evaluated the interaction between these genes and two environmental factors previously associated with PD: childhood trauma and parental bonding.

Methods - This is a case-control candidate gene study (107 PD patients and 125 controls). Diagnoses were confirmed by M.I.N.I and clinical interview. Childhood trauma was evaluated by the Childhood Trauma Questionnaire (CTQ) and Parental Bonding Instrument (PBI) was used to evaluate parenting. Genes were screened using a set-based test in PLINK followed by single marker association tests and haplotype test for genes that reached experiment-wide significance. Logistic regression was used to model the relationships between genotype in the dominant model and trauma, optimal father parenting and optimal mother parenting, independently in order to evaluate gene-environment interaction. We addressed multiple comparisons at two levels of significance correction: gene-wide (p_1) and experiment-wide (p_2).

Results – Only *HTR1A* was experiment-wide associated with PD in set-based test (p_2=0.027). Three SNPs of this gene were nominally significantly associated with PD, but only one (rs6449693) remained significant after correction for multiple testing. Additionally, one *HTR1A* haplotype was associated with PD (p=0.008), and another haplotype was protective (p=0.004). In the interaction analysis with optimal

father parenting, both interaction terms of SNPs of HTR2A (rs6311 and rs6313) were nominally associated with PD and rs6311 remained significant after Bonferroni correction. Among subjects with TT/TC genotype in rs6311 the protection effect of fathers with high care and low overprotection was higher than the protection effect among subjects with CC genotype (β=0.134, t=-2.678, p=0.007).

Conclusion – PD was associated with HTR1A, but not with 5-HTTLPR even considering interaction with childhood trauma. We also reinforce evidence of gene-environment interaction in HTR2A gene with parenting, maybe influencing the capacity of subjects to use familiar experiences as environmental support.

Introduction

Twin studies have established that genes contribute to panic disorder (PD), (1), but efforts to identify the specific genes involved have had mixed results (2, 3). Among the most widely studied candidate genes have been those involved in serotonergic neurotransmission (4), reflecting evidence of serotonergic dysfunction in PD and the fact that medications modulating the serotonin system are first-line treatments (5). The serotonin transporter (*5-HTTLPR*), serotonin receptor 1A (*HTR1A*) and serotonin receptor 2A (*HTR2A*) polymorphisms have been associated with PD in some studies (4, 6-8) but not in others (6, 9, 10). In addition, previous studies of the *5-HTTLPR* polymorphism have not examined a recently described functional SNP within the insertion/deletion polymorphism (11).

On the other hand, environmental risk factors such as childhood experiences have been systematically associated with psychopathology (1, 12-14). For instance, childhood sexual abuse elevates the risk for adult generalized anxiety disorder and panic disorder, as well as other psychiatric and substance use disorders (12, 13). Some authors also suggest that relationship with the primary caregiver is related to psychopathology (15-17). Studies have reported that PD is associated with parental lower care and higher overprotection scores than healthy control (17-21) .

Although childhood experiences seem to be strongly associated with PD, not all people who had childhood trauma develop PD in adulthood. Diathesis-stress theories of depression and anxiety suggest that individuals' sensitivity to stressful events depends on their genetic makeup (22-26). Besides this, heritability

estimated for anxiety symptoms were larger among individuals who had reported stressor life events as compared to those without stressors, suggesting environmental moderation of genetic effects (27). Association studies attempt to identify both environmental and genetic risk factors as well as their interaction to test if a specific genotype moderates the exposure to an environmental factor (28). Serotoninergic genes have been implicated in a gene-environment interaction for depression (22, 29) and for anxiety (30). However no study have accounted for gene-environment interactions in PD.

The aim of this study is to evaluate the association between *HTR1A*, *HTR2A* and *5-HTTLPR* in PD patients and controls. In addition, we evaluated the interaction between these genes and two environmental factors previously associated with PD: childhood trauma and parental bonding.

Methods

This is a case-control candidate-gene study where PD patients were recruited from the Anxiety Disorder Outpatient Clinic at the Hospital de Clínicas de Porto Alegre. Controls were employees from HCPA that were recruited between June 2006 and April 2007. The inclusion criteria were as follows: (1) PD with or without agoraphobia for patients and absence of psychiatric disorder for controls; (2) at least eighteen years old; (3) European-Brazilian ethnicity. Patients with comorbidities common to PD were included in the study provided that the symptoms were not clinically more relevant than the PD symptoms. Blood was collected from participants for DNA extraction after written informed consent.

Institutional review board approval was obtained from the ethics committee of the Hospital de Clínicas de Porto Alegre (Number 04-272).

Psychometric testing

Subjects were evaluated by a clinical interview and the Mini International Neuropsychiatry Interview (MINI) - Brazilian version (31). A semi-structured interview was used to assess sociodemographic data and clinical history.

Childhood trauma was evaluated by the Childhood Trauma Questionnaire (CTQ) – Brazilian version (32), which evaluates five subscales: childhood emotional, physical and sexual abuse and childhood physical and emotional neglect. CTQ is a self-report questionnaire with 28 items with Likert-type questions (one to five points). Each subscale score ranges from 5 to 25, with higher scores indicative of greater maltreatment. A total score (ranging from 25 to 125) was calculated by summing the values obtained on the five subscales. Previous studies indicates that CTQ remained stable after 6-months of therapy for child abuse or neglect, and subscales demonstrated good internal consistency and convergent validity with trauma-specific measures of distress and therapist rating of abuse (33, 34). Dill et al (35) showed that self-report instruments to evaluate childhood trauma are more likely to elicit truthful responses than clinical interviews. Since all subscales were associated with PD (Mann-Whitney U, $p<0.01$) and were intercorrelated ($r>0.4$), CTQ was only assessed as the global index to avoid multiple comparison (36).

Parenting was assessed by the Parental Bonding Inventory (PBI) – Brazilian Version (37). The original PBI (38) was intended to measure the perceived parental

rearing styles of overprotection and warmth as remembered by the respondents during their first 16 years of life. It consists of 25 items to be assessed separately for mother and father. In a number of studies the PBI proved to be a reliable and valid measure of actual and not merely perceived parenting (39-42). The long-term stability of the PBI has been demonstrated by Wilhelm et al. (43) in a 20-year cohort study. Recently some studies have used confirmatory factor analyses to show the pre-eminence of three-factor models of the PBI with the additional dimension of authoritarianism (44-46). In a previous study we were able to confirm the three factor structure in the Brazilian sample (unpublished data). Both low warmth and high overprotection were associated with PD (Mann-Whitney U, $p<0.01$), but no association was found with authoritarianism in neither father nor mother scales. In order to avoid multiple comparisons, we generated a combined variable for father and mother scales, respectively, creating an ideally parenting subtype named “optimal father” and “optimal mother” characterized by high warmth and low overprotection scores.

Genotyping

A total amount of 1μg of genomic DNA (diluted in 1X TE buffer at 50ng/μl) from studied subjects was equally interleaved on 96-well master plates to ensure technical uniformity during the laboratory process. DNA quality control metrics were assessed prior to genotyping. Quantity of stranded DNA was assessed using PicoGreen® (Molecular Probes, Oregon, USA).

Genotyping of HTR1A and HTR2A was performed with the methods previously described with a few modifications (47). Primers were designed with SpectroDESIGNER software (Sequenom, San Diego, California). Polymerase chain reaction (PCR) was performed, followed by single base extension (SBE) reaction. Samples were analyzed in automated mode by a MassARRAY RT mass spectrometer (Sequenom) (48). The resulting spectra were analyzed by SPECTROTYPER software (Sequenom) after baseline correction and peak identification.

Genotyping was performed for HapMap single nucleotide polymorphisms (SNPs) selected within each gene and 10kb flanking region. For *5-HTTLPR* we examined functional polymorphism including the embedded SNP (rs25531). The Tagger program (49) (http://www.broad.mit.edu/mpg/tagger/) was used to identify tag SNPs with a minor allele frequency of greater than 5% and minimum r^2 of 0.8. A total of 9 markers were thus selected for genotyping using mass spectrometry as previously described (50).

Genotyping of the *5-HTTLPR* was performed with the following protocol - Genomic DNA (1.4 ng) was amplified in a 7 µl reaction using KlenTaq DNA Polymerase (0.2 U), the proprietary KlenTaq Buffer (1X), dNTPs (200 µM each), 5% glycerol, Betaine (1 M) and the marker specific primers (0.2 µM). Amplification was performed with thirteen cycles of denaturation for 30 seconds at 93ºC, annealing for 30 seconds beginning at 61.5ºC and dropped 0.5º C every cycle and primer extension at 72ºC for 30 second, followed by 37 cycles of denaturation for 30 seconds at 93ºC, annealing for 30 seconds at 55º C and primer extension at

72ºC for 30; 72ºC for 1 hour. The amplified product (1µl) was combined with size standard (LIZ-250) before being analyzed on an ABI-3730. The long allele appears as a product of about 412 while the short allele shows a band at about 370. The genotype of the SNP embedded in the 5-HTTLPR (51) (rs25531) was assayed by digesting the PCR product with the restriction enzyme, *Msp*I (11). This 10 µl reaction contains 2 µl of the PCR product, 1 µl of 10X restriction buffer (New England Biolabs), 1 µl of *Msp*I enzyme and 6 µl of water. The reaction was incubated at 37ºC for 1 hour. The digested product (1µl) was then combined with size standard (LIZ-250) before being analyzed on an ABI-3730. Post-digestion, the long allele with the A SNP allele appears as a product of about 320, the G SNP allele is indicated by the presence of a band at 148, while the short allele shows a band at about 277. The final genotype was determined from the information from the analysis of both the digested and undigested PCR product.

Markers were retained only if they met the following quality control criteria: (1) >90% genotype call rate; (2) minor allele frequency (MAF) >5%; (3) Hardy Weinberg equilibrium $P > 0.001$. Three duplicates were assessed in the experiment with 100% concordance. After applying these quality control filters, 8 markers were retained for the association analysis. Four individuals with genotyping call rates less than 90% were excluded resulting in 232 subjects included in the analysis.

Statistical Analysis

PBI and CTQ Scales

Normal distribution and sphericity were assessed previously to any statistical analysis with Kolmogorov-Smirnov test and Levene's test. Since the majority of CTQ and PBI scales are not normally distributed, the CTQ and PBI scores were dichotomized using ROC curves to maximize the accuracy of the cut-off to predict presence of PD in adulthood for risk factors, or predict absence of PD in adulthood for protection factors. The Youden's index *J* was used to define the optimal cut-point for the continuous variables and Area Under the Curve (AUC) was used to test significance. The best cut-points for CTQ Global Score was ≥41 (AUC=0.695; p<0.001). The best cut-points for PBI scales were: ≥6 for mother overprotection (AUC=0.621; p=0.002) and ≥4 for father overprotection (AUC=0.631; p=0.001) - defined in risk factors direction; and ≥16 for mother warmth (AUC=0.615; p=0.003) and ≥13 for father warmth (AUC=0.600; p=0.001) - defined in protection direction.

Genetic analysis plan

A. Association

For an initial analysis, we screened each gene using the set-based test in PLINK (52) (http://pngu.mgh.harvard.edu/purcell/plink/) followed by single marker association for genes that reached experiment-wide significance. The set-based test is based on calculating the average test statistic for the best three SNPs per gene region, and evaluating the significance of these set-statistics by permutation (10,000). Haplotype analysis was performed for genes that reached the experiment-wide significance. The initial haplotype test is an omnibus test for any joint effect of all haplotypes observed (minor allele frequencies [MAF] < .01).

Where the omnibus test was significant, we performed haplotype-specific tests of each haplotype versus all others.

B. Interaction

Logistic regression techniques in Plink were used to model the relationships between genotype in the dominant model and trauma, optimal father parenting and optimal mother parenting, independently. Each model included: the SNP, one of the three environmental factors and their interaction term (SNP*environment).

C. Level of significance and corrections for multiple testing

The overall level of significance adopted was $\alpha=0.05$. Nominal p-values (uncorrected) were named p_0. We addressed multiple comparisons at two levels of significance correction: gene-wide and experiment-wide. Gene-wide significant accounts for the SNPs within the gene, (named p_1) whereas experiment-wide (named p_2), the more restrict one, accounts for all the SNPs evaluated. For single marker association, permutation was used to correct for multiple testing yielding both gene-wise (p_1) and experiment-wise (p_2) p values. Haplotype analysis was corrected for multiple testing using a Bonferroni correction for the number of haplotypes. The interaction analysis was corrected with Bonferroni's correction accounting for the three environment covariates evaluated. Only the dominant model was evaluated for the interaction analysis. All tests were two-tailed.

Results

The sample comprised 107 patients (76.6% woman, mean age 39.94, SD 10.17 years) and 125 controls (70.4% woman, mean age 36.93, SD 9.78 years).

The mean age of PD onset was 32 (SD=10.38) years, and mean of PD duration of 7.94 year (SD=8.45). PD comorbidities included agoraphobia (89.7%), major depression (29%), generalized anxiety disorder (37.4%), social anxiety disorder (17.8%), and dysthymia (15.9%).

Table 1 depicts the set-based test for PD and shows that only *HTR1A* achieved experiment-wise significant association with PD. Table 2 shows single-marker association and haplotype association with PD. Three SNPs included in the analysis were nominally associated with PD, however only rs6449693 remained significant at experiment-wide threshold. In the haplotype analysis, Hap2 was significantly associated with PD (asymptotic p-value=0.008), whereas Hap4 was significantly protective for PD (asymptotic p-value=0.004) and remained significant after Bonferroni correction.

We could not find any significant interaction between all SNPs evaluated, optimal mother parenting or trauma in childhood and PD in adulthood (data not shown). However, optimal father parenting and rs6313 interaction term (β=0.176, t= -2.403, p=0.016) and rs6311 (β=0.134, t=-2.678, p=0.007) was nominally associated with PD, and both association remained significant after Bonferroni correction. The genotypes in rs6311 and rs6313 moderate the effect of optimal father parenting in childhood and PD in adulthood. Figure 1 illustrates this association. Among subjects with TT/TC genotype in rs6311 the protection effect of fathers with high warmth and low overprotection was higher than the protection effect among subjects with CC genotype.

Discussion

As reported in previous studies (6, 53), we observed significant association between *HTR1A* and PD. Moreover we described specific haplotypes in *HTR1A* related to this disorder. We also provide new data about gene-environment interaction between childhood experiences, PD and *HTR2A*, a gene previously associated with maternal nurturance and symptoms of major depression (29).

Preclinical (54) and clinical studies have implicated *HTR1A* in the pathogenesis of PD (55, 56). Studies in *HTR1A* knockout mice demonstrated an increased anxiety-like behavior in several tests (54, 57, 58). Besides this, PD has been associated with a reduction on both pre-synaptic and post-synaptic *HTR1A* receptors as assessed by positron emission tomography (59). However, association studies evaluating rs6295 and PD have found controversial results. Rothe et al (6) found an association with the G allele for PD with agoraphobia and Huang et al (53) found an association with panic attack, while Maron et al. (7) found an association between PD and the C allele. In our study, rs6295, rs4521432 and rs6449693 were associated with PD, but only the last SNP remained significant at experiment-wide levels. Additionally we could detect one protective haplotype and another risk haplotype significantly associated with PD even after correction.

Two independent studies have found association with *HTR2A* and PD (7, 8); however this finding was not replicated in two additional studies (6, 9). In our study, no association was found between PD and *HTR2A*, but we found a gene-environmental interaction with *HTR2A* and optimal father parenting in childhood

and PD in adulthood. This gene-environment interaction was also found with both rs6311 and rs6313. Since rs6311 and rs6313 were in high linkage disequilibrium in our sample, these two SNPs can bring redundant information to this interaction. A recent study found that individuals carrying the TT/TC genotype of rs6313 *HTR2A* were responsive to the protective aspects of nurturing mothering, so that in the presence of high maternal nurturance they expressed low levels of depressive symptoms (29). The authors hypothesize a path via the prefrontal cortex, since *HTR2A* are involved in the functioning of the prefrontal cortex involved in cognitive control and regulation of negative emotions (29). Anxiety and depression are frequently comorbidities, and studies in families have shown a high susceptibility for depression in families with PD (60), suggesting a common risk factors to these disorders (61). In our study we were not able to find a significant association with optimal mother parenting, although there was a trend in the interaction term in rs6311 (p-nominal=0.081). Lack of statistical power could explain this result. In spite of this, in the presence of father with high warmth and low overprotection, individuals carrying the TT/TC genotype of rs6313 and rs6311 were more responsive to optimal father parenting protection against PD.

The association between parenting and psychopathology seems to be influenced by cultural aspects. For instance, a study that evaluated parental bonding in six European countries found a different role to father/mother warmth and protection. In France no aspects of parental bonding were associated with PD, while in Belgium only father warmth and in Germany only mother overprotection

seems to be associated to PD (18). Maybe the *HTR2A* influences the ability of individuals to use environmental support according to cultural aspects and could be not linked to one parental figure itself.

Although *5-HTTLPR* has been associated with anxiety traits in healthy volunteers (62), no association was found with this polymorphism with PD in a recent meta-analysis (3) and our study corroborates this previous finding. This is the first study that evaluated the triallelic form (11) of *5-HTTLPR* and PD and found no association with this phenotype. Gene-environment interaction with *5-HTTLPR* for depression has been shown in many studies (22, 63-70), but not in all of them (71, 72). Besides, some studies have contradictory results (64, 65, 73). In anxiety, Stein et al (74) found an interaction between emotional abuse and the short allele with anxiety sensitivity, but not with neuroticism. In our study, we evaluate a combined trauma index instead of the subscales to avoid multiple comparisons, and no gene-environmental interaction was found with none of the SNPs evaluated in PD.

Our results should be interpreted in light of some limitations. Most importantly, the small sample size means that our analyses may be subject to Type II error. The possible confounding due to population stratification in Brazil was addressed by restricting our analyses to Caucasian subjects (75), therefore these findings are restricted to this population. The assessment of maltreatment childhood and parental bonding was retrospective and at the same time as the phenotypic evaluation. Although PBI has a long-term stability (43), biased recall could not be ruled out (28). Genes selection was performed in a no-systematic

search, and some serotoninergic genes previously associated with PD were not evaluated (4, 7, 76). The interaction with optimal father parenting should be interpreted carefully since we corrected for multiple testing only considering the three phenotypes and not all SNPs included. However as this finding is similar to what has been described for depression and mother nurturance (29), the chance of spurious association by multiple testing is reduced.

In sum, we were able to demonstrate an association between *HTR1A* and PD, and a lack of association between *5-HTTLPR* and PD even considering childhood trauma or parental bonding. Besides, this study is the first analysis of gene-environment interaction in PD. We observed that fathers with high warmth and low overprotection interact with variants in *HTR2A* protecting for PD in adulthood. Clearly, additional larger studies are needed to further define this interaction effect.

References

1. Hettema JM, Prescott CA, Myers JM, Neale MC, Kendler KS. The structure of genetic and environmental risk factors for anxiety disorders in men and women. Arch Gen Psychiatry. 2005 Feb;62(2):182-9.
2. Domschke K, Deckert J, O'Donovan M C, Glatt SJ. Meta-analysis of COMT val158met in panic disorder: ethnic heterogeneity and gender specificity. Am J Med Genet B Neuropsychiatr Genet. 2007 Jul 5;144(5):667-73.
3. Blaya C, Salum GA, Lima MS, Leistner-Segal S, Manfro GG. Lack of association between the Serotonin Transporter Promoter Polymorphism (5-HTTLPR) and Panic Disorder: a systematic review and meta-analysis. Behav Brain Funct. 2007;3:41.
4. Maron E, Lang A, Tasa G, Liivlaid L, Toru I, Must A, et al. Associations between serotonin-related gene polymorphisms and panic disorder. Int J Neuropsychopharmacol. 2005 Jun;8(2):261-6.
5. Graeff FG. Serotonin, the periaqueductal gray and panic. Neurosci Biobehav Rev. 2004 May;28(3):239-59.
6. Rothe C, Gutknecht L, Freitag C, Tauber R, Mossner R, Franke P, et al. Association of a functional 1019C>G 5-HT1A receptor gene polymorphism with panic disorder with agoraphobia. Int J Neuropsychopharmacol. 2004 Jun;7(2):189-92.
7. Maron E, Nikopensius T, Koks S, Altmae S, Heinaste E, Vabrit K, et al. Association study of 90 candidate gene polymorphisms in panic disorder. Psychiatr Genet. 2005 Mar;15(1):17-24.
8. Inada Y, Yoneda H, Koh J, Sakai J, Himei A, Kinoshita Y, et al. Positive association between panic disorder and polymorphism of the serotonin 2A receptor gene. Psychiatry Res. 2003 May 1;118(1):25-31.
9. Fehr C, Schleicher A, Szegedi A, Anghelescu I, Klawe C, Hiemke C, et al. Serotonergic polymorphisms in patients suffering from alcoholism, anxiety disorders and narcolepsy. Prog Neuropsychopharmacol Biol Psychiatry. 2001 Jul;25(5):965-82.
10. Hamilton SP, Heiman GA, Haghighi F, Mick S, Klein DF, Hodge SE, et al. Lack of genetic linkage or association between a functional serotonin transporter polymorphism and panic disorder. Psychiatr Genet. 1999 Mar;9(1):1-6.
11. Hu XZ, Lipsky RH, Zhu G, Akhtar LA, Taubman J, Greenberg BD, et al. Serotonin transporter promoter gain-of-function genotypes are linked to obsessive-compulsive disorder. Am J Hum Genet. 2006 May;78(5):815-26.
12. Fergusson DM, Lynskey MT, Horwood LJ. Childhood sexual abuse and psychiatric disorder in young adulthood: I. Prevalence of sexual abuse and factors associated with sexual abuse. J Am Acad Child Adolesc Psychiatry. 1996 Oct;35(10):1355-64.
13. Kendler KS, Bulik CM, Silberg J, Hettema JM, Myers J, Prescott CA. Childhood sexual abuse and adult psychiatric and substance use disorders in women: an epidemiological and cotwin control analysis. Arch Gen Psychiatry. 2000 Oct;57(10):953-9.

14. Kendler KS, Neale MC, Kessler RC, Heath AC, Eaves LJ. Childhood parental loss and adult psychopathology in women. A twin study perspective. Arch Gen Psychiatry. 1992 Feb;49(2):109-16.
15. Leckman JF, Feldman R, Swain JE, Eicher V, Thompson N, Mayes LC. Primary parental preoccupation: circuits, genes, and the crucial role of the environment. J Neural Transm. 2004 Jul;111(7):753-71.
16. Repetti RL, Taylor SE, Seeman TE. Risky families: family social environments and the mental and physical health of offspring. Psychol Bull. 2002 Mar;128(2):330-66.
17. Faravelli C, Panichi C, Pallanti S, Paterniti S, Grecu LM, Rivelli S. Perception of early parenting in panic and agoraphobia. Acta Psychiatr Scand. 1991 Jul;84(1):6-8.
18. Heider D, Matschinger H, Bernert S, Alonso J, Brugha TS, Bruffaerts R, et al. Adverse parenting as a risk factor in the occurrence of anxiety disorders : A study in six European countries. Soc Psychiatry Psychiatr Epidemiol. 2008 Jan 14.
19. Wiborg IM, Dahl AA. The recollection of parental rearing styles in patients with panic disorder. Acta Psychiatr Scand. 1997 Jul;96(1):58-63.
20. Silove D, Parker G, Hadzi-Pavlovic D, Manicavasagar V, Blaszczynski A. Parental representations of patients with panic disorder and generalised anxiety disorder. Br J Psychiatry. 1991 Dec;159:835-41.
21. Pacchierotti C, Bossini L, Castrogiovanni A, Pieraccini F, Soreca I, Castrogiovanni P. Attachment and panic disorder. Psychopathology. 2002 Nov-Dec;35(6):347-54.
22. Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, et al. Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science. 2003 Jul 18;301(5631):386-9.
23. Mann JJ, Oquendo M, Underwood MD, Arango V. The neurobiology of suicide risk: a review for the clinician. J Clin Psychiatry. 1999;60 Suppl 2:7-11; discussion 8-20, 113-6.
24. Costello EJ, Erkanli A, Fairbank JA, Angold A. The prevalence of potentially traumatic events in childhood and adolescence. J Trauma Stress. 2002 Apr;15(2):99-112.
25. Monroe SM, Simons AD. Diathesis-stress theories in the context of life stress research: implications for the depressive disorders. Psychol Bull. 1991 Nov;110(3):406-25.
26. Lau JY, Gregory AM, Goldwin MA, Pine DS, Eley TC. Assessing gene-environment interactions on anxiety symptom subtypes across childhood and adolescence. Dev Psychopathol. 2007 Fall;19(4):1129-46.
27. Silberg J, Rutter M, Neale M, Eaves L. Genetic moderation of environmental risk for depression and anxiety in adolescent girls. Br J Psychiatry. 2001 Aug;179:116-21.
28. Moffitt TE, Caspi A, Rutter M. Strategy for investigating interactions between measured genes and measured environments. Arch Gen Psychiatry. 2005 May;62(5):473-81.

29. Jokela M, Keltikangas-Jarvinen L, Kivimaki M, Puttonen S, Elovainio M, Rontu R, et al. Serotonin receptor 2A gene and the influence of childhood maternal nurturance on adulthood depressive symptoms. Arch Gen Psychiatry. 2007 Mar;64(3):356-60.
30. Fox NA, Nichols KE, Henderson HA, Rubin K, Schmidt L, Hamer D, et al. Evidence for a gene-environment interaction in predicting behavioral inhibition in middle childhood. Psychol Sci. 2005 Dec;16(12):921-6.
31. Amorim P. Mini International Neuropsychiatric Interview (MINI): validação de entrevista breve para diagnóstico de transtornos mentais. Rev Bras Psiquiatr. 2000;22(3):106-15.
32. Grassi-Oliveira R, Stein LM, Pezzi JC. [Translation and content validation of the Childhood Trauma Questionnaire into Portuguese language]. Rev Saude Publica. 2006 Apr;40(2):249-55.
33. Paivio SC. Stability of retrospective self-reports of child abuse and neglect before and after therapy for child abuse issues. Child Abuse Negl. 2001 Aug;25(8):1053-68.
34. Bernstein DP, Stein JA, Newcomb MD, Walker E, Pogge D, Ahluvalia T, et al. Development and validation of a brief screening version of the Childhood Trauma Questionnaire. Child Abuse Negl. 2003 Feb;27(2):169-90.
35. Dill DL, Chu JA, Grob MC, Eisen SV. The reliability of abuse history reports: a comparison of two inquiry formats. Compr Psychiatry. 1991 Mar-Apr;32(2):166-9.
36. Savitz JB, van der Merwe L, Newman TK, Solms M, Stein DJ, Ramesar RS. The relationship between childhood abuse and dissociation. Is it influenced by catechol-O-methyltransferase (COMT) activity? Int J Neuropsychopharmacol. 2008 Apr;11(2):149-61.
37. Hauck S, Schestatsky S, Terra L, Knijnik L, Sanchez P, Ceitlin LH. Cross-cultural adaptation of Parental Bonding Instrument (PBI) to Brazilian Portuguese Rev Psiquiatr Rio Gd Sul. 2006;28(2):162-8.
38. Parker G, Tupling H, Brown LB. Parental bonding instrument. Br J Med Psychol. 1979;52:1-10.
39. Mackinnon AJ, Henderson AS, Scott R, Duncan-Jones P. The Parental Bonding Instrument (PBI): an epidemiological study in a general population sample. Psychol Med. 1989 Nov;19(4):1023-34.
40. Parker G. Parental reports of depressives. An investigation of several explanations. J Affect Disord. 1981 Jun;3(2):131-40.
41. Parker G. The Parental Bonding Instrument: psychometric properties reviewed. Psychiatr Dev. 1989 Winter;7(4):317-35.
42. Plantes MM, Prusoff BA, Brennan J, Parker G. Parental representations of depressed outpatients from a U.S.A. sample. J Affect Disord. 1988 Sep-Oct;15(2):149-55.
43. Wilhelm K, Niven H, Parker G, Hadzi-Pavlovic D. The stability of the Parental Bonding Instrument over a 20-year period. Psychol Med. 2005 Mar;35(3):387-93.

44. Kendler KS, Sham PC, MacLean CJ. The determinants of parenting: an epidemiological, multi-informant, retrospective study. Psychol Med. 1997 May;27(3):549-63.
45. Sato T, Narita T, Hirano S, Kusunoki K, Sakado K, Uehara T. Confirmatory factor analysis of the Parental Bonding Instrument in a Japanese population. Psychol Med. 1999 Jan;29(1):127-33.
46. Cox BJ, Enns MW, Clara IP. The Parental Bonding Instrument: confirmatory evidence for a three-factor model in a psychiatric clinical sample and in the National Comorbidity Survey. Soc Psychiatry Psychiatr Epidemiol. 2000 Aug;35(8):353-7.
47. Smoller JW, Rosenbaum JF, Biederman J, Kennedy J, Dai D, Racette SR, et al. Association of a genetic marker at the corticotropin-releasing hormone locus with behavioral inhibition. Biol Psychiatry. 2003 Dec 15;54(12):1376-81.
48. Buetow KH, Edmonson M, MacDonald R, Clifford R, Yip P, Kelley J, et al. High-throughput development and characterization of a genomewide collection of gene-based single nucleotide polymorphism markers by chip-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Proc Natl Acad Sci U S A. 2001 Jan 16;98(2):581-4.
49. de Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D. Efficiency and power in genetic association studies. Nat Genet. 2005 Nov;37(11):1217-23.
50. Sklar P, Gabriel SB, McInnis MG, Bennett P, Lim YM, Tsan G, et al. Family-based association study of 76 candidate genes in bipolar disorder: BDNF is a potential risk locus. Brain-derived neutrophic factor. Mol Psychiatry. 2002;7(6):579-93.
51. Wendland JR, Martin BJ, Kruse MR, Lesch KP, Murphy DL. Simultaneous genotyping of four functional loci of human SLC6A4, with a reappraisal of 5-HTTLPR and rs25531. Mol Psychiatry. 2006 Mar;11(3):224-6.
52. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 2007 Sep;81(3):559-75.
53. Huang YY, Battistuzzi C, Oquendo MA, Harkavy-Friedman J, Greenhill L, Zalsman G, et al. Human 5-HT1A receptor C(-1019)G polymorphism and psychopathology. Int J Neuropsychopharmacol. 2004 Dec;7(4):441-51.
54. Parks CL, Robinson PS, Sibille E, Shenk T, Toth M. Increased anxiety of mice lacking the serotonin1A receptor. Proc Natl Acad Sci U S A. 1998 Sep 1;95(18):10734-9.
55. Broocks A, Meyer T, Opitz M, Bartmann U, Hillmer-Vogel U, George A, et al. 5-HT1A responsivity in patients with panic disorder before and after treatment with aerobic exercise, clomipramine or placebo. Eur Neuropsychopharmacol. 2003 May;13(3):153-64.
56. Lesch KP, Wiesmann M, Hoh A, Muller T, Disselkamp-Tietze J, Osterheider M, et al. 5-HT1A receptor-effector system responsivity in panic disorder. Psychopharmacology (Berl). 1992;106(1):111-7.

57. Dulawa SC, Gross C, Stark KL, Hen R, Geyer MA. Knockout mice reveal opposite roles for serotonin 1A and 1B receptors in prepulse inhibition. Neuropsychopharmacology. 2000 Jun;22(6):650-9.
58. Ramboz S, Oosting R, Amara DA, Kung HF, Blier P, Mendelsohn M, et al. Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. Proc Natl Acad Sci U S A. 1998 Nov 24;95(24):14476-81.
59. Neumeister A, Bain E, Nugent AC, Carson RE, Bonne O, Luckenbaugh DA, et al. Reduced serotonin type 1A receptor binding in panic disorder. J Neurosci. 2004 Jan 21;24(3):589-91.
60. Maier W, Minges J, Lichtermann D. The familial relationship between panic disorder and unipolar depression. J Psychiatr Res. 1995 Sep-Oct;29(5):375-88.
61. Wittchen HU, Kessler RC, Pfister H, Lieb M. Why do people with anxiety disorders become depressed? A prospective-longitudinal community study. Acta Psychiatr Scand Suppl. 2000(406):14-23.
62. Sen S, Burmeister M, Ghosh D. Meta-analysis of the association between a serotonin transporter promoter polymorphism (5-HTTLPR) and anxiety-related personality traits. Am J Med Genet. 2004 May 15;127B(1):85-9.
63. Mandelli L, Serretti A, Marino E, Pirovano A, Calati R, Colombo C. Interaction between serotonin transporter gene, catechol-O-methyltransferase gene and stressful life events in mood disorders. Int J Neuropsychopharmacol. 2007 Aug;10(4):437-47.
64. Eley TC, Sugden K, Corsico A, Gregory AM, Sham P, McGuffin P, et al. Gene-environment interaction analysis of serotonin system markers with adolescent depression. Mol Psychiatry. 2004 Oct;9(10):908-15.
65. Grabe HJ, Lange M, Wolff B, Volzke H, Lucht M, Freyberger HJ, et al. Mental and physical distress is modulated by a polymorphism in the 5-HT transporter gene interacting with social stressors and chronic disease burden. Mol Psychiatry. 2005 Feb;10(2):220-4.
66. Jacobs N, Kenis G, Peeters F, Derom C, Vlietinck R, van Os J. Stress-related negative affectivity and genetically altered serotonin transporter function: evidence of synergism in shaping risk of depression. Arch Gen Psychiatry. 2006 Sep;63(9):989-96.
67. Kaufman J, Yang BZ, Douglas-Palumberi H, Houshyar S, Lipschitz D, Krystal JH, et al. Social supports and serotonin transporter gene moderate depression in maltreated children. Proc Natl Acad Sci U S A. 2004 Dec 7;101(49):17316-21.
68. Kendler KS, Kuhn JW, Vittum J, Prescott CA, Riley B. The interaction of stressful life events and a serotonin transporter polymorphism in the prediction of episodes of major depression: a replication. Arch Gen Psychiatry. 2005 May;62(5):529-35.
69. Wilhelm K, Mitchell PB, Niven H, Finch A, Wedgwood L, Scimone A, et al. Life events, first depression onset and the serotonin transporter gene. Br J Psychiatry. 2006 Mar;188:210-5.
70. Zalsman G, Huang YY, Oquendo MA, Burke AK, Hu XZ, Brent DA, et al. Association of a triallelic serotonin transporter gene promoter region (5-HTTLPR)

polymorphism with stressful life events and severity of depression. Am J Psychiatry. 2006 Sep;163(9):1588-93.
71. Gillespie NA, Whitfield JB, Williams B, Heath AC, Martin NG. The relationship between stressful life events, the serotonin transporter (5-HTTLPR) genotype and major depression. Psychol Med. 2005 Jan;35(1):101-11.
72. Surtees PG, Wainwright NW, Willis-Owen SA, Luben R, Day NE, Flint J. Social adversity, the serotonin transporter (5-HTTLPR) polymorphism and major depressive disorder. Biol Psychiatry. 2006 Feb 1;59(3):224-9.
73. Zammit S, Owen MJ. Stressful life events, 5-HTT genotype and risk of depression. Br J Psychiatry. 2006 Mar;188:199-201.
74. Stein MB, Schork NJ, Gelernter J. Gene-by-environment (serotonin transporter and childhood maltreatment) interaction for anxiety sensitivity, an intermediate phenotype for anxiety disorders. Neuropsychopharmacology. 2008 Jan;33(2):312-9.
75. Zembrzuski VM, Callegari-Jacques SM, Hutz MH. Application of an African Ancestry Index as a genomic control approach in a Brazilian population. Ann Hum Genet. 2006 Nov;70(Pt 6):822-8.
76. Deckert J, Catalano M, Syagailo YV, Bosi M, Okladnova O, Di Bella D, et al. Excess of high activity monoamine oxidase A gene promoter alleles in female patients with panic disorder. Hum Mol Genet. 1999 Apr;8(4):621-4.

Table 1 Set-based test for the association between Polymorphisms (PMs) and Panic Disorder (PD n=107, Controls n=125)

Set	PMs in the Set	PMs	T-value	Empirical p-value 0	Empirical p-value 1	Empirical p-value 2
HTR1A	P1	rs6449693	7.495	0.0159	0.0203	0.0555
	P2	rs4521432	7.226	0.0103	0.0137	0.0383
	P3	rs6295	7.059	0.0089	0.0111	**0.0303**
HTR2A	P1	rs6311	0.335	0.6337	0.6493	0.9673
	P2	rs6313	0.238	0.6415	0.6493	0.9677
5-HTTLPR	P1	Biallelic	0.318	0.5975	0.5975	0.9597
SLC6A4	P1	Triallelic	0.225	0.6469	0.6469	0.9717

A multi-marker set-based test implemented in PLINK was used for the analysis of all genes. Sets that have an experiment-wide association p-value ($p<0.05$) are shown in bold. In each set, P1 is the best PMs associated with the outcome; P2 is the second one and P3 is the third one. Maximum computed permutations were 10000.
Empirical p-value p 0: p-value for average chi-square without correction;
Empirical p-value p 1: p-value corrected for all tests within this set/ gene
Empirical p-value p 2: p-value corrected for all tests in all sets
Abbreviations: PMs, Polymorphisms; CGI, Clinical Global Impression; HTR2A, Serotonin receptor 2A; HTR1A, Serotonin receptor 1A; 5-HTTLPR, Serotonin transporter gene.

Table 2 HTR1A single-marker association and haplotype association with Panic Disorder (107 cases and 125 controls)

	rs4521432	rs6449693	rs6295	rs13361335	Frequency in PD	Frequency in Controls	χ^2 (df=1)	Asymptotic p-value	Corrected p-value
Hap1	C	A	C	G	0.12	0.11	0.11	0.736	>0.999
Hap2	T	G	G	T	0.55	0.42	6.97	**0.008**	**0.032**
Hap3	C	A	G	T	0.01	0.02	0.03	0.871	>0.99
Hap4	C	A	C	T	0.32	0.45	8.29	**0.004**	**0.016**
						Ominbus .032			
Role	Downstream	Downstream	Promoter	Promoter					
HWE	0.695	0.794	0.794	>0.999					
Allele change	C/T	A/G	C/G	G/T					
Minor allele	T	G	G	G					
Frequency in PD	0.55	0.55	0.56	0.12					
Frequency in Controls	0.42	0.42	0.44	0.11					
χ^2 (df=1)	6.96	7.49	6.72	0.09					
Asymptotic p-value	**0.008**	**0.006**	**0.009**	0.764					
Empirical p-value 1	**0.024**	**0.015**	**0.026**	0.969					
Empirical p-value 2	0.053	**0.041**	0.066	0.999					
OR	1.64	1.67	1.62	1.09					

Abbreviations: PD, Panic Disorder; HTR1A, Serotonin Receptor 1A; SNP, Single Nucleotide Polymorphism; HWE, Hardy Weinberg Equilibrium p value; OR, Odds Ratio; χ^2 chi-square; df, degrees of freedom. **Bold** means p<0.05.
Asymptotic p-value: p-value for chi-square without correction;
Empirical p-value P 1: p-value corrected for all tests within this set/ gene
Empirical p-value P 2: p-value corrected for all tests in all sets

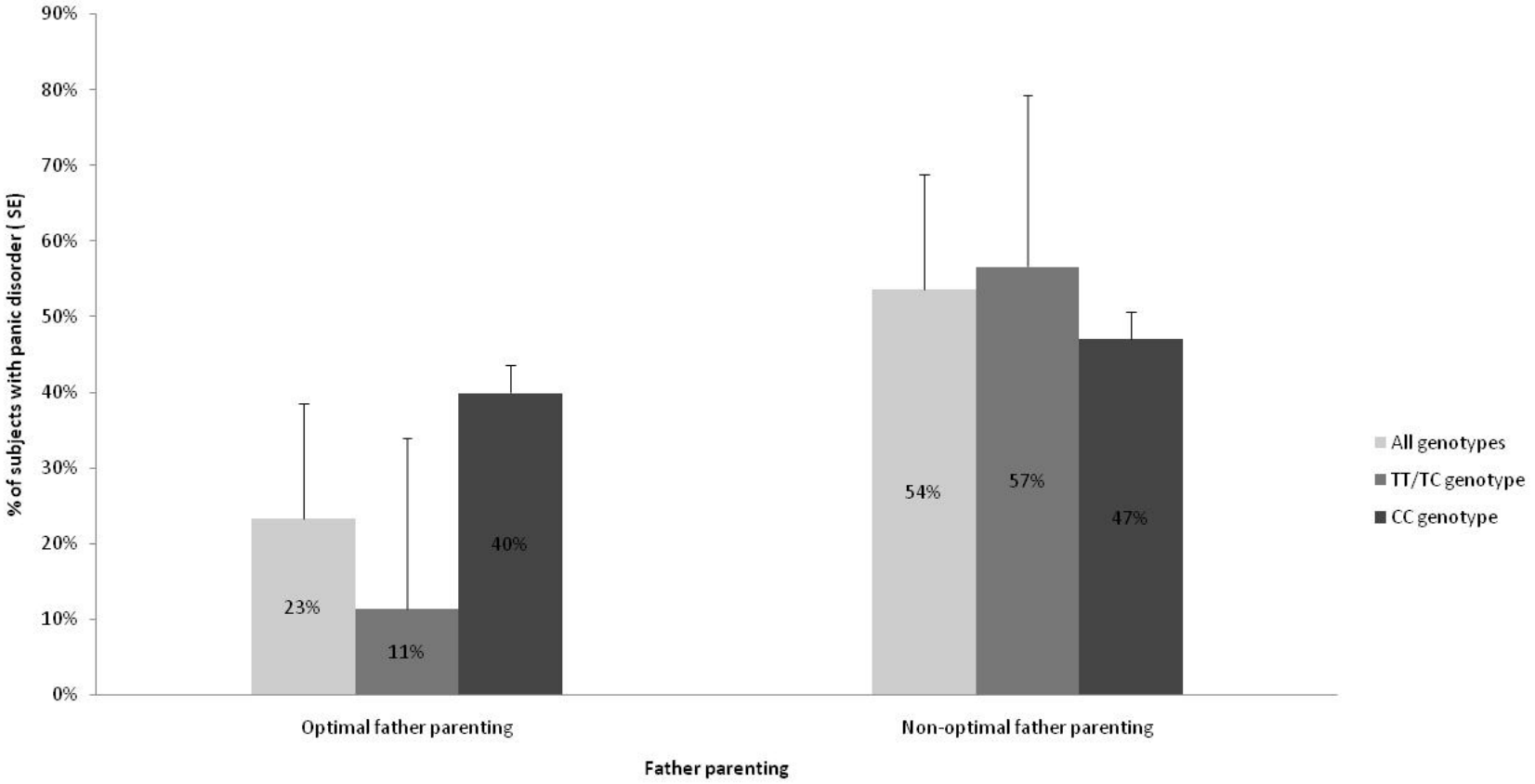

Figure 1. Relative frequency of PD striatified for optimal father parenting (hight warm and low overprotection) and rs6311 genotype
Abbreviations. SE, Standard Error.

Artigo 3 – *Association between EFHC2 with Panic Disorder, Harm Avoidance and Behavioral Inhibition*

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Anxiety-proneness is known to be influenced by genes (Munafo et al., 2004). Recent evidence suggests that anxiety traits and anxiety disorders are associated with altered limbic reactivity to fearful stimuli (Pezawas et al., 2005; Pillay et al., 2007). Previous studies have related the X-linked genes to fear recognition in which amygdale, a key structure of the limbic system, plays a critical role (Good et al., 2003). Weiss et al (Weiss et al., 2007) performed a regression-based association mapping in women with Turner syndrome and found an association between fear recognition and EF-hand domain containing 2 (*EFHC2*). This finding makes this a candidate gene for involvement in anxiety-proneness and perhaps disorder.

With this study we aim to examined whether *EFHC2* also affects non-syndromic anxiety-related traits that have been shown to reflect altered fear recognition, e.g. harm avoidance (HA) (Pezawas et al., 2005) and behavioral inhibition (BI) (Schwartz et al., 2003). We also aim to find if *EFHC2* is related to panic disorder (PD), a disorder in which reactivity to fearful stimuli has also been implicated (Pillay et al., 2007). Since the power of this study is likely to be greater for the quantitative intermediate phenotypes, we run HA and BI as a primary analyses and PD as a secondary analysis.

This is a case-control study where 127 Caucasian PD patients (75.5% females, mean age 39.05 ± 10.12 years) were recruited from the Outpatient Clinic and 132 Caucasian employees from HCPA (73.5% females mean age 36.93 ± 9.75 years) were recruited as controls. Blood was collected from

participants for DNA extraction after informed consent was signed. Institutional review board approval was obtained from the ethics committee (Number 04-272).

The Mini International Neuropsychiatry Interview - Brazilian version was used to confirm the psychiatric diagnoses. HA was assessed with the Cloninger's Temperament and Character Inventory (Cloninger, 1986). HA is described as a heritable tendency to inhibit or stop behaviors in response to signals of aversive stimuli in order to avoid punishment. The Self-Report Scale of Behavioral Inhibition - Retrospective Version (Reznick et al., 1992) was used to assess childhood BI. BI is a strong predictor of anxiety disorder and has an estimated heritability exceeding that of the anxiety disorders themselves.

Single nuIcleotide polymorphisms (SNPs) were selected within the *EFHC2* gene and 10KB flanking region. Genotyping of SNPs was performed according to Weiss et al. (Weiss et al., 2007) with minor modifications. Markers were retained only if they met the following quality control criteria: >90% genotype call rate, minor allele frequency (MAF) >5%, Hardy-Weinberg Equilibrium (HWE) p > 0.001. One SNP was excluded due to departure from HWE.

We used PLINK software (Purcell et al., 2007) to perform the basic single SNP association test for alleles. For each SNP, we calculated empirical significant value based on a Wald test and empirical significant value based on 10,000 permutations. SNPs that achieve significance in permuted analysis are safer regarding false findings due to multiple testing. Genotypic tests were

performed when allelic tests show significant in the permuted analysis. We considered an α=0.05 as significance level.

Single marker tests of 8 *EFHC2* SNPs and HA showed one nominally significant association at rs1562875 and remained significant in the permuted analysis (p=0.031). In the genotype analysis, rs1562875 was also nominally associated with HA (Mean±SD: AA 23.6±5.8, AT 21.7±5.9 and TT 15.6±6.5, nominal p-value=0.004; permuted p-value=0.033). In the BI analysis, rs1562875 and rs17146914 were associated with this phenotype, but did not remained significant in the permuted analysis (p=0.137 and p=0.184, respectively) (table 1). In the PD analysis, the same SNP (rs1562875) was associated with this disorder (10.7% cases vs. 4.3% controls; OR 2.64, CI95% 1.23-5.66, p=0.009). However this association did not remain significant in the permuted analysis (p=0.059). Multiple logistic regression could not be performed because of high colinearity between the three phenotypes evaluated (tolerance values < 1 - r^2).

This is the first study that evaluates *EFHC2* and anxiety traits, a gene previously associated with fear recognition (Weiss et al., 2007). In our study, we found that rs1562875 minor allele is nominally associated with higher scores in HA and BI and is more frequent in PD than in controls. The strength of this study is the congruent finding of these three highly associated phenotypes that could not be isolated in regression analysis. Spurious association regarding PD and BI should be considered due to multiple testing since only HA passed in permuted adjusted analysis.

EFHC2 may be associated with anxiety by interfering in fear process related to amygdale function. Pillay et al (2007) showed that PD patients produced more activation of amygdale than controls in response to fearful faces using fMRI. However, the SNP found in this association study was not the same and neither it is in linkage disequilibrium (LOD<0.5) with the one that has been reported to be associated with fear recognition in Turner Syndrome (Weiss et al., 2007). *EFHC2* is a candidate to be involved in neural circuits; however its role in fear circuits has not been properly understood.

Our results should be interpreted in light of some limitations. Most importantly, the small sample size means that our analyses may be subject to Type II error. Besides this, the possibility of spurious association must be considered. Finally, p values were corrected only by permutation and not for the three phenotypes evaluated with Bonferroni's correction.

Regardless the small sample size and the unknown RNA expression implications of polymorphisms in this gene, the association described between *EFHC2* and anxiety, if replicated, has potential implication in elucidating a possible neurobiological path of fear.

References

Cloninger, CR (1986) A unified biosocial theory of personality and its role in the development of anxiety states. *Psychiatr Dev,* 4(3): 167-226.

Good, CD, Lawrence, K, Thomas, NS, Price, CJ, Ashburner, J, Friston, KJ, Frackowiak, RS, Oreland, L, Skuse, DH (2003) Dosage-sensitive X-linked locus influences the development of amygdala and orbitofrontal cortex, and fear recognition in humans. *Brain,* 126(Pt 11): 2431-2446.

Munafo, MR, Clark, T, Flint, J (2004) Does measurement instrument moderate the association between the serotonin transporter gene and anxiety-related personality? A meta-analysis. *Mol Psychiatry,* 0: 1-5.

Pezawas, L, Meyer-Lindenberg, A, Drabant, EM, Verchinski, BA, Munoz, KE, Kolachana, BS, Egan, MF, Mattay, VS, Hariri, AR, Weinberger, DR (2005) 5-HTTLPR polymorphism impacts human cingulate-amygdala interactions: a genetic susceptibility mechanism for depression. *Nat Neurosci,* 8(6): 828-834.

Pillay, SS, Rogowska, J, Gruber, SA, Simpson, N, Yurgelun-Todd, DA (2007) Recognition of happy facial affect in panic disorder: an fMRI study. *J Anxiety Disord,* 21(3): 381-393.

Purcell, S, Neale, B, Todd-Brown, K, Thomas, L, Ferreira, MA, Bender, D, Maller, J, Sklar, P, de Bakker, PI, Daly, MJ, Sham, PC (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet,* 81(3): 559-575.

Reznick, JS, Hegeman, JM, Kaufman, ER, Woods, SW, Jacobs, M (1992) Retrospective and concurrent self-report of behavioral inhibition and their relation to adult mental health. *Dev Psychopathol,* 4: 301-321.

Schwartz, CE, Wright, CI, Shin, LM, Kagan, J, Whalen, PJ, McMullin, KG, Rauch, SL (2003) Differential amygdalar response to novel versus newly familiar neutral faces: a functional MRI probe developed for studying inhibited temperament. *Biol Psychiatry,* 53(10): 854-862.

Weiss, LA, Purcell, S, Waggoner, S, Lawrence, K, Spektor, D, Daly, MJ, Sklar, P, Skuse, D (2007) Identification of EFHC2 as a quantitative trait locus for fear recognition in Turner syndrome. *Hum Mol Genet,* 16(1): 107-113.

Table 1. Allelic Association between individual SNPs in EFHC2 with Behavioral Inhibition and Harm Avoidance

SNP Characteristic					Behavioral Inhibition				Harm Avoidance			
SNP	**Role**	**Allele Change**	**MAF**	**HWE p**	**B**	**r^2**	**p-value (asymptotic)**	**p-value corrected**	**β**	**r^2**	**p-value (asymptotic)**	**p-value corrected**
rs287779	Intron	A/**G**	0.24	0.54	-0.059	0.005	0.267	0.849	-0.237	<0.001	0.690	0.999
rs12557505	Intron	**A**/T	0.10	0.70	0.066	0.002	0.410	0.961	0.888	0.004	0.309	0.894
rs12014680	Intron	**A**/G	0.18	0.47	0.081	0.006	0.210	0.761	1.091	0.009	0.119	0.536
rs2208592	Exon	G/**T**	0.12	0.53	0.151	0.015	0.055	0.301	1.154	0.007	0.178	0.698
rs7055196	Intron	A/**G**	0.18	0.48	0.089	0.007	0.170	0.681	0.032	<0.001	0.964	1.000
rs17146914	Exon	**C**/T	0.09	1	0.204	0.019	0.031*	0.184	1.232	0.005	0.236	0.800
rs1562875	Intron	**A**/T	0.08	1	0.206	0.021	0.022*	0.137	2.798	0.031	0.005*	0.032*
rs5906930	Promoter	**C**/G	0.40	0.54	0.011	<0.001	0.814	1.000	-0.206	<0.001	0.694	0.999

Abbreviations: SNP, Single Nucleotide Polymorphism; EFHC2, EF-hand domain containing 2; MAF, Minimal allele frequency; HWE, Hardy-Weinberg equilibrium; β, regression coefficient; r^2 regression r-squared; p, Wald-test asymptomatic p-value.

Statistic tests: Wald test;

Note: **Bold** indicates minor allele. * indicates p<0.05.

Artigo 4 – *CREB1 as a predictor of long term response to cognitive-behavior therapy for pharmacotherapy-resistant panic disorder patients*

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Submetido no *Genes, Brain and Behavior*

Abstract

OBJECTIVES: While numerous studies have investigated the role of genetic variation in predicting response to psychotropic medications, none have examined whether genes influence response to Cognitive Behavior Therapy (CBT). The aim of this study was to evaluate the role of variants in 7 genes previously implicated in the etiology or treatment of anxiety: *BDNF, CREB1, RGS2, CRHR1, SLC6A4, HTR2A, HTR1A* and response to CBT in pharmacological-resistant panic disorder (PD) patients.

METHODS: Seventy-four Caucasian patients with PD resistant to antidepressant treatment received group CBT for 4 months. Baseline illness severity was assessed using the Clinical Global Impression (CGI) scale, and treatment response was indexed by change in CGI at the end of treatment and at 1-year follow-up. Additionally, the remission criteria (CGI≤2 and no panic attack) were also evaluated. We examined single nucleotide polymorphisms (SNPs) using either known functional variants or tag SNPs within each gene and 10 kb flanking sequence which yielded a total of 47 SNPs. For *SLC6A4*, we examined the 5HTTLPR promoter variation including the embedded SNP (rs25531). Genes were screened using a set-based test in PLINK followed by single marker association tests for genes that reached gene-wide significance.

RESULTS: In the set-based test, *CREB1* achieved gene-wide significant association with improvement at 1-year follow-up (empirical p-value = 0.016). Out of the 5 *CREB1* single markers SNPs, two showed nominally significant

associations (rs7594560, p-value 0.003 and rs2253206, p-value 0.021), that did not remain significant after 10,000 permutations. In the haplotype analysis, we identified one haplotype significantly associated with 1-year response (p=0.0002). We did not observe experiment-wide association between CBT outcome and the remaining genes for either acute or 1-year response or remission.

CONCLUSION: Although limited by small sample size, these results provide preliminary evidence that variations in *CREB1* may be related to long-term CBT response among pharmacological-resistant patients with PD.

Introduction

Cognitive-behavioral therapy (CBT) has been established as an effective treatment for panic disorder (PD), and may be superior to pharmacotherapy for maintaining long-term treatment gains (Gould *et al.*, 1995). Despite the consistent evidence for the efficacy of CBT (Gould *et al.*, 1995, Otto & Deveney, 2005), many patients fail to respond to treatment. The nature of factors that underlie these individual differences in treatment response are not well understood (Gould *et al.*, 1995, Rosenbaum *et al.*, 1996).

The burgeoning field of pharmacogenetics has provided evidence that specific genetic variants may explain differential response and toxicity to medication treatment. Previous research has shown that individual response to pharmacotherapy in major depression (Kim *et al.*, 2006, Kraft *et al.*, 2005, Mcmahon *et al.*, 2006), schizophrenia (Wang *et al.*, 2007), generalized social anxiety disorder (Stein *et al.*, 2006), and other psychiatric disorders may be related to genetic variation. However, no study has evaluated the role of these genetic markers in panic disorder patients and CBT response.

The biological mechanism by which CBT exerts its therapeutic effect is still unknown. However, Linden (2006) proposed similarities in the biological mechanisms of psycho- and pharmacotherapy. Neuroimage studies have shown regional metabolic changes with psychotherapy that are similar to pharmacological treatment in patients with major depression (Brody *et al.*, 2001, Martin *et al.*, 2001) and anxiety disorders including obsessive compulsive

disorder (Baxter *et al.*, 1992, Schwartz *et al.*, 1996) and specific phobias (Paquette *et al.*, 2003). Hence, genes implicated in etiology and pharmacological treatments of anxiety disorders are plausible candidates to be evaluated in the CBT response for PD.

PD has been associated with polymorphisms in serotonin receptor 1A (HTR1A) (Broocks *et al.*, 2003), serotonin receptor 2A (HTR2A) (Maron *et al.*, 2005) and regulator of G-protein signalling 2 (RGS2) (Leygraf *et al.*, 2006) in humans. Animal models have shown that anxiety is related to cAMP responsive-element binding protein (CREB1) (Blendy, 2006), corticotropin-releasing hormone receptor (CRHR1) (Timpl *et al.*, 1998) and brain derived neurotrophic factor (BDNF) (Chen *et al.*, 2006). Regarding response to pharmacological treatment, Perna et al. showed that paroxetine treatment response in PD is related to the serotonin transporter gene (SLC6A4) (Perna *et al.*, 2005).

The aim of this study was to evaluate the association between CBT response in PD patients resistant to previous pharmacological treatment and variants in 7 genes previously implicated in the etiology or treatment of anxiety: BDNF, CREB1, RGS2, CRHR1, SLC6A4, HTR2A, HTR1A.

Methods

We examined the role of these genes in a cohort of PD patients treated in an open study of CBT with one year of naturalistic follow-up. Participants were recruited from the Anxiety Outpatient Clinic at the Hospital de Clínicas de Porto

Alegre from 1998 to 2006. All participants were referred to be treated with cognitive behavioral group therapy (CBGT) as an augmentation strategy after resistant pharmacological treatment i.e. patients had to have residual symptoms of PD such as panic attacks, anticipatory anxiety and phobic avoidance despite being on stable dose of medication for at least 4 months. Patients were advised not to change pharmacological treatment during CBT. CBGT was an adaptation of the procedures manualized (Otto *et al.*, 1996) and sessions are described detailed elsewhere (Heldt *et al.*, 2003). Patients were treated in one of the twelve groups (with a mean of 7 patients per group) offering 12-sessions of treatment over 4 months. Blood was collected from participants for DNA extraction during 2005 to 2006 after written informed consent. Institutional review board approval was obtained from the ethics committee (Number 04-272).

The inclusion criteria were as follows: (1) PD with or without agoraphobia, according to DSM-IV diagnostic criteria (Heldt *et al.*, 2003); (2) at least eighteen years old; (3) Clinical GIobal Impressions (CGI) ≥ 3 despite being on a stable dose of medications for at least 4 months (selective serotonin reuptake inhibitor, tricyclic antidepressant or venlafaxine, with or without benzodiazepines). Patients with mental retardation, dementia or other organic brain syndrome, psychotic disorders and disabling chronic disease were excluded. Patients with comorbidities common to PD were included in the study provided that the symptoms were not clinically more prominent than the PD symptoms.

Of the 110 patients that entered the treatment protocol, DNA was collected from 91 subjects. Only 82 Caucasians patients were included in order to minimize confusion due to population stratification. Ethnicity was defined based on phenotypic characteristics (Zembrzuski *et al.*, 2006) and ethnic background. Eight individuals with genotyping call rates less than 90% were excluded resulting on 74 patients included in the analysis. Figure 1 displays how the analytic sample was selected. The sample was predominantly female (79.7%), with a mean age of 39.51 (SD=11.26) years, mean age of PD onset of 29.83 (SD=10.47) years, and median PD duration of 8 year (P25=3 to P75=11). Patients included had been on pharmacological treatment for a median of 2 years (p25=1 to p75=4) before starting the CBGT. Five patients spontaneously stop their medication during the acute treatment, and eighteen patients stop it during the follow-up period. Thirteen patients (72%) that stop the medication were in remission in the 1-year follow-up.

The Mini International Neuropsychiatry Interview (MINI) - Brazilian version (Amorim, 2000) was used to confirm the PD diagnosis and establish comorbidity diagnoses. A semi-structured interview was used to access sociodemographic data and clinical history. Comorbidities included agoraphobia (90.54%), generalized anxiety disorder (33.78%), major depression (25.68%), social anxiety disorder (18.92%), and dysthymia (14.86%).

The Clinical Global Impressions scale (CGI) (Guy, 1976) and Panic Inventory (PI) (Heldt *et al.*, 2003) were used to access severity at baseline, after

the 12th session, and 1 year post treatment. At study entry, ratings were completed by the clinicians running the group blinded to genotype. Outcome ratings at the end of the treatment and at follow-up evaluations were completed by trained clinicians who did not participate in the group therapy or knew the results of the baseline ratings and the genotyping.

Two end-points were evaluated in this study: acute treatment (at the end of 12-session therapy) and one-year follow-up. Treatment response was defined by change in CGI score at each endpoint relative to baseline; and remission was defined as the absence panic attacks and CGI ≤ 2. Since this sample is composed by resistant patients, the 1-year endpoint could be a most adequate moment to evaluate CBT outcome because some patients could need more time to recover (Heldt *et al.*, 2006).

Genotyping was performed for HapMap single nucleotide polymorphisms (SNPs) selected within each gene and 10kb flanking region. For the CREB1 gene, SNPs were selected based on previous studies (Perlis *et al.*, 2007b, Perlis *et al.*, 2007c). For SLC6A4 we examined functional polymorphism including the embedded SNP (rs25531). For BDNF we examined functional Val66Met polymorphism. The Tagger program (De Bakker *et al.*, 2005) (http://www.broad.mit.edu/mpg/tagger/) was used to identify tag SNPs with a minor allele frequency of greater than 5% and minimum r^2 of 0.8. A total of 47 SNPs were thus selected for genotyping using mass spectrometry as previously described (Sklar *et al.*, 2002).

Genotyping of the polymorphism in the promoter of the serotonin transporter (5-HTTLPR) was performed with the following protocol - Genomic DNA (1.4 ng) was amplified in a of 7 µl reaction using KlenTaq DNA Polymerase (0.2 U), the proprietary KlenTaq Buffer (1X), dNTPs (200 µM each), 5% glycerol, Betaine (1 M) and the marker specific primers (0.2 µM). Amplification was performed with thirteen cycles of denaturation for 30 seconds at 93ºC, annealing for 30 seconds beginning at 61.5ºC and dropped 0.5º C every cycle and primer extension at 72ºC for 30 second, followed by 37 cycles of denaturation for 30 seconds at 93ºC, annealing for 30 seconds at 55º C and primer extension at 72ºC for 30; 72ºC for 1 hour. The amplified product (1µl) was combined with size standard (LIZ-250) before being analyzed on an ABI-3730. The long allele appears as a product of about 412 while the short allele shows a band at about 370. The genotype of the SNP embedded in the 5-HTTLPR (Wendland *et al.*, 2006) (rs25531) was assayed by digesting the PCR product with the restriction enzyme, *Msp*I (Hu *et al.*, 2006). This 10 µl reaction contains 2 µl of the PCR product, 1 µl of 10X restriction buffer (New England Biolabs), 1 µl of *Msp*I enzyme and 6 µl of water. The reaction was incubated at 37ºC for 1 hour. The digested product (1µl) was then combined with size standard (LIZ-250) before being analyzed on an ABI-3730. Post-digestion, the long allele with the A SNP allele appears as a product of about 320, the presence of the G SNP allele is indicated by the presence of a band at 148, while the short allele shows a band

at about 277. The final genotype was determined from the information from the analysis of both the digested and undigested PCR product.

In addition, markers were retained only if they met the following quality control criteria: (1) >90% genotype call rate; (2) minor allele frequency (MAF) >5%; (3) Hardy Weinberg equilibrium $P > 0.001$. Three duplicates were assessed in the experiment with 100% concordance. After applying these quality control filters, 42 SNPs were retained for the association analysis.

For an initial analysis, we screened each gene using the set-based test in PLINK (Purcell *et al.*, 2007) (http://pngu.mgh.harvard.edu/purcell/plink/) followed by single marker association tests for genes that reached gene-wide significance. The set-based test is based on calculating the average test statistic for the best three SNPs per gene region, and evaluating the significance of these set-statistics by permutation (10,000). Gene-wide accounts for multiple comparison within the gene, whereas experiment-wide significance accounts for multiple comparisons, controlling for the 42 polymorphisms evaluated by permutation. Haplotype analysis was performed for genes that reached the gene-wide significance. Haplotype analysis was corrected for number of haplotypes observed (minor allele frequencies [MAF] < .01) with Bonferroni's correction. The overall level of significance adopted was $\alpha=0.05$.

Results

Patients who provided DNA were generally similar to the full sample treated with CBGT. Since the DNA sample collection began after CBGT

longitudinal study initiation, those providing DNA samples were the ones in follow-up. However, there were no differences between baseline characteristics, outcome measures or co adjuvant treatments of patients included and those who did not provide blood sample (n=19).

A set-based test was performed with delta CGI and remission at acute treatment and 1-year follow-up. No association was found with the set-based test in remission at both end-points and neither for delta-CGI after acute-treatment. On the other hand, for the delta-CGI 1-year follow-up, a gene-wide association was found in CREB1 (Table 1). Single marker tests of 5 *CREB1* tag SNPs (Figure 2) showed two nominally significant associations (Table 2), which did not remain significant after 10,000 permutations. In light of previous studies indicating sex-specific effects of *CREB1* (Perlis *et al.*, 2007a, Perlis *et al.*, 2007b), we also tested for sex-related heterogeneity. No interaction was found in the gene-by-sex analysis (data not shown).

In the haplotype analysis, Hap4 was significantly associated with delta-CGI evaluated at 1-year (p=0.0002) that remained significant after Bonferroni´s correction, and two haplotypes were associated with worse response, but this association did not remain significant after Bonferroni´s correction (Table 2).

Discussion

This is the first study that evaluates the role of genetic markers in CBT response. Two SNPs in *CREB1* were nominally associated with improvement at

1 year follow-up treatment. *CREB1* is a nuclear transcription factor that mediates the effects of cAMP on gene expression (Montminy *et al.*, 1990) and has been previously related to anger and antidepressant-emergent suicidal ideation in depressed patients (Perlis *et al.*, 2007b, Perlis *et al.*, 2007c).

By what mechanism might *CREB1* influence response to CBT? One possibility is that *CREB1* mediate anger, a domain associated with impulsivity, aggressive and goal-directed behaviors (Lara & Akiskal, 2006). Recently, Lara & Akiskal (2006) have proposed a bidimensional model based on fear and anger traits which conceive mood, behavior and personality concomitantly. Interestingly, Perlis *et al.* (2007b) found an association between a haplotype and anger that is similar to the one found to be associated with PD improvement. Anger would be associated with desinhibited exploration and low perception of cues of danger (Lara & Akiskal, 2006), and probably facilitate exposition in CBT for PD. Moreover anxiety and stress is related to the "flight or fight response" (Mcewen, 2007) and PD patients who fight (anger) could have better improvement as compared to those that flight (fear). Besides, *CREB1* is also related to memory – an important hallmark for CBT (Garakani *et al.*, 2006). The connection between *CREB1* and long-term memory was established in several organisms (Garakani *et al.*, 2006), however the mechanisms that underlie the ability of CREB1 to facilitate memory are not completely understood (Carlezon *et al.*, 2005). *CREB1* activity in the amygdale serves as a molecular switch for the formation of long-term memory in fear conditioning in rats (Josselyn *et al.*, 2001).

The association between *CREB1* and CBT response might be mediated through personal characteristics as well as through memory modulation that can interfere in desensitization and exposure during the follow up treatment period.

The association with *CREB1* was found only in 1-year follow-up. Although outcomes cannot be attributed unequivocally to CBGT, the likelihood that our results are due to non-specific factors or time are unlikely given the history of chronicity of the illness (median duration of the disease was 8 years) and inadequate response to open pharmacotherapy for this sample (median 2 years of pharmacotherapy before starting CBT). Besides, it is also unlikely that this association is due to modification in medication status, since almost 30% of those who were in remission have spontaneously stopped their medication. It is possible that due to patient´s severity, they need more time to perform the exposure exercise and that is the reason why we found the association only in follow-up period. Another possibility is *CREB1* influence some characteristics toward “fight response”, that could be related to anger. These personal characteristics would become more important to patients’ recover in follow-up period, when exercise depends on their own motivation and not on the therapist.

There was no significant association between the remaining genes (*BDNF*, *SLC6A4*, *RGS2*, and *CRHR1*) and CBT response. We examined these genes in light of prior evidence that they may contribute to anxiety-related or treatment response phenotypes. For example, serum *BDNF* level has been related to CBT response in a sample of PD (Kobayashi *et al.*, 2005). *SLC6A4*

and other genes in the serotonin pathway (Broocks *et al.*, 2003) (Stahl, 1998) have been implicated in PD, although results have been inconclusive (Blaya *et al.*, 2007, Maron *et al.*, 2004, Perna *et al.*, 2005). A recent study (Leygraf *et al.*, 2006) reported association between four SNPs in *RGS2* and PD. Finally, extrahypothalamic CRH systems mediate a broad range of behavioral stress and anxiety responses (Heinrichs & Koob, 2004) and the CRH receptor 1 (*CRHR1*) is implicated in anxiogenic CRH actions (Contarino *et al.*, 1999, Muller & Wurst, 2004). We found no evidence that these loci are associated with response to CBGT for PD, although the small sample size examined here does not allow us to exclude a role for these genes.

Our results should be interpreted in light of some limitations. Most importantly, the small sample size means that our analyses may be subject to Type II error. Besides this, the association was found only in the follow-up period with the empirical p value and not after 10,000 permutations. The possible confounding due to population stratification in Brazilian was addressed by restricting our analyses to Caucasian subjects (Zembrzuski *et al.*, 2006), therefore these findings are restricted to this population. Gene's selection was performed in a no-systematic search, and association previously related to PD was not evaluated (Deckert *et al.*, 1999, Domschke *et al.*, 2003). Additionally, our finding could be restricted only to pharmacological resistant PD. Finally, the *CREB* polymorphisms examined here were chosen to tag genetic variation across the gene but are not known to have direct functional effects.

In sum, this study is the first analysis of genetic influences on response to CBT. We observed preliminary evidence that variants in *CREB1*, a gene implicated in the biological basis of anger, anxiety and memory, are associated with CBT outcome at 1 year. Clearly, additional larger studies are needed to further define the effect of *CREB1*, if any, on CBT response. However, if replicated, these findings may have implications for elucidating the neurobiology of CBT and identifying a potential subgroup of PD patients more likely to improve with this treatment modality.

References

Amorim, P. (2000) Mini International Neuropsychiatric Interview (MINI): validação de entrevista breve para diagnóstico de transtornos mentais. *Rev Bras Psiquiatr,* **22,** 106-115.

Baxter, L.R., Jr., Schwartz, J.M., Bergman, K.S., Szuba, M.P., Guze, B.H., Mazziotta, J.C., Alazraki, A., Selin, C.E., Ferng, H.K., Munford, P. & et al. (1992) Caudate glucose metabolic rate changes with both drug and behavior therapy for obsessive-compulsive disorder. *Arch Gen Psychiatry,* **49,** 681-689.

Blaya, C., Salum, G.A., Lima, M.S., Leistner-Segal, S. & Manfro, G.G. (2007) Lack of association between the Serotonin Transporter Promoter Polymorphism (5-HTTLPR) and Panic Disorder: a systematic review and meta-analysis. *Behav Brain Funct,* **3,** 41.

Blendy, J.A. (2006) The role of CREB in depression and antidepressant treatment. *Biol Psychiatry,* **59,** 1144-1150.

Brody, A.L., Saxena, S., Stoessel, P., Gillies, L.A., Fairbanks, L.A., Alborzian, S., Phelps, M.E., Huang, S.C., Wu, H.M., Ho, M.L., Ho, M.K., Au, S.C., Maidment, K. & Baxter, L.R., Jr. (2001) Regional brain metabolic changes in patients with major depression treated with either paroxetine or interpersonal therapy: preliminary findings. *Arch Gen Psychiatry,* **58,** 631-640.

Broocks, A., Meyer, T., Opitz, M., Bartmann, U., Hillmer-Vogel, U., George, A., Pekrun, G., Wedekind, D., Ruther, E. & Bandelow, B. (2003) 5-HT1A responsivity in patients with panic disorder before and after treatment with aerobic exercise, clomipramine or placebo. *Eur Neuropsychopharmacol,* **13,** 153-164.

Carlezon, W.A., Jr., Duman, R.S. & Nestler, E.J. (2005) The many faces of CREB. *Trends Neurosci,* **28,** 436-445.

Chen, Z.Y., Jing, D., Bath, K.G., Ieraci, A., Khan, T., Siao, C.J., Herrera, D.G., Toth, M., Yang, C., McEwen, B.S., Hempstead, B.L. & Lee, F.S. (2006) Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science,* **314,** 140-143.

Contarino, A., Heinrichs, S.C. & Gold, L.H. (1999) Understanding corticotropin releasing factor neurobiology: contributions from mutant mice. *Neuropeptides,* **33,** 1-12.

de Bakker, P.I., Yelensky, R., Pe'er, I., Gabriel, S.B., Daly, M.J. & Altshuler, D. (2005) Efficiency and power in genetic association studies. *Nat Genet,* **37,** 1217-1223.

Deckert, J., Catalano, M., Syagailo, Y.V., Bosi, M., Okladnova, O., Di Bella, D., Nothen, M.M., Maffei, P., Franke, P., Fritze, J., Maier, W., Propping, P., Beckmann, H., Bellodi, L. & Lesch, K.P. (1999) Excess of high activity monoamine oxidase A gene promoter alleles in female patients with panic disorder. *Hum Mol Genet,* **8,** 621-624.

Domschke, K., Kuhlenbaumer, G., Schirmacher, A., Lorenzi, C., Armengol, L., DiBella, D., Gratacos, M., Garritsen, H.S., Nothen, M.M., Franke, P., Sand, P., Fritze, J., Perez, G., Maier, W., Sibrowski, W., Estivill, X., Bellodi, L., Ringelstein, E.B., Arolt, V., Martin-Santos, R., Catalano, M., Stogbauer, F. & Deckert, J. (2003) Human nuclear transcription factor gene CREM: genomic organization, mutation screening, and association analysis in panic disorder. *Am J Med Genet B Neuropsychiatr Genet,* **117,** 70-78.

Garakani, A., Mathew, S.J. & Charney, D.S. (2006) Neurobiology of anxiety disorders and implications for treatment. *Mt Sinai J Med,* **73,** 941-949.

Gould, R., Otto, M.W. & Pollack, M. (1995) A meta-analysis of treatment outcome for panic disorder. *Clin Psychol Review,* **15,** 819-844.

Guy, M. (1976) *Assessment Manual for Psychopharmachology,* National Institute of Mental Health, Rockville, MD.

Heinrichs, S.C. & Koob, G.F. (2004) Corticotropin-releasing factor in brain: a role in activation, arousal, and affect regulation. *J Pharmacol Exp Ther,* **311,** 427-440.

Heldt, E., Gus Manfro, G., Kipper, L., Blaya, C., Isolan, L. & Otto, M.W. (2006) One-year follow-up of pharmacotherapy-resistant patients with panic disorder treated with cognitive-behavior therapy: Outcome and predictors of remission. *Behav Res Ther,* **44,** 657-665.

Heldt, E., Manfro, G.G., Kipper, L., Blaya, C., Maltz, S., Isolan, L., Hirakata, V.N. & Otto, M.W. (2003) Treating medication-resistant panic disorder: predictors and outcome of cognitive-behavior therapy in a Brazilian public hospital. *Psychother Psychosom,* **72,** 43-48.

Hu, X.Z., Lipsky, R.H., Zhu, G., Akhtar, L.A., Taubman, J., Greenberg, B.D., Xu, K., Arnold, P.D., Richter, M.A., Kennedy, J.L., Murphy, D.L. & Goldman, D. (2006) Serotonin transporter promoter gain-of-function genotypes are linked to obsessive-compulsive disorder. *Am J Hum Genet,* **78,** 815-826.

Josselyn, S.A., Shi, C., Carlezon, W.A., Jr., Neve, R.L., Nestler, E.J. & Davis, M. (2001) Long-term memory is facilitated by cAMP response element-binding protein overexpression in the amygdala. *J Neurosci,* **21,** 2404-2412.

Kim, W., Choi, Y.C., Yoon, K.S., Cho, D.Y., Pae, C.U. & Woo, J.M. (2006) Tryptophan hydroxylase and serotonin transporter gene polymorphism does not affect the diagnosis, clinical features and treatment outcome of panic disorder in the Korean population. *Prog Neuropsychopharmacol Biol Psychiatry,* **30,** 1413-1418.

Kobayashi, K., Shimizu, E., Hashimoto, K., Mitsumori, M., Koike, K., Okamura, N., Koizumi, H., Ohgake, S., Matsuzawa, D., Zhang, L., Nakazato, M. & Iyo, M. (2005) Serum brain-derived neurotrophic factor (BDNF) levels in patients with panic disorder: as a biological predictor of response to group cognitive behavioral therapy. *Prog Neuropsychopharmacol Biol Psychiatry,* **29,** 658-663.

Kraft, J.B., Slager, S.L., McGrath, P.J. & Hamilton, S.P. (2005) Sequence analysis of the serotonin transporter and associations with antidepressant response. *Biol Psychiatry,* **58,** 374-381.

Lara, D.R. & Akiskal, H.S. (2006) Toward an integrative model of the spectrum of mood, behavioral and personality disorders based on fear and anger traits: II. Implications for neurobiology, genetics and psychopharmacological treatment. *J Affect Disord,* **94,** 89-103.

Leygraf, A., Hohoff, C., Freitag, C., Willis-Owen, S.A., Krakowitzky, P., Fritze, J., Franke, P., Bandelow, B., Fimmers, R., Flint, J. & Deckert, J. (2006) Rgs 2 gene polymorphisms as modulators of anxiety in humans? *J Neural Transm,* **113,** 1921-1925.

Linden, D.E. (2006) How psychotherapy changes the brain--the contribution of functional neuroimaging. *Mol Psychiatry,* **11,** 528-538.

Maron, E., Nikopensius, T., Koks, S., Altmae, S., Heinaste, E., Vabrit, K., Tammekivi, V., Hallast, P., Koido, K., Kurg, A., Metspalu, A., Vasar, E., Vasar, V. & Shlik, J. (2005) Association study of 90 candidate gene polymorphisms in panic disorder. *Psychiatr Genet,* **15,** 17-24.

Maron, E., Tasa, G., Toru, I., Lang, A., Vasar, V. & Shlik, J. (2004) Association between serotonin-related genetic polymorphisms and CCK-4-induced panic attacks with or without 5-hydroxytryptophan pretreatment in healthy volunteers. *World J Biol Psychiatry,* **5,** 149-154.

Martin, S.D., Martin, E., Rai, S.S., Richardson, M.A. & Royall, R. (2001) Brain blood flow changes in depressed patients treated with interpersonal psychotherapy or venlafaxine hydrochloride: preliminary findings. *Arch Gen Psychiatry,* **58,** 641-648.

McEwen, B.S. (2007) Physiology and neurobiology of stress and adaptation: central role of the brain. *Physiol Rev,* **87,** 873-904.

McMahon, F.J., Buervenich, S., Charney, D., Lipsky, R., Rush, A.J., Wilson, A.F., Sorant, A.J., Papanicolaou, G.J., Laje, G., Fava, M., Trivedi, M.H., Wisniewski, S.R. & Manji, H. (2006) Variation in the gene encoding the serotonin 2A receptor is associated with outcome of antidepressant treatment. *Am J Hum Genet,* **78,** 804-814.

Montminy, M.R., Gonzalez, G.A. & Yamamoto, K.K. (1990) Regulation of cAMP-inducible genes by CREB. *Trends Neurosci,* **13,** 184-188.

Muller, M.B. & Wurst, W. (2004) Getting closer to affective disorders: the role of CRH receptor systems. *Trends Mol Med,* **10,** 409-415.

Otto, M.W. & Deveney, C. (2005) Cognitive-behavioral therapy and the treatment of panic disorder: efficacy and strategies. *J Clin Psychiatry,* **66 Suppl 4,** 28-32.

Otto, M.W., jones, J.C., Craske, M.G. & Barlow, D.H. (1996) *Stopping anxiety medication: Panic control therapy*

for benzodiazepine discontinuation (therapist guide). , Oxford University Press., Oxford.

Paquette, V., Levesque, J., Mensour, B., Leroux, J.M., Beaudoin, G., Bourgouin, P. & Beauregard, M. (2003) "Change the mind and you change the brain": effects of cognitive-behavioral therapy on the neural correlates of spider phobia. *Neuroimage,* **18,** 401-409.

Perlis, R.H., Beasley, C.M., Jr., Wines, J.D., Jr., Tamura, R.N., Cusin, C., Shear, D., Amsterdam, J., Quitkin, F., Strong, R.E., Rosenbaum, J.F. & Fava, M. (2007a) Treatment-associated suicidal ideation and adverse effects in an open, multicenter trial of fluoxetine for major depressive episodes. *Psychother Psychosom,* **76,** 40-46.

Perlis, R.H., Purcell, S., Fagerness, J., Cusin, C., Yamaki, L., Fava, M. & Smoller, J.W. (2007b) Clinical and genetic dissection of anger expression and CREB1 polymorphisms in major depressive disorder. *Biol Psychiatry,* **62,** 536-540.

Perlis, R.H., Purcell, S., Fava, M., Fagerness, J., Rush, A.J., Trivedi, M.H. & Smoller, J.W. (2007c) Association between treatment-emergent suicidal ideation with citalopram and polymorphisms near cyclic adenosine monophosphate response element binding protein in the STAR*D study. *Arch Gen Psychiatry,* **64,** 689-697.

Perna, G., Favaron, E., Di Bela, D., Bussi, R. & Bellodi, L. (2005) Antipanic efficacy of paroxetine and polymorphism within the Promoter of the Serotonin Transporter Gene. *Neuropsychopharmacology,* **30,** 2230-2235.

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A., Bender, D., Maller, J., Sklar, P., de Bakker, P.I., Daly, M.J. & Sham, P.C. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet,* **81,** 559-575.

Rosenbaum, J.F., Pollack, M.H. & Pollock, R.A. (1996) Clinical issues in the long-term treatment of panic disorder. *J Clin Psychiatry,* **57 Suppl 10,** 44-48; discussion 49-50.

Schwartz, J.M., Stoessel, P.W., Baxter, L.R., Jr., Martin, K.M. & Phelps, M.E. (1996) Systematic changes in cerebral glucose metabolic rate after successful behavior modification treatment of obsessive-compulsive disorder. *Arch Gen Psychiatry,* **53,** 109-113.

Sklar, P., Gabriel, S.B., McInnis, M.G., Bennett, P., Lim, Y.M., Tsan, G., Schaffner, S., Kirov, G., Jones, I., Owen, M., Craddock, N., DePaulo, J.R. & Lander, E.S. (2002) Family-based association study of 76 candidate genes in bipolar disorder: BDNF is a potential risk locus. Brain-derived neutrophic factor. *Mol Psychiatry,* **7,** 579-593.

Stahl, S.M. (1998) Mechanism of action of serotonin selective reuptake inhibitors. Serotonin receptors and pathways mediate therapeutic effects and side effects. *J Affect Disord,* **51,** 215-235.

Stein, M.B., Seedat, S. & Gelernter, J. (2006) Serotonin transporter gene promoter polymorphism predicts SSRI response in generalized social anxiety disorder. *Psychopharmacology (Berl),* **187,** 68-72.

Timpl, P., Spanagel, R., Sillaber, I., Kresse, A., Reul, J.M., Stalla, G.K., Blanquet, V., Steckler, T., Holsboer, F. & Wurst, W. (1998) Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. *Nat Genet,* **19,** 162-166.

Wang, L., Yu, L., He, G., Zhang, J., Zhang, A.P., Du, J., Tang, R.Q., Zhao, X.Z., Ma, J., Xuan, J.K., Xiao, Y., Gu, N.F., Feng, G.Y., Xu, M.Q., Xing, Q.H. & He, L. (2007) Response of risperidone treatment may be associated with polymorphisms of HTT gene in Chinese schizophrenia patients. *Neurosci Lett,* **414,** 1-4.

Wendland, J.R., Martin, B.J., Kruse, M.R., Lesch, K.P. & Murphy, D.L. (2006) Simultaneous genotyping of four functional loci of human SLC6A4, with a reappraisal of 5-HTTLPR and rs25531. *Mol Psychiatry,* **11,** 224-226.

Zembrzuski, V.M., Callegari-Jacques, S.M. & Hutz, M.H. (2006) Application of an African Ancestry Index as a genomic control approach in a Brazilian population. *Ann Hum Genet,* **70,** 822-828.

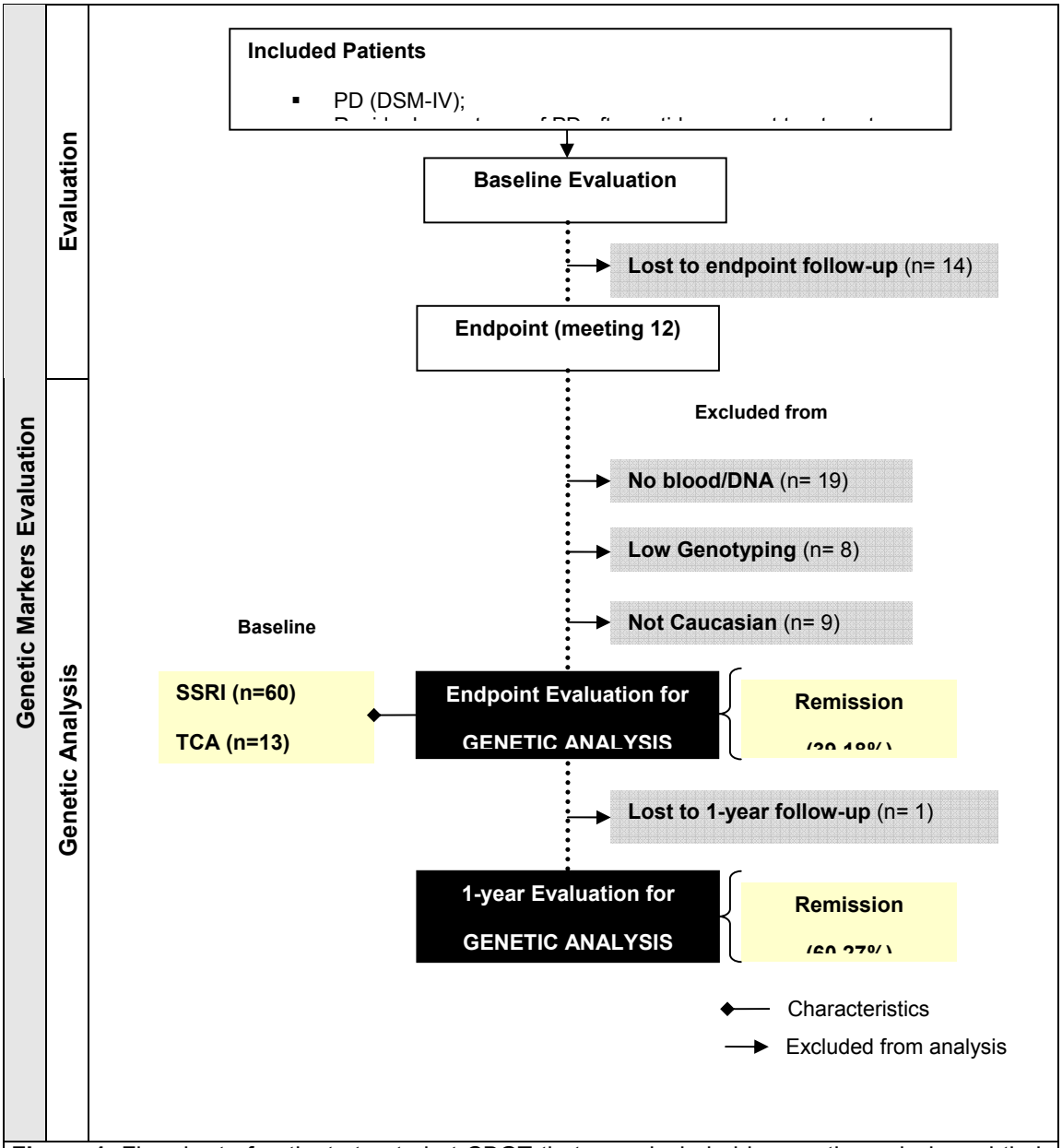

Figure 1. Flowchart of patients treated at CBGT that were included in genetic analysis and their characteristics.

Abbreviations: SSRI, Selective serotonin reuptake inhibitor; TCA, Tryciclic Antidepressant.

Table 1 Set-based test for the association between Polymorphisms (PMs) and Delta-CGI (Baseline CGI – 1-year CGI) at 1-year follow-up (n=73)

Set	PMs in the Set	PMs	T-value	Empirical p-value 0	Empirical p-value 1	Empirical p-value 2
BDNF	P1	rs6265	0.006	1	1	1
CREB	P1	rs7594560	9.961	0.013	0.020	0.111
	P2	rs2253206	7.765	0.009	0.013	0.079
	P3	rs1862952	5.839	0.011	**0.016**	0.098
CRHR1	P1	rs9892359	1.316	0.915	0.931	1
	P2	rs17690314	1.13	0.917	0.932	1
	P3	rs242944	0.867	0.941	0.953	1
HTR1A	P1	rs6295	0.0381	0.995	0.996	1
	P2	rs4521432	0.0238	0.995	0.995	1
	P3	rs6449693	0.0159	0.995	0.995	1
HTR2A	P1	rs6313	0.789	0.472	0.485	0.994
	P2	rs6311	0.621	0.441	0.455	0.988
RGS2	P1	rs2746073	0.723	0.867	0.886	1
	P2	rs3856223	0.720	0.827	0.850	1
	P3	rs10921267	0.689	0.796	0.820	1
SLC6A4	P1	Biallelic	0.802	0.380	0.380	0.979
SLC6A4	P1	Triallelic	0.079	0.805	0.805	1

A multi-marker set-based test implemented in PLINK was used for the analysis of all the genes. Sets that have gene-wide association p-value ($p<0.05$) are shown in bold. In each set, P1 is the best PMs associated with the outcome; P2 is the second one and P3 is the third one. Maximum computed permutations were 10000.
Empirical p-value P_0: p-value for average chi-square without correction.
Empirical p-value P_1: p-value corrected for all tests within this gene.
Empirical p-value P_2: p-value corrected for all tests within the experiment.
Abbreviations: PMs, Polymorphisms; CGI, Clinical Global Impression; BDNF, Brain Derived Neurotrophic Factor; CREB, cAMP responsive-element binding protein; RGS2, regulator of G-protein signalling g; CRHR1, corticotropin-releasing hormone receptor; HTR2A, Serotonin receptor 2A; HTR1A, Serotonin receptor 1A; SLC6A4, Serotonin transporter gene.

Table 2 Association between individual SNP and Haplotype in CREB gene and delta-CGI at 1-year follow-up (n=73)

SNP	rs1862952	rs2253206	rs7569963	rs7594560	rs4675690	Frequency	β	R^2	Wald (t)	p-value (asymptotic)	p-value corrected
Hap1	A	A	A	T	T	0.01	0.798	0.006	0.681	0.498	>0.999
Hap2	T	G	G	T	T	0.02	-0.452	0.004	-0.550	0.584	>0.999
Hap3	A	A	G	T	T	0.09	-0.435	0.019	-1.176	0.244	>0.999
Hap4	A	G	G	C	T	0.09	1.111	0.171	3.832	**0.0002**	**0.002**
Hap5	A	G	G	T	T	0.18	-0.036	<0.001	-0.124	0.902	>0.999
Hap6	T	A	A	T	C	0.06	0.017	<0.001	0.042	0.967	>0.999
Hap7	A	A	A	T	C	0.19	-0.067	<0.001	-0.256	0.799	>0.999
Hap8	T	A	G	T	C	0.02	-1.985	0.086	-2.592	**0.012**	0.12
Hap9	A	A	G	T	C	0.04	-1.695	0.080	-2.484	**0.015**	0.15
Hap10	A	G	G	T	C	0.29	0.061	<0.001	0.240	0.811	>0.999
HWE	1	0.34	0.76	0.14	0.47						
Allele change	A/T	A/G	A/G	C/T	C/T						
Minor allele	T	A	A	C	T						
MAF	0.11	0.43	0.26	0.10	0.40						
β	-0.439	-0.478	-0.016	0.873	0.282						
SE	0.311	0.203	0.230	0.277	0.208						
R^2	0.027	0.073	<0.001	0.123	0.025						
Wald (t)	-1.410	-2.360	-0.068	3.156	1.358						
p-value (asymptotic)	0.163	**0.021**	0.946	**0.002**	0.179						
p-value (empirical, corrected)	>0.999	0.998	>0.999	0.929	>0.999						

Abbreviations: CGI, Clinical Global Impression; CREB, cAMP responsive-element binding protein; SNP, Single Nucleotide Polymorphism; HWE- Hardy Weinberg Equilibrium p value; MAF, Minimal Allele Frequency; β, regression coefficient; r^2 regression r-squared. **Bold** means p<0.05.

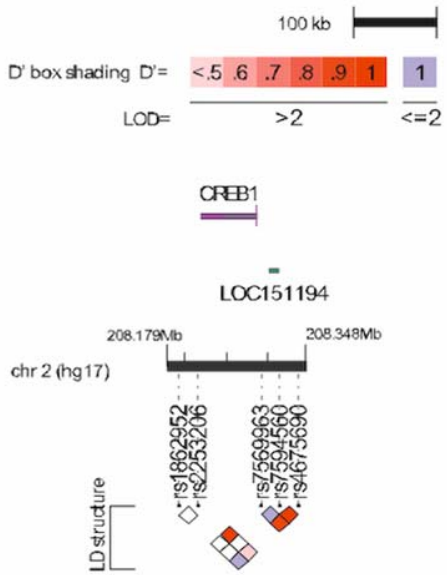

Figure 2. Location of single nucleotide polymorphisms (SNPs) that span the CREB1 gene. LOD indicates logarithmic odds; Chr, chromosome; Hg 17, Human Genome Sequence 17 (Single Nucleotide Polymorphisms database, National Center for Biotechology Information, build 35, May 2004); LD, linkage disequilibrium; and Mb, megabases.

14. Considerações finais

Dentre os diversos fatores etiológicos envolvidos no TP, a vulnerabilidade genética está bem estabelecida como um fator de risco, uma vez que a herdabilidade para esse transtorno é de 28 (14) a 43% (10). No entanto, os estudos com genes candidatos não demostraram resultados muito consistentes.

O gene transportador da serotonina, que é um gene candidato natural para ser investigado nos Transtornos de Ansiedade devido a sua associação aos Transtornos de Humor (71) e ao temperamento de evitação de danos (75), não foi associado ao TP em diversos estudos prévios. Alguns autores freqüentemente argumentam que a ausência de associação nos estudos em genética pode ser por falta de poder dos estudos, pois se espera um efeito muito pequeno para um único polimorfismo conferir risco a um transtorno. No entanto, no estudo 1 pode-se constatar com a meta-análise a ausência de associação do polimorfismo 5-HTTLPR com o Transtorno do Pânico (RC=0,91, IC95% 0,80 a 1,03, p=0,14). Três subanálises foram realizadas nessa meta-análise divididas por etnia, qualidade do grupo controle e comorbidade com Agorafobia, e também não foi identificada nenhuma associação com esse gene. Entretanto, o tamanho amostral incluído nesse estudo foi de cerca de 1000 pacientes com TP, justificando então a realização de outros estudos para vir a identificar um possível efeito do gene no TP com menor magnitude. Além disso, nenhum estudo havia avaliado o *5-HTTLPR* na sua forma trialélica no TP.

Realizamos então o estudo 2 com um dos objetivos de avaliar o *5-HTTLPR* na forma bialélica e trialélica no TP e, assim como na meta-análise, não encontramos qualquer associação. O diferencial desse estudo foi que ele contemplou a medida de interação do gene com traumas na infância. Ao contrário do que ocorreu na depressão (71), não encontramos qualquer interação do gene-ambiente. Esses resultados devem ser considerados com cautela, pois esse é o primeiro estudo no TP que avaliou esse tipo de interação. Um estudo prévio encontrou associação entre abuso emocional ou abuso físico e sensibilidade à ansiedade (193), um fenótipo intermediário que está intimamente ligado ao TP. Diante desses achados é possível levantar algumas hipóteses. A primeira é de que nosso estudo não possui poder suficiente para identificar a interação do *5-HTTLPR* com trauma, uma vez que foram incluídos apenas 232 sujeitos. Curiosamente, o achado de Stein *et al* (193) foi descrito em apenas 150 sujeitos, mas como o fenótipo avaliado era uma medida contínua, e não dicotômica como no nosso estudo, é possível que esse tamanho amostral tenha sido suficiente.

Outra hipótese é de que a interação gene-ambiente com o *5-HTTLPR* ocorre apenas para um aspecto ligado ao TP – a sensibilidade à ansiedade – e não ao transtorno como um todo. Isso fundamenta a pesquisa de fenótipos intermediários, que possivelmente são mais adequados para os estudos de associação em comparação com a nosologia psiquiátrica proposta pelo DSM-IV (1). No estudo 2 optou-se por não avaliar fenótipos intermediários, uma vez que

o número excessivo de testes implicaria uma correção ainda maior do valor p. Determinamos uma única medida para cada covariável avaliada na interação (pai protetor, mão protetora e trauma), mas como foram testados 8 marcadores, o número de testes já nos pareceu excessivo para incluir também os fenótipos intermediários. Nossa abordagem difere da adotada por Stein *et al* (193) que foi utilizar duas subescalas do CTQ, ao invés de avaliar uma medida mais global, em dois fenótipos intermediários: neuroticismo e sensibilidade à ansiedade. Apesar de realizar 12 testes (sensibilidade à ansiedade, escala de sintomas físicos da sensibilidade à ansiedade e neuroticismo para a forma bialélica e trialélica e duas covariáveis, abuso emocional e físico), os autores não fizeram correção para múltiplos testes. Baseado nos achados dos estudos 1 e 2, concluímos que o *5-HTTLPR* não apresenta um papel maior na genética do TP nem mesmo quando considerada sua forma trialélica ou a interação com trauma, apesar de existir evidência de influenciar fenótipos intermediários associados à ansiedade.

Outros genes foram também pesquisados, e naturalmente os receptores da serotonina *HTR1A* e *HTR2A* são genes candidatos. Um polimorfismo do *HTR1A* havia sido associado previamente ao TP, mas com resultados contraditórios (88, 95). No nosso achado replicamos a associação do alelo G do polimorfismo localizado no promotor (rs6295) com TP e identificamos outros 2 SNPs do *HTR1A* associados ao TP. Os alelos de menor freqüência desses polimorfismos constituem um haplótipo de risco para o TP, enquanto que os

alelos complementares conferem proteção ao transtorno. As associações encontradas com o *HTR1A* não sofreram interação com nenhuma medida ambiental avaliada no nosso estudo. Possivelmente, estudos de meta-análise poderão avaliar qual a real contribuição desse gene.

Já o *HTR2A* havia sido associado ao TP em 2 estudos independentes (12, 95), mas esse achado não foi replicado em 2 outros estudos (88, 96). No nosso estudo não identificamos associação entre esse gene e o TP. No entanto, quando considerada a interação com pais pouco superprotetores e muito afetivos, a presença de alelo T em dois polimorfismos do *HTR2A* conferiu proteção ao TP. Esse achado replica parcialmente o que foi recentemente reportado para a Depressão por Jokela *et al* (196). Ansiedade e Depressão são comorbidades freqüentes, e alguns autores sugerem que ambas compartilham fatores de risco comuns (218). Apesar de o achado prévio ter sido descrito com mães e o nosso com pais, os achados nos parecem congruentes, pois existe uma contribuição diferente para o cuidado materno e paterno dentro de cada cultura (21). Parece também que variações no *HTR2A* influenciam o indivíduo a aproveitar o suporte do ambiente familiar (podendo ser tanto o pai, quanto à mãe) para evitar o desenvolvimento tanto de Ansiedade quanto Depressão.

Além de replicar associações com genes previamente associados ao TP, essa tese sugere que o *EFHC2* está associado ao TP e a dois fenótipos intermediários associados à ansiedade (comportamento inibido e temperamento evitador de dano). A força desse achado está na congruência da associação

com esses três fenótipos. O *EFHC2* havia sido previamente associado à percepção do medo (180), levando então ao desenvolvimento do nosso estudo 3. Caso esse achado seja replicado, possivelmente poderá contribuir para a compreensão do neurocircuito do medo.

Já o estudo 4 propõe pela primeira vez avaliar a influência de variações gênicas na resposta à Terapia Cognitivo-Comportamental. Nesse estudo avaliamos variações no *5-HTTLPR, HTR1A, HTR2A, BDNF, CREB1, RGS2* e *CRHR1* e encontramos que variações no *CREB1* estavam associadas à melhor resposta à TCC. O *CREB1* havia sido previamente associado à raiva, e na análise de haplótipos encontramos que o haplótipo associado à raiva foi também associado com a melhor resposta à TCC. A ansiedade está associada à resposta de “luta ou fuga”, e nosso estudo sugere que aqueles pacientes que lutam (raiva) respondem melhor à TCC que os que fogem (medo).

Por fim, essa tese versa sobre alguns aspectos relacionados ao TP, abrangendo desde a revisão sistemática da literatura, até estudos de associação, de interação gene-ambiente e de resposta à TCC. Esses estudos estão de acordo com a proposta de estudos para o século 21 (219), incluindo a pesquisa de vários genes e análise de múltiplos lócus. Como perspectiva futura, essa linha de pesquisa será ampliada, com o aumento do tamanho amostral, a pesquisa de novos genes candidatos, a avaliação de outros fenótipos intermediários e a análise de interação gene-ambiente e gene-gene na busca de um melhor entendimento da genética do TP.

15. Anexo A: Termo de Consentimento Informado

Termo de Consentimento Informado para Pacientes

Estamos realizando uma pesquisa sobre a influência genética na evolução de pacientes com Transtorno do Pânico. A causa do Transtorno do Pânico é multifatorial, ou seja, existem alguns fatores genéticos e ambientais que predispõe à doença. O objetivo desse estudo é verificar se existe alguma predisposição genética que influencie no modo como os indivíduos lidam com os estressores ambientais.A avaliação constará de uma entrevista psiquiátrica e um exame de sangue que identificará alguns genes que são associados a ansiedade. O tratamento será escolhido de acordo com as indicações clínicas e desejo do paciente, podendo ser farmacológico ou psicoterápico. Os pacientes serão reavaliados clinicamente anualmente e inquiridos quanto a estressores ambientais no ano anterior à entrevista. A não participação do estudo não acarretará em nenhum prejuízo no seu atendimento no HCPA.

Eu ..... fui informado dos objetivos acima descritos e da justificativa para qual estão sendo utilizadas as escalas de forma clara e detalhada. Sei que poderei solicitar novos esclarecimentos e que, a qualquer momento, terei liberdade de retirar meu consentimento da participação da pesquisa, sem prejudicar meu atendimento no HCPA.

O (a) pesquisador(a) ..... certificou-me de que as informações por mim fornecidas terão caráter confidencial e no caso de divulgação serão sem identificação e unicamente para fins de pesquisa.

Porto Alegre, ... de ..... de ..... .

Assinatura do paciente .....

Assinatura do pesquisador .....

Contato com pesquisadora: Carolina Blaya – Fone: 3316 82 72

Pesquisador Responsável: Gisele Gus Manfro

Adendo ao Termo de Consentimento Informado para Pacientes

Estamos realizando uma pesquisa sobre a influência genética na evolução de pacientes com Transtorno do Pânico que você já está participando conforme Consentimento prévio. Atualmente estamos desenvolvendo uma parceria com uma Universidade do exterior que dispõe de maior tecnologia para o estudo desses fatores genéticos. Com essa parceria, iremos avaliar um outro fator genético que vem sendo relacionado ao Transtorno do Pânico. Esses estudos serão desenvolvidos em parceria entre o nosso grupo de pesquisa e essa instituição do Exterior.

Eu ..... fui informado dos objetivos acima descritos e da justificativa para qual minha amostra será enviada para o exterior e concordo.

Como existem estudos novos na área da genética a cada ano, o seu sangue poderá ser armazenado para futuras pesquisas. No entanto, a cada nova pesquisa, os pesquisadores entrarão em contato com você para obter um novo consentimento informado.

Concordo com o armazenamento do material biológico ( ) sim

( ) não

O (a) pesquisador(a) ..... certificou-me de que as informações por mim fornecidas terão caráter confidencial e no caso de divulgação serão sem identificação e unicamente para fins de pesquisa.

Porto Alegre, ... de ..... de ..... .

Assinatura do paciente .....

Assinatura do pesquisador .....

Contato com pesquisadora: Carolina Blaya – Fone: 2101 82 72

Pesquisador Responsável: Gisele Gus Manfro

Termo de Consentimento Informado para os Controles

Estamos realizando um banco de dados genéticos de indivíduos sem patologia psiquiátrica. Sabe-se que a etiologia dos transtornos psiquiátricos é multifatorial, ou seja, existem alguns fatores genéticos e ambientais que predispõe às doenças. O objetivo desse estudo é estabelecer um banco de dados genéticos de indivíduos normais para que possa ser utilizado como controle nas pesquisas psiquiátricas. A avaliação constará de uma entrevista psiquiátrica e um exame de sangue que identificará alguns genes que estão sendo estudados nos transtornos psiquiátricos. Se for identificado algum problema psiquiátrico, você será encaminhado para tratamento. O anonimato dos dados será garantido através de um banco de códigos, ou seja, os seus dados não serão identificados pelo seu nome, e sim por um número. A não participação do estudo não acarretará em nenhum prejuízo no seu trabalho no HCPA.

Eu ..... fui informado dos objetivos acima descritos e da justificativa para qual serão utilizados os dados de forma clara e detalhada. Sei que poderei solicitar novos esclarecimentos e que, a qualquer momento, terei liberdade de retirar meu consentimento da participação da pesquisa, sem prejudicar meu trabalho no HCPA. O (a) pesquisador(a) ..... certificou-me de que as informações por mim fornecidas terão caráter confidencial e no caso de divulgação serão sem identificação e unicamente para fins de pesquisa.

Porto Alegre, ... de ..... de ..... .

Assinatura do paciente .....

Assinatura do pesquisador .....

Pesquisadora: Carolina Blaya – Fone: 2101 82 72 - 3333 40 78

Pesquisador Responsável: Gisele Gus Manfro

Adendo ao Termo de Consentimento Informado para Controles

Conforme consentimento informado prévio, você faz parte de um banco de controles de patologia psiquiátrica. Estamos realizando uma pesquisa sobre a influencia genética no Transtorno do Pânico, para verificar se os pacientes com esse transtorno possuem alguma variação no material genético. Para isso, precisamos comparar os resultados dos pacientes com um grupo controle.

Eu……..fui informado dos objetivos acima descritos e concordo que avaliem marcadores genéticos na minha amostra do meu material genético.

Como existem estudos novos na área de genética a cada ano, o seu material genético poderá ser armazenado para futuras pesquisas. No entanto, a cada nova pesquisa, os pesquisadores entrarão em contato com você para obter um novo consentimento informado.

Concordo com o armazenamento do material biológico ( ) sim

( ) não

O pesquisador(a) …….. certificou-me de que as informações por mim fornecidas terão caráter confidencial e no caso de divulgação serão sem a identificação e unicamente para fins de pesquisa.

Porto Alegre, ……. de …… de …….

Assinatura do paciente ……..

Assinatura do pesquisador …….

Contato com pesquisadora – Carolina Blaya – Fone 2101 82 72

Pesquisador Responsável – Gisele Gus Manfro

16. Anexo B: Produção científica durante o período do doutorado

Com o objetivo de aumentar a amostra de pacientes com TP para os estudos de associação em genética, foi desenvolvido um ensaio clínico aberto com Milnaciprano. Esse estudo resultou em uma publicação em anexo nessa tese.

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Original article 1

The efficacy of milnacipran in panic disorder: an open trial

Carolina Blaya, Ana Carolina Seganfredo, Marina Dornelles, Mariana Torres, Angela Paludo, Elizeth Heldt and Gisele Gus Manfro

The aim of this study is to evaluate the efficacy of milnacipran in the acute treatment of patients with panic disorder. Thirty-one patients who met *Diagnostic and Statistical Manual of Mental Disorders-IV* criteria for panic disorder with or without agoraphobia were included in the study. Patients were initially treated with milnacipran 25 mg twice daily and then 50 mg twice daily until the 10th week. The treatment outcome and panic disorder severity were determined by the Panic Disorder Severity Scale, Panic Inventory, Clinical Global Impression and Hamilton Anxiety Scale, all of which were applied during every evaluation interview. Quality of life (WHOQOL-*bref*) was evaluated at baseline and at the end of the study. Missing data were handled by using the last observation carried forward for all participants who had taken at least one dose of study medication. Intention-to-treat was used in the analyses. Pharmacological treatment resulted in a clinically and statistically significant mean reduction in all severity measures. Remission (Clinical Global Impression ≤ 2) was obtained in 58.1% of the sample. Regarding WHOQOL, we found a significant improvement ($P < 0.05$) across treatment in all the domains studied. Although results may be influenced by the open design of this pilot study and by the small sample size, our findings suggest that milnacipran may be effective for the treatment of panic disorder and justify further research. *Int Clin Psychopharmacol* 00:000–000 © 2007 Lippincott Williams & Wilkins.

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Keywords: efficacy, milnacipran, open-trial, panic disorder

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Introduction

Panic disorder (PD) is characterized by panic attacks, which are recurrent and followed by anticipatory anxiety and phobic avoidance (Association, 1994). This disorder usually has a chronic course, complicated by agoraphobia, impairing participants' quality of life (Candilis *et al.*, 1999; Mendlowics and Stein, 2000).

The lifetime prevalence of PD is between 1.5 and 3.5% and is two to three times more frequent in women, usually beginning in young adulthood (Kessler *et al.*, 1994).

Serotoninergic dysfunction is present in PD (Inada *et al.*, 2003). Panic patients have a decrease in this neurotransmitter in some cerebral regions (Versiani *et al.*, 2002). In addition, the response shown by panic patients when treated with selective serotonin reuptake inhibitors (SSRIs) and the worsening when using serotoninergic agonist have corroborated this serotoninergic dysfunction hypothesis (Versiani *et al.*, 2002; Inada *et al.*, 2003). Nowadays the SSRI are the most commonly used medication for PD (Bakker *et al.*, 2002; Baldwin *et al.*, 2005).

There is also a considerable body of evidence to implicate norepinephrine in the pathophysiology of PD. Indeed, the locus coeruleus, which contains the highest concentration of norepinephrine cell bodies in the brain, is known to be involved in mediating fear and anxiety responses (Charney, 1998; Coplan and Lydiard, 1998). Studies in patients with PD using agents with some degree of selectivity for the noradrenergic system have shown efficacy. Lofepramine, a norepinephrine selective tricyclic antidepressant (TCA), was shown to be at least as effective as clomipramine in reducing both the symptoms and the number of panic attacks experienced by patients (Fahy *et al.*, 1992). On the other hand, desipramine, which is also mainly selective on norepinephrine reuptake, was less effective than clomipramine in a double-blind crossover trial (Sasson *et al.*, 1999). Reboxetine, a selective norepinephrine reuptake inhibitor, has significantly improved the clinical profile of patients with PD (Versiani *et al.*, 2002), but maprotiline had almost no effect in PD (Den Boer and Westenberg, 1988).

It has been suggested that there may be an imbalance between norepinephrine and serotonin systems leading to heterogeneity in PD. Thus, one group of patients may experience panic as a result of dysfunction of the norepinephrine system, and others as a result of dysfunction on the serotonin system (Versiani *et al.*, 2002). Alternatively, dysfunction at the level of a

downstream pathway common to both the serotonin and norepinephrine (SNRI) systems may be responsible, and therefore agents acting on either system specifically could be effective. Venlafaxine, a dual-action drug that selectively inhibits the reuptake of SNRI, has been shown to be effective in PD (Pollack *et al.*, 1996) and is considered by some authors as a first line treatment for PD (Baldwin *et al.*, 2005).

Milnacipran is SNRI that generally has similar efficacy to TCAs and SSRI in the acute and long-term treatment of depression (Spencer and Wilde, 1998). Unlike the TCA, however, and owing to its singular chemical structure, which is directly associated to an inhibitory action at norepinephrine and serotonin neuronal reuptake systems, milnacipran shows no relevant affinity to other neurotransmitter receptors (postsynaptic adrenergic, muscarinic and histamine receptors). Therefore, this new antidepressant has a potential tolerance profile superior to that of the TCA, with a reduced propensity to produce undesirable antimuscarinic effects.

The pharmacokinetic profile of milnacipran is characterized by rapid absorption, high bioavailability, low protein binding and rapid elimination. This gives milnacipran certain pharmacokinetic advantages, such as low interindividual variation in plasma levels, low potential for drug interactions and limited impact on hepatic cytochrome P450 systems (Puozzo *et al.*, 2002). When comparing milnacipran to venlafaxine and mirtazapin, which were proved to be efficient in the PD treatment (Pollack *et al.*, 1996; Sarchiapone *et al.*, 2003; Manfro and Blaya, 2004), it was found that venlafaxine and mirtazapin in lower doses have a predominantly serotoninergic action, contrasting with the predominantly serotoninergic and noradrenergic action of milnacipran.

Recent studies in animal models have raised the idea that milnacipran may be effective in anxiety disorders (Bourin *et al.*, 2005; Moojen *et al.*, 2006). No open or controlled trials, however, evaluating the efficacy of milnacipran in the treatment of patients with PD exist. Although controlled trials are trustworthy to assure efficacy of a medication, they can only be justified after open trials are performed.

The aim of the present study was to determine the efficacy and effectiveness of milnacipran in the acute treatment of patients with PD with or without agoraphobia.

Method

Study design

Milnacipran was administered in an open 10-week trial using a flexible dosing design. All participants, after 1 week of placebo, started on a regimen of milnacipran 25 mg twice daily. This dose was increased to 50 mg twice daily, when tolerated. The decision to increase or decrease a participant's dose was based on the treating psychiatrist's judgment of clinical response assessed by the rating scales and the absence of side effects. No other psychotropic drug or psychotherapy treatment was allowed during this trial. All patients gave their informed consent. The study was approved by the local ethical committee (number 04-225).

Study procedures

Participants in the study were recruited from clinical referrals and local media advertisements. Patients referred to the study were prescreened by an interview to determine eligibility and willingness to participate. Screening included a clinical interview, which was confirmed with the structured clinical interview Mini International Neuropsychiatric Interview – Portuguese version 5.0. (Amorim, 2000). Patients who met the inclusion criteria and agreed to participate in the study were eligible to the study.

Patient selection

Inclusion criteria

Outpatients from 18–65 years old who met *Diagnostic and Statistical Manual of Mental Disorders-IV* criteria for primary diagnosis of PD were eligible for the study. Patients should have presented at least 1 panic attack a week in the 4 weeks before entering in the study and at least 1 complete attack in the week before the study. Patients with comorbidity with mild major depression or anxiety disorders could be included if their major symptoms were related to PD. Patients should have Hamilton Anxiety (HAM-A) score above 18 and Hamilton Depression (HAM-D) below 18, be in good clinical condition, and be using safe contraceptive methods. Patients with previous pharmacological treatment could be enrolled in the study after a wash-out of three half-lives. Patients with panic attacks and Clinical Global Impression (CGI) above 2 after 1-week placebo treatment were to be included in this trial.

Exclusion criteria

Individuals were excluded if they had (i) comorbidities involving any psychotic disorder, bipolar disorder, mental retardation or any neurological disease; (ii) alcohol or drugs dependence or abuse during the previous 6 months; (iii) any history of hypersensitivity to milnacipran; (iv) Current cognitive-behavioral or psychodynamic treatment for PD (individual or group); (v) patients with other anxiety or mood disorders whose symptoms were more severe than those of PD; (vi) patients with suicide risk in the 6 months before the study; (vii) patients taking psychotropic medications in the 4 weeks before the study and whose interruption was not recommended; (viii) patients who were currently breastfeeding, pregnant or unwilling or unable to take adequate contraceptive

precautions; (ix) patients with a HAM-D score equal to or above 18.

Assessment

The CGI determines the global severity of the disease according to the frequency and intensity of panic attacks, level of anticipatory anxiety, level of phobic avoidance, a decrease in quality of life as well as the environmental maladaptation to the disorder. Its scores vary from 1 (normal, not ill) to 7 (extremely ill) (Guy, 1976).

The Panic Inventory is an instrument that characterizes panic attacks as spontaneous, situational, complete or incomplete. It also characterizes their intensity, duration and frequency, and the severity of the agoraphobia and the anticipatory anxiety.

The Hamilton Anxiety scale (HAM-A) (Hamilton, 1959) quantifies anxiety through its 14 items and scores vary from 0 to 4, according to severity. The Hamilton Depression (Hamilton, 1969) scale evaluates the severity of depressive symptoms (17 items). Participants with scores < 7 are normal, 7–17 are mild, 18–24 are moderate and > 25 are severe.

The Panic Disorder Severity Scale (Shear *et al.*, 1997) is composed of seven items with five alternatives that evaluate frequency and intensity of panic attacks, anticipatory anxiety, phobic avoidance, physical avoidance, and professional and social impairment.

The WHOQOL-*bref* evaluates quality of life. It is a self-applied instrument composed of 26 items that evaluate four domains of quality of life (physical, psychological, social relationships and environmental) (Anonymous, 1998; Fleck *et al.*, 2000).

Efficacy evaluation

The primary efficacy variables were the CGI scores, which were assessed at weeks 1, 2, 4, 6, 8 and 10.

Secondary variables included scores on HAM-A, Panic Disorder Severity Scale, Panic Inventory and WHOQOL-*bref*. All severity scales were assessed at weeks 1, 2, 4, 6, 8 and 10. The WHOQOL-*bref* was assessed at baseline and at week 10.

Remission was defined as CGI < 2 (Rapaport *et al.*, 2001) and improvement was defined according to decrease of 50% on the severity scales.

Safety measure

Side effects were assessed by spontaneous report and by a brief checklist of those commonly associated with SNRI. The checklist comprised 24 items. Each item was scored on a 4-point scale, with higher scores indicating greater distress (0 = none, 1 = mild, 2 = moderate, 3 = severe). The checklist was completed at each session, including the first and the second interviews (baseline and after 1-week of placebo, respectively).

Statistical analysis

Missing data were handled by using the last observation carried forward for all participants who had taken at least one dose of the study medication. On the quality of life analysis, only patients who had completed the protocol were included. Participants who dropped out during the placebo run-in were excluded from the analyses ($n = 5$). Changes in baseline scores on the outcome measures were analyzed with either a paired-samples t-test or a repeated-measures analysis of variances. Categorical data were compared using χ^2 test (with Yates correction) and Fisher's exact test; Levene's test and Kolmogorov–Smirnov test were used for normality and homogeneity of variances. Effect size for treatment outcome was calculated with the Cohen formula ($ES = M_1 - M_2 / \sqrt{SD_1^2 + SD_2^2 - 2 \times r \times SD_1 \times SD_1}$). All analyses were conducted using the Statistical Package for the Social Sciences 14.0 version for Windows and all statistical tests were two-tailed with $\alpha = 0.05$.

Results

A flow diagram of the patients in the study is presented in Fig. 1. Out of a total of 77 screened PD patients, 37 patients were treated with 1 week of placebo treatment. One patient was excluded owing to response to placebo and five patients were excluded owing to intolerance to placebo side effects. Thirty-one patients (10 male, mean age 37 + 12 years) were finally eligible for the study and were included in the intention-to-treat efficacy analyses and safety population. Two patients withdrew from the study owing to non-compliance and one patient had a clinical intercurrence and was not able to remain in the study. The mean drop-out time was 6 weeks (range 2–8 weeks). The mean milnacipran dosage was 91 mg which was achieved by week 4.

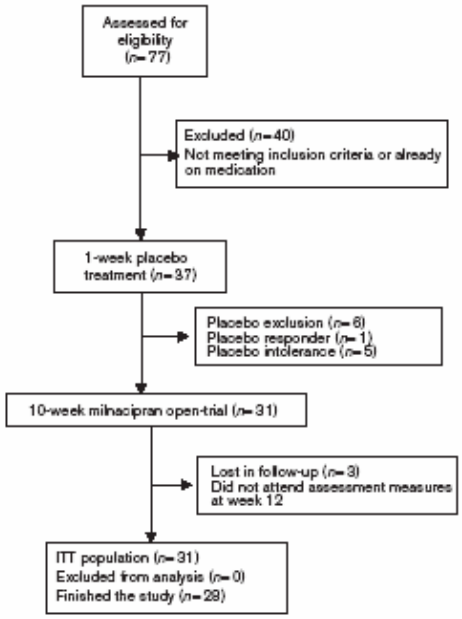
Efficacy measures

Table 1 depicts treatment outcome measures on all severity scales. Fifteen patients (48.4%) became free of panic attacks after the treatment. Pharmacological treatment resulted in a clinically and statistically significant mean reduction in all severity measures. Remission (CGI ≤ 2) was obtained in 18 patients (58.1% of the sample). When using a stricter criterion of remission (CGI < 2 and no panic attacks) (Heldt *et al.*, 2003), 14 patients (45.2%) achieved this criterion. Improvement (i.e. decrease higher than 50% of initial symptoms) was obtained in 14 patients (45.2%) when using the Hamilton Anxiety scale, 19 patients (61.3%) when using the agoraphobia measure and 18 patients (58.1%) when using the anticipatory anxiety measure.

Regarding WHOQOL, we found a significant improvement ($P < 0.05$) across treatment in all domains studied (Table 2).

The analysis of the remission predictors resulted in no significant effect related to mean age, age of onset, sex, baseline severity or mean milnacipran dose (χ^2 and independent t-test, P = NS).

Fig. 1



Flowchart of patients' progress through the phases of the study. ITT, intention-to-treat.

Safety measures

No unexpected adverse effects occurred. Most of the treatment-emergent adverse events reported during the study were mild in severity. Table 3 lists the most commonly reported treatment-emergent adverse-effects, that is, reported by at least 5% of the treated patients. Only four patients had severe side effects (two had dysuria, one had nausea and one had constipation). After a dose decrease, such effects became mild and all the patients remained in the study.

Discussion

This open trial provides preliminary evidence that milnacipran may be efficacious and well tolerated for the treatment of PD. Patients had improvement in all efficacy measures and 58% of the participants were in remission by the end of the 10-week trial. A statistically significant improvement in CGI was observed from the fourth week of treatment, and the improvement continued progressively until the end of the study. Furthermore, the drop-out numbers during milnacipran open-trial were in agreement with data found in literature (Kimura *et al.*, 2002) and were below those which were found in the placebo week. The drop-out numbers seen in our study were below those reported with TCAs and SSRI (Bakker *et al.*, 2002).

In general, milnacipran was well tolerated in this group of participants. Only four patients had serious side effects, which were easily controlled with dose decrease. Side effects, such as constipation and dry mouth, were primarily related to the anticholinergic properties of milnacipran. We also found a high incidence of dysuria which is consistent with previous reports on milnacipran (Puech *et al.*, 1997). This effect can be explained by the facilitation of sympathetic neurotransmission caused by norepinephrine reuptake inhibition in the periphery (Deakin and Dursun, 2002).

It is interesting to emphasize the effect size found with milnacipran in anticipatory anxiety which was above 2.0 (see Table 1). On the other hand, only 48% of the patients became free of panic attacks after milnacipran treatment, a result that differs from a meta-analysis (Bakker *et al.*, 2002) that reported that 60% of patients treated with TCA and 55% with SSRI became free of

Table 1 Treatment outcome measures on severity scales

	Baseline mean (SD)	Week 1 mean (SD)	Week 2 mean (SD)	Week 4 mean (SD)	Week 6 mean (SD)	Week 8 mean (SD)	Week 10 mean (SD)	F(6,180)	P	Effect size[a]
PDSS	15.97 (3.43)[b]	12.84 (4.40)[b]	11.03 (5.55)[b]	8.42 (5.01)[b]	6.48 (4.82)[b]	4.81 (4.37)[b]	4.90 (4.85)[b]	59.762	<0.001	1.71
Hamilton Anxiety	24.29 (7.70)[b]	19.74 (7.00)[b]	17.61 (7.73)[b]	14.61 (7.12)[b]	12.87 (6.14)[b]	11.97 (7.07)[b]	11.42 (8.32)[b]	30.534	<0.001	1.08
Agoraphobia	7.39 (2.68)[b]	6.77 (2.99)[b]	5.94 (3.73)[b]	5.35 (3.29)[b]	4.03 (3.97)[b]	3.61 (3.77)[b]	3.2 (3.4)[b]	13.952	<0.001	1.08
Anticipatory anxiety	8.65 (2.19)[b]	8.81 (1.16)[b]	7.32 (3.16)[b]	6.13 (3.23)[b]	5.26 (3.16)[b]	4.39 (3.74)[b]	3.87 (3.04)[b]	18.772	<0.001	2.14
CGI	4.39 (0.66)[b]	3.97 (0.83)[b]	3.61 (0.91)[b]	3.19 (0.91)[b]	2.77 (0.84)[b]	2.61 (0.71)[b]	2.35 (0.87)[b]	44.096	<0.001	1.89

CGI, clinical global impression; PDSS, panic Disorder Severity Scale.
[a]Effect size was calculated based on Hedt between week 1 and week 10.
[b]Equal letters means did not differ in Bonferroni adjustment.

Table 2 **Changes in quality of life after milnacipran treatment**

	Baseline mean (SD)	Week 10 mean (SD)	*t*	df.	*P*[a]	Effect size
Physical domain	47.8 (18.7)	67.3 (19.4)	−5.90	27	<0.001	1.02
Psychological domain	46.3 (16.9)	62.8 (18.9)	−6.59	27	<0.001	0.92
Social domain	56.8 (21.8)	65.8 (17.5)	−3.17	27	0.004	0.45
Environmental domain	49.4 (14.3)	58.4 (13.9)	−3.88	27	0.001	0.63
General Quality of life	52.7 (17.5)	62.5 (18.0)	−2.92	27	0.007	0.55

[a]Paired *t*-test.

Table 3 **Most common treatment-emergent adverse events (new or worsened symptoms)**

Adverse event	Frequency *N*(%)
Constipation[b]	18 (58.1)
Hot flashes	18 (58.1)
Nausea[b]	18 (58.1)
Anxiety	18 (58.1)
Dyspepsia	17 (54.8)
Tiredness	16 (51.6)
Agitation	16 (51.6)
Sweating	15 (48.4)
Headache	15 (48.4)
Abdominal pain	13 (41.9)
Nervousness	13 (41.9)
Dry mouth	14 (45.2)
Sexual dysfunction	12 (38.7)
Insomnia	12 (38.7)
Somnolence	12 (38.7)
Palpitation	12 (38.7)
Erectile dysfunction[a]	4 (40.0)
Dizziness	11 (35.5)
Decreased libido	10 (32.3)
Ejaculation impairment[a]	3 (30.0)
Tremors	9 (29.0)
Vomiting	7 (22.6)
Dysuria[b]	4 (12.9)
Anorgasmia	5 (16.1)

[a]On the basis of the number of men.
[b]Patient had severe side effects and had to decrease medication dosage.

panic attacks. Studies in rodents (Moret *et al.*, 1985) and humans (Vaishnavi *et al.*, 2004) showed that milnacipran preferentially blocks the uptake of norepinephrine when compared with serotonin by approximately 2:1. Perhaps the lower activity of milnacipran in serotonin is what led to this intermediate result in panic attacks which is close to some placebo reports (Rapaport *et al.*, 2000). The norepinephrine action may have led to a better response in anticipatory anxiety. These findings provide data that may contribute to elucidate the role of neurotransmitters in different clinical aspects of PD.

Quality of life has been a new valuable variable used in clinical trials to evaluate response (Rapaport *et al.*, 2000). Some studies suggested that quality of life in patients with PD is as impaired as that for patients with major depression, and worse than in other chronic diseases such as hypertension, type 2 diabetes, chronic obstructive pulmonary disease and osteoarthritis (Candilis *et al.*, 1999). That is the reason why changes in this measure are an important outcome for clinical studies in PD, potentially capturing changes associated with active treatment compared with placebo (Rapaport *et al.*, 2000). Our study provides evidence that milnacipran is effective in improving quality of life of patients with PD despite having a small improvement in panic attacks. This is in accordance with a previous study which suggested that symptoms in anticipatory anxiety and avoidance appear to be more important than episodic panic attacks in affecting quality of life (Heldt *et al.*, 2006).

The analysis of remission predictors did not show any variable related to this outcome. This data differ from a previous study with sertraline in which age of onset and severity were predictors of treatment outcome (unpublished data). Maybe the small sample size of this open trial has not enabled the identification of the usual predictors of treatment response.

The major limitations of this study are the small number of patients, the absence of a placebo control group and of randomization procedures, all of which limited the generalizability of these findings. In addition, an extension to 12 or more weeks could have resulted in further improvement in the partial responders.

Despite the limitations common to all open-label studies, the results of this trial suggest that milnacipran may be an effective short-term treatment for PD. Findings from this study, however, suggest that a more rigorous randomized, double-blind, placebo-controlled trial to access the efficacy of milnacipran in the treatment of PD is justified.

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References

Amorim P (2000). Mini International Neuropsychiatric Interview (MINI): validação de entrevista breve para diagnóstico de transtornos mentais. *Rev Bras Psiquiatr* 22:106–115.

Anonymous (1998). Development of the World Health Organization WHOQOL-BREF quality of life assessment. The WHOQOL Group. *Psychol Med* 28:551–558.

Association AP (1994). *Diagnostic and statistical manual of mental disorders (DSM-IV)*. 4th ed. Washington, DC: American Psychiatric Association.

Bakker A, van Balkom AJ, Spinhoven P (2002). SSRIs vs. TCAs in the treatment of panic disorder: a meta-analysis. *Acta Psychiatr Scand* 106:163–167.

Baldwin DS, Anderson IM, Nutt DJ, Bandelow B, Bond A, Davidson JR, et al. (2005). Evidence-based guidelines for the pharmacological treatment of anxiety disorders: recommendations from the British Association for Psychopharmacology. *J Psychopharmacol* 19:567–596.

Bourin M, Masse F, Dailly E, Hascoet M (2005). Anxiolytic-like effect of milnacipran in the four-plate test in mice: mechanism of action. *Pharmacol Biochem Behav* 81:645–656.

Candilis PJ, McLean RY, Otto MW, Manfro GG, Worthington JJ, Penava SJ, et al. (1999). Quality of life in patients with panic disorder. *J Nerv Ment Dis* 187:429–434.

Charney DS (1998). Monoamine dysfunction and the pathophysiology and treatment of depression. *J Clin Psychiatry* 59 (Suppl 14):11–14.

Coplan JD, Lydiard RB (1998). Brain circuits in panic disorder. *Biol Psychiatry* 44:1264–1276.

Deakin B, Duraun S (2002). Optimizing antidepressant treatment: efficacy and tolerability. *Int Clin Psychopharmacol* 17 (Suppl 1):S13–S24.

Den Boer JA, Westenberg HG (1988). Effect of a serotonin and noradrenaline uptake inhibitor in panic disorder; a double-blind comparative study with fluvoxamine and maprotiline. *Int Clin Psychopharmacol* 3:59–74.

Fahy GT, Easty DL, Cdlum LM, Benedict-Smith A, Hillery M, Parsons DG (1992). Randomised double-masked trial of lodoxamide and sodium cromoglycate in allergic eye disease. A multicentre study. *Eur J Ophthalmol* 2:144–149.

Fleck MP, Louzada S, Xavier M, Chachamovich E, Vieira G, Santos L, Pinzon V (2000). Application of the Portuguese version of the abbreviated instrument of quality life WHOQOL-bref. *Rev Saude Publica* 34:178–183.

Guy M (1976). *Assessment manual for psychopharmacology*. Rockville, MD: National Institute of Mental Health.

Hamilton M (1959). The assessment of anxiety states by rating. *Br J Med Psychol* 32:50–55.

Hamilton M (1969). Standardised assessment and recording of depressive symptoms. *Psychiatr Neurol Neurochir* 72:201–205.

Heldt E, Blaya C, Isolan L, Kipper L, Teruchkin B, Otto MW, et al. (2006). Quality of life and treatment outcome in panic disorder: cognitive behavior group therapy effects in patients refractory to medication treatment. *Psychother Psychosom* 75:183–186.

Heldt E, Manfro GG, Kipper L, Blaya C, Maltz S, Isolan L, et al. (2003). Treating medication-resistant panic disorder: predictors and outcome of cognitive-behavior therapy in a Brazilian public hospital. *Psychother Psychosom* 72:43–48.

Inada Y, Yoneda H, Koh J, Sakai J, Himei A, Kinoshita Y, et al. (2003). Positive association between panic disorder and polymorphism of the serotonin 2A receptor gene. *Psychiatry Res* 118:25–31.

Kessler RC, McGonagle KA, Zhao S, Nelson CB, Hughes M, Eshleman S, et al. (1994). Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. *Arch Gen Psychiatry* 51:8–19.

Kimura M, Kanetani K, Imai R, Suzuki H, Isayama K, Endo S (2002). Therapeutic effects of milnacipran, a serotonin and noradrenaline reuptake inhibitor, on post-stroke depression. *Int Clin Psychopharmacol* 17:121–125.

Manfro GG, Blaya C (2004). ITranstorno do Pânico. In: Cordioli A, editor. *Psicofármacos*. Porto Alegre: Artmed. pp.

Mendlowicz MV, Stein MB (2000). Quality of life in individuals with anxiety disorders. *Am J Psychiatry* 157:669–682.

Moojen VK, Martins MR, Reinke A, Feier G, Agostinho FR, Cechin EM, Quevedo J (2006). Effects of milnacipran in animal models of anxiety and memory. *Neurochem Res* 31:571–577.

Moret C, Charveron M, Finberg JP, Couzinier JP, Briley M (1985). Biochemical profile of midalcipran (F 2207), 1-phenyl-1-diethyl-aminocarbonyl-2-amino-methyl-cyclopropane (Z) hydrochloride, a potential fourth generation anti-depressant drug. *Neuropharmacology* 24:1211–1219.

Pollack MH, Worthington JJ 3rd, Otto MW, Maki KM, Smoller JW, Manfro GG, et al. (1996). Venlafaxine for panic disorder: results from a double-blind, placebo-controlled study. *Psychopharmacol Bull* 32:667–670.

Puech A, Montgomery SA, Prost JF, Sdlea A, Briley M (1997). Milnacipran, a new serotonin and noradrenaline reuptake inhibitor: an overview of its antide-pressant activity and clinical tolerability. *Int Clin Psychopharmacol* 12:99–108.

Puozzo C, Panconi E, Deprez D (2002). Pharmacology and pharmacokinetics of milnacipran. *Int Clin Psychopharmacol* 17 (Suppl 1):S25–S35.

Rapaport MH, Pollack M, Wolkow R, Mandelian J, Clary C (2000). Is placebo response the same as drug response in panic disorder? *Am J Psychiatry* 157:1014–1016.

Rapaport MH, Wolkow R, Rubin A, Hackett E, Pollack M, Ota KY (2001). Sertraline treatment of panic disorder: results of a long-term study. *Acta Psychiatr Scand* 104:289–298.

Sarchiapone M, Amore M, De Risio S, Carli V, Faia V, Potenzo F, et al. (2003). Mirtazapine in the treatment of panic disorder: an open-label trial. *Int Clin Psychopharmacol* 18:35–38.

Sasson Y, Iancu I, Fux M, Taub M, Dannon PN, Zohar J (1999). A double-blind crossover comparison of clomipramine and desipramine in the treatment of panic disorder. *Eur Neuropsychopharmacol* 9:191–196.

Shear MK, Brown TA, Barlow DH, Money R, Sholomskas DE, Woods SW, et al. (1997). Multicenter collaborative panic disorder severity scale. *Am J Psychiatry* 154:1571–1575.

Spencer CM, Wilde MI (1998). Milnacipran. A review of its use in depression. *Drugs* 56:405–427.

Vaishnavi SN, Nemeroff CB, Plott SJ, Rao SG, Kranzler J, Owens MJ (2004). Milnacipran: a comparative analysis of human monoamine uptake and transporter binding affinity. *Biol Psychiatry* 55:320–322.

Versiani M, Cassano G, Perugi G, Benedetti A, Mastalli L, Nardi A, Savino M (2002). Reboxetine, a selective norepinephrine reuptake inhibitor, is an effective and well-tolerated treatment for panic disorder. *J Clin Psychiatry* 63:31–37.