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ORIGINAL PAPER

Parent of origin effect and allelic expression imbalance of the serotonin transporter in bipolar disorder and suicidal behaviour

Crystal Pinto · Renan P. Souza · Diane Lioult · Mawahib Semeralul · James L. Kennedy · Jerry J. Warsh · Albert H. Wong · Vincenzo De Luca

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Abstract Suicide and suicidal behaviour are a major health concern worldwide particularly in patients with mood disorders. Family, adoption and twin studies show that genetics influences suicidal behaviour. The serotonin transporter (5HTT) plays an important role in the pathophysiology of mood disorders and may also be involved in suicidal behaviour since 5HTT binding is decreased in the brain of suicide completers. Because the effect of genomic imprinting in the 5HTT gene on suicidal behaviour has not been investigated, we analysed the parent-of-origin effect (POE) of four 5HTT markers and the differential expression of the 5HTT G2651T (rs1042173) alleles in suicide attempters affected by bipolar disorder. We performed a family based association study and ETDT/QTDT analyses of the rs25531, HTTLPR, VNTR-2 and G2651T polymorphisms in 312 nuclear families with at least one subject affected by bipolar disorder. The main outcomes investigated in this study are bipolar disorder diagnosis, suicide attempts, suicidal behaviour severity and age at onset of bipolar disorder. We also compared the allele-specific mRNA levels in lymphoblastoid cells from 13 bipolar suicide attempters and 8 bipolar non-suicide attempters. Allele 2651T was transmitted significantly more often to bipolar patients ($P = 0.042$). There was no significant difference between maternal and paternal transmission ratios. Furthermore, there was no significant difference in the ratio of T/G-specific mRNA expression between bipolar attempters and non-attempters. These data do not support a role for differential allelic expression of 5HTT for suicidal behaviour in bipolar disorder. Small sample size and the fact that RNA was obtained from lymphoblastoid cell lines were some of the limitations of this study.

Keywords Suicide · 5HTT · Imprinting · Parent of origin effect · Bipolar disorder · TDT

A. Wong and V. De Luca have contributed equally to the preparation of this manuscript.

C. Pinto · D. Lioult · M. Semeralul · A. H. Wong · V. De Luca
Neurobiology of Schizophrenia Section, Neuroscience Department, Centre for Addiction and Mental Health Toronto, Toronto, ON, Canada

C. Pinto · R. P. Souza · J. L. Kennedy · J. J. Warsh · A. H. Wong · V. De Luca
Department of Psychiatry, University of Toronto, Toronto, ON, Canada

R. P. Souza · J. L. Kennedy · V. De Luca
Neurogenetics Section, Neuroscience Department, Centre for Addiction and Mental Health Toronto, Toronto, ON, Canada

J. J. Warsh · A. H. Wong
Department of Pharmacology, University of Toronto, Toronto, ON, Canada

J. J. Warsh
Cellular and Molecular Pathophysiology Section, Neuroscience Department, Centre for Addiction and Mental Health Toronto, Toronto, ON, Canada

A. H. Wong (✉)
Centre for Addiction and Mental Health, 250 College Street, Room 711, Toronto, ON M5T 1R8, Canada
e-mail: albert.wong@utoronto.ca

Introduction

Suicide is an important contributor to morbidity and mortality in individuals with mental disorders. Mental disorders, especially depression, are present in more than 90%

of suicides [13]. The lifetime suicide risk is 19% in bipolar disorder patients [10]. Between 25 and 60% of BD patients make at least one suicide attempt during the course of their illness [4]. Furthermore, in BD, the duration of untreated illness is associated with suicide attempts and multiple attempts [2] and interventions aimed to treat mood disorders significantly reduce the rate of suicide and suicide attempts [7].

The serotonin (5-HT) neurotransmitter system has been implicated in the pathogenesis of mood disorders, and the 5-HT transporter (5-HTT) is a major target for antidepressants [15]. The 5-HTT plays a significant role in the action of antidepressants [19] and as such has been widely studied. Specifically, the 5-HTT gene has been considered a candidate for bipolar disorder [1], with an association between bipolar disorder and the 5-HTT gene-linked polymorphic region (5-HTTLPR) and the intron 2 variable number of tandem repeat (VNTR) polymorphism [5]. Several studies have demonstrated an association of the 5-HTTLPR polymorphism with depression in the context of stressful life events [3, 9], though a recent meta-analysis of published studies did not confirm these findings [18]. The 5-HTTLPR polymorphism has also been associated with depression in patients with chronic psychotic disorders [6]. Because the 5-HTT gene is important in the study of mood disorders, we investigated the effects of 5-HTT polymorphisms on the clinical manifestations of bipolar disorder.

The 5-HTTLPR polymorphism may also affect risk of suicide or suicidal behaviour [11], so this phenotype was examined in the current study as the primary outcome variable. Specifically, the 5-HTTLPR, rs25531, intron 2 VNTR and 3′UTR polymorphisms were studied for their association with suicide attempts in patients with bipolar disorder. Although suicidal behaviour is the main focus of this study, we also examined the potential relationship between 5-HTT polymorphisms and age of onset as an indicator of disease severity [20].

Finally, we sought to determine whether variation in the 5-HTT gene was associated with risk of bipolar disorder itself, by studying 312 families with at least one member diagnosed with bipolar disorder type I or type II. Although previous studies have not shown a parent-of-origin effect on the transmission of bipolar disorder [8], we hypothesize here that polymorphic genomic imprinting may also affect risk of bipolar disorder. Thus, we examined differential allelic expression of the 5-HTT gene in lymphoblastoid cell lines from patients with bipolar disorder. Despite previous evidence of differential mRNA expression between 5-HTT alleles [12], it is not clear whether this differential expression is related to clinical variables. Thus, we investigated the effect of 5-HTT allelic imbalance on suicide attempts.

Methodology

Patient selection and inclusion

Ascertainment and clinical characterization of the sample have previously been described [16]. In brief, the subjects investigated were from 312 nuclear families, each with at least one proband (118 men and 194 women) affected with DSM-IV bipolar disorder type I or type II. In addition, 26 siblings with bipolar disorder were included in the family based analysis. The total sample consisted of 1,043 subjects: 350 bipolar disorder patients (131 men and 219 women) and 693 unaffected relatives. Suicidal behaviour was assessed as the presence/absence of a suicide attempt at any time (lifetime suicidal behaviour). Furthermore, we assessed suicidal behaviour as a continuous variable from 0 to 5 considering the severity of suicidal behaviour as follows: 0 = absence of suicidal behaviour; 1 = thoughts of death; 2 = suicidal ideation; 3 = suicide plan; 4 = suicide attempt and 5 = violent suicide attempt.

There were 91 patients with a diagnosis of bipolar type II. The mean (SD) age at contact was 35 (10.69) and 36 (10.67) years for male and female patients, respectively. The overall mean (SD) age at onset was 20.22 (7.61) years. There were 86 patients that attempted suicide at least once and most attempts were during a major depressive episode after the onset of the illness (Table 1; Fig. 1).

Genetic analysis in the family sample

The 3′UTR polymorphism (rs1042173) genotyping was performed using commercially available TaqMan® allelic discrimination assays on the ABI 7500 Sequence Detection System (ABI), following the manufacturer's protocol for the SNP markers. The HTTLPR and the VNTR-2 repeat polymorphisms were typed using agarose gel electrophoresis, and the rs25531 was typed by RFLP (MspI).

Table 1 Clinical and demographics in suicide attempters and non-attempters and allelic expression data

	Attempters	Non-Attempters
n	13	8
Men/women	4/9	2/6
Age	38.57 ± 9.8	44.8 ± 7.7
Onset	16.2 ± 7.7	21.8 ± 8.7
T/G ratio	1.37 ± 0.83	1.26 ± 0.6
Alcohol use comorbidity (Y/N)	2/11	3/5
Drug use comorbidity (Y/N)	3/10	3/5

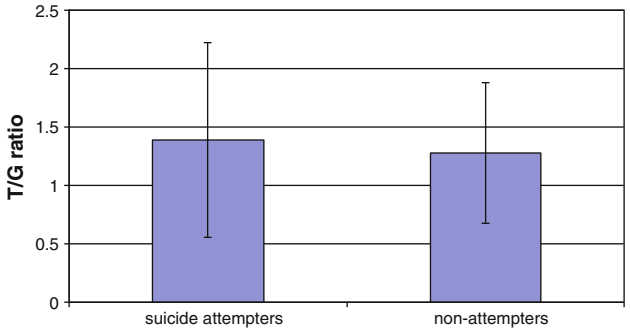

Fig. 1 Differential allelic expression comparison between suicide attempters and non-attempters. Data are displayed as means and standard deviations

Statistical analysis in the family sample

Genetic association tests and transmission disequilibrium test (TDT) analyses of the 3′UTR SNP and HTLPR repeat were performed using ETDT for the binary trait and QTDT for the quantitative trait, thus permitting separate analyses of paternal and maternal transmissions. Differences in effect size between maternal and paternal transmission ratios were assessed by calculating Pearson's χ^2 values.

RNA and DNA analysis in lymphoblastoid cells

RNA and genomic DNA were extracted from transformed B lymphoblasts derived from 21 bipolar disorder subjects. B lymphoblast cell lines were generated by Epstein–Barr virus induced transformation. Diagnosis was established using a SCID interview (DSM-IV criteria), supplemented by review of medical records. Complementary DNA (cDNA) was synthesized according to the Omniscript Protocol for Reverse Transcription (Qiagen). Allele-specific expression of 3′UTR mRNA was measured with quantitative PCR (qPCR) using the TaqMan® Assays-on Demand. Differential allelic-specific expression analysis was carried out on an ABI Prism 7500 Sequence Detection System (Applied Biosystems Inc., Foster City, CA). All reactions were performed in quadruplicate, and the investigators performing the PCR (D.L. and M.S.) were blind to the diagnosis of the tissue donors until after the data were analysed. Differential allele expression was calculated by subtracting qPCR threshold cycle (*C*t) values for the two alleles (*C*t-T−*C*t-G = delta*C*t). The allele 1 probe (G) was marked with FAM, and allele 2 probe (T) was marked with VIC. With assays based on allelic-specific probes, the heterozygote ratio may deviate from one because of differential binding efficiencies of the probes, rather than differences in expression levels. To correct for this possible bias, the average delta*C*t of the genomic DNA from a control sample (reference sample or calibrator) was subtracted from the delta*C*t of the cDNA sample from brain (deltadelta*C*t). The formula for deltadeltaCt calculation was deltadelta*C*t = (*C*t-T−*C*t-G) sample − (*C*t-T−*C*t-G) calibrator. Relative levels of T to G allele mRNA (i.e. T/G ratio) were calculated by the 2−deltadeltaCt method.

Statistical analysis in lymphoblastoid cells

The T/G 3′UTR mRNA expression ratio for each diagnostic group was expressed as the mean ± standard deviation. Differences in means between groups were evaluated using the independent *t* tests. Correlations between mRNA levels and potential confounding factors were evaluated using the Pearson's coefficient (age and sex). All test *P* values are two-tailed, and the level of significance was set at $P = 0.05$. The tests were not corrected for multiple testing because the analysis was exploratory in nature.

Results

Primary outcome analysis: suicidal behaviour

When we examined suicidal behaviour as the phenotype, none of the polymorphisms studied were significantly associated with severity of suicidal ideation, although the 3′UTR polymorphism approached significance ($P = 0.056$). The G allele of the 3′UTR polymorphism showed a trend towards being associated with greater suicidal ideation severity ($r = 0.164$). For maternally transmitted alleles, allele 10 of the VNTR was significantly associated with reduced suicidal ideation ($r = -0.083$, $P = 0.043$), while allele 12 of VNTR was associated with a trend towards higher suicidal ideation scores that approached significance ($r = 0.233$, $P = 0.051$). None of the other maternally transmitted alleles had a statistically significant association with suicidal behaviour scores. Furthermore, there were no statistically significant relationships between paternally transmitted alleles and suicidal behaviour as calculated by QTDT. Of the polymorphisms tested, none was significantly associated with suicide attempts. Similarly, there was no statistically significant imprinting from maternal or paternal alleles when suicide attempts were analysed.

Secondary outcome analysis: bipolar disorder and age at onset

Of the 5-HTT polymorphisms studied, only the 3′UTR polymorphism was significantly associated with bipolar disorder ($P = 0.042$). Allele T of the 3′UTR was the risk allele, with a relative risk ratio of 1.346 compared to allele G. None of the other polymorphisms were significantly

associated with the diagnosis of bipolar disorder. The association of allele T of the 3′UTR was significant and stronger for paternally transmitted alleles (RR = 1.728, $P = 0.038$). For maternally transmitted alleles, none of the polymorphisms showed any significant association with the diagnosis of bipolar disorder.

Regarding the age of onset of bipolar disorder, the short allele of the HTTLPR polymorphism was significantly associated with earlier onset of disease ($r = -0.170$, $P = 0.0209$), when compared to the long allele. Furthermore, when considering the phased haplotype for the markers rs25531 and HTTLPR, the association between age at onset and the haplotype A-Long approached significance ($P = 0.0545$) with a trend towards later onset ($r = 0.007$). The haplotype G-Long was not significantly associated. There were no statistically significant relationships between maternally or paternally transmitted alleles and age of onset, when considered individually. Finally, we calculated the D′ and r^2 between the 3′UTR SNP and the markers in the parental DNA only. The 3′UTR and the VNTR-2 were in moderate linkage with a D′ of 0.626 and 0.614 with the 10 repeat and 12 repeat allele, respectively. On the other hand, the 3′ SNP showed a very weak linkage with the promoter markers 0.224 and 0.158 with the HTLPR and the rs25531, respectively.

Differential allelic expression analysis

To assess differential allele expression, we examined RNA samples from 21 lymphoblastoid cell lines derived from bipolar disorder subjects. All 21 patients have diagnosis of bipolar disorder type I; there were 6 men and 15 women; the mean age at the time of the assessment was 40.96 ± 9.37, and the mean age at onset was 18.38 ± 8.39. There were 13 patients with at least one lifetime suicide attempt and eight patients who never attempted suicide. There was no association between gender and T/G ratio ($t = 0.873$; $df = 19$; $P = 0.394$). When we considered gender, the ratio difference was 0.314 and the 95%CI was between −0.438 and 1.066. The ratio was 1.58 ± 0.91 in the men and 1.26 ± 0.67 in women. Furthermore the T/G ratio did not correlate with the age at time of the assessment ($r = -115$; $P = 0.619$).

When we examined suicide attempts and allele-specific expression ratios, we found that there was no significant difference when we compared the mean ratio in suicide attempters (1.39 ± 0.835) and non-suicide attempters (1.28 ± 0.60). We performed the ANCOVA incorporating age and sex as covariates and the test was not significant ($F_{1/17} = 0.006$; $P = 0.938$). There was no correlation between age at onset and T/G ratio, based on the non-parametric Spearman's test ($r = -0.194$; $P = 0.400$).

Discussion

We did not find significant differences in 5HTT T/G allele mRNA levels between bipolar patients with or without suicide attempts. Although cDNA analysis showed a lower T/G allele ratio in the non-attempter group, there is no evidence indicating that epigenetic mechanisms are altering expression of this gene in lymphoblastoid cells from bipolar patients. However, few studies have explored the epigenetics of 5HTT in the context of suicide and no one has focused on POE or a potential allelic imbalance.

To our knowledge, this is the first study to examine 5HTT *T/G* mRNA ratios in bipolar patients. However, no evidence of genomic imprinting (complete inactivation of one allele) was found in our sample of lymphoblastoid cell lines that were heterozygous at the G2651T 3′UTR SNP. Unfortunately, our suicide attempt analysis was limited to only 21 subjects, so the power to detect allele-specific expression differences is low. Consistent with the lack of association with the G2651T mRNA ratio, there were no significant differences between paternal and maternal transmission ratios in suicide attempters for the 5HTT alleles we examined. However, the small number of families does not provide sufficient statistical power to detect POE differences, even though our family collection is one of the largest in the world.

Nevertheless, when we examined the severity of suicidal behaviour as quantitative trait, we were able to analyse a larger number of families, and showed that the G allele in the 3′UTR polymorphism is associated with greater severity of suicidal behaviour. When only maternal meioses were examined, we found that that the 10 repeat allele of the 2-VNTR is associated with higher suicidal behaviour severity. Furthermore, this is one the first studies that used a family based association approach for analysing quantitative measures of severity of suicidal behaviour and variation in the 5HTT gene.

Regarding the secondary outcomes of bipolar disorder and age of onset, the T allele in the 3′UTR showed increased transmission to bipolar patients overall. However, when maternal meioses or paternal meioses were considered individually, there were no longer significant differences in transmission. One hypothesis we did not test is that differential methylation of the 5HTT gene may occur in suicide attempters in comparison with non-suicide attempters. Furthermore, the T/G polymorphism seems unlinked from the HTLPR, so the allelic ratio cannot be influenced by these cis-acting elements. This may indicate that other regulatory mechanisms are involved in this gene.

The differential expression of 5HTT T/G alleles in the lymphoblastoid cells could be the result of differential methylation patterns affecting gene transcription. It is unclear whether the allele-specific expression levels in

these transformed peripheral cells are similar to that in brain. Although the effect of the cis-acting elements such as the HTTLPR may be similar in different tissues, we did not find any association between the T/G ratio and other phenotypes related to bipolar disorder.

There are several limitations to the present study: small sample size, drug treatment effects, the ability to examine only the effect of the T/G SNP and the fact that the lymphoblastoid cells were obtained from an independent sample. However, the high heterozygosity of the rs1042173 SNP allowed the analysis of a fair number of bipolar disorder subjects. Drug treatment may have confounded our expression results because selective serotonin reuptake inhibitors (SSRIs) influence the 5HTT mRNA levels [14, 17] and patients in the lymphoblastoid cell sample were not drug-free.

Direct comparisons between this study and the findings of Lim et al. [12] are not possible because we used a different technique to analyse the T/G allelic imbalance. They reported no correlation between allelic expression of serotonin transporter (SERT) mRNA and the HTTLPR in human pons. Of course, it is difficult to obtain blood and brain tissues from the same subject, but that would be the best strategy to address this limitation. In addition to possible drug treatment effects, other unknown group differences could account for differences in mRNA ratios. We found no significant effect of basic demographic variables on mRNA ratios, but did not have more detailed information on other potentially important group differences that could affect gene expression. Allele-specific expression can be affected by multiple *cis*-regulatory elements, but the markers in the 5HTT promoter are in very weak LD with the 3′UTR SNP. We cannot exclude the possibility that differences between our study and the Lim study may be due to different LD between regulatory elements and the rs1042173 SNP in the two sample sets [12].

In conclusion, we are reasonably confident that genomic imprinting is not a confounding factor in genetic association studies of the 5HTT gene and suicide attempts in bipolar disorder. Therefore, inconsistencies in genetic association studies could be attributed to other epigenetic phenomena or environmental factors interacting with the 5HTT gene. Our results suggest that differential allele-specific expression of the rs1042173 SNP is not involved in suicidal behaviour. Further analysis of allele-specific expression of this polymorphic site may identify the functional meaning of the genetic associations reported for various neuropsychiatric diseases. It would be useful to investigate postmortem samples with regard to bipolar disorder and suicidality. The analysis of relationships between methylation at the 5HTT promoter and suicidal behaviour would also be of interest.

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Conflict of interest None.

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