Receptor mediated effect of serotonergic transmission in patients with bipolar affective disorder

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Bipolar affective disorder (BPD), a common severe mood disorder characterised by manic and depressive episodes, has an estimated lifetime prevalence of 0.1%–1% in various populations, including that of Taiwan.¹ Although previous studies have strongly suggested the involvement of genetic factors in the aetiology of BPD, the search for predisposing genes using classical linkage analysis has been fraught with difficulty. Association studies have been shown to be effective in mapping genes for complex diseases and have therefore been widely applied to studies of many psychiatric traits, including BPD.^{2–3} The results suggest that several genes with minor effects might be involved in the pathogenesis of BPD and that genes involved in neurotransmitter metabolism or signal transduction are possible candidates for an association with BPD.^{3–5}

Serotonin (5-hydroxytryptamine; 5-HT), a major neurotransmitter in the central nervous system, is involved in various physiological events, such as mood control, sleep, thermoregulation, learning, and memory.⁶ Disruption of serotonergic function has been implicated in the pathogenesis of many psychiatric disorders, including BPD.^{7–8} Serotonin exerts its diverse functions through multiple receptor (HT receptor; HTR) subtypes.⁹ These receptors, localised on the post- or pre-synaptic neuronal membrane, bind released serotonin and either transmit the signal to the post-synaptic neurone or regulate serotonin production by the neurone itself. Seven classes of HTRs (HTR1–7), made up of 15 functionally and pharmacologically distinct receptor subtypes,^{10–11} have been identified in mammalian species in the past few years. Except for HTR3, which is a ligand gated ion channel receptor, all known HTR family members are guanine nucleotide binding protein (G-protein) coupled receptors.

Of these 15 subtypes, several, which are abundantly expressed in the limbic system and have a high affinity for antipsychotic drugs, are related to mood control.¹² Studies on knockout or transgenic mice have shown that impaired mood regulation is caused by dysfunction of certain HTRs. For example, *HTR1B* knockout mice exhibit more aggressive and reactive behaviour and less anxious behaviour.^{13–15} Conversely, *HTR1A* knockouts show less reactive behaviour, more anxious behaviour, and possibly less aggressive behaviour.^{13–15} Moreover, mice with mutations in the *HTR2C* or *HTR5A* gene exhibit, respectively, an obesity syndrome or a reduced locomotor stimulatory effect.^{13–15} In addition to this strong evidence from animal studies, previous studies have shown that suicidal patients have a higher number of platelet *HTR2A*,^{16–17} and BPD patients have significantly decreased brain levels of the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA).^{16–17} Furthermore, it has been reported that treatment with *HTR1A* agonists can affect neurotransmitter release and cause behavioural change.¹⁸ The results of these studies suggest that the HTRs implicated in mood regulation are good candidates for involvement in BPD pathogenesis.

Key points

- Bipolar affective disorder (BPD) is a common affective disorder characterised by episodes of mania and depression. The complex phenotypes imply that the disease probably involves multiple genes and gene-environment interactions.
- Serotonin (5-hydroxytryptamine; 5-HT) is a key neurotransmitter in the central nervous system, and dysfunction of the serotonergic system has been implicated in several psychiatric diseases, such as affective and anxiety disorders. Serotonin exerts its diverse effects through multiple receptors, and serotonin receptors (HTRs) are therefore the subject of extensive research on the pathophysiology of many diseases. To determine whether specific HTRs are involved in the aetiology of BPD in Taiwan, single nucleotide polymorphisms located in the coding or regulatory regions of several HTRs were examined in 100 unrelated BPD subjects and 106 matched normal controls.
- Statistically significant associations were found between *HTR2C* and *HTR7* gene polymorphisms and BPD. In addition, logistic regression analysis suggested that a model including *HTR2C*, *HTR7*, *HTR2A*, and *HTR6* was best for predicting BPD disease status.
- *HTR2C* and *HTR7* are G-protein coupled receptors that regulate the expression of downstream genes through two major intracellular signal transduction pathways. The results of this study suggest that the *HTR2C* and *HTR7* genes may play important roles in the pathophysiology of BPD by affecting signalling networks as a consequence of an altered interaction of the HTR with serotonin.
- A study on the effects of sequence changes in these *HTRs* on the physiological status in vivo is ongoing and should provide valuable information for understanding the biological and functional significance of these *HTR* genes in BPD pathogenesis. This knowledge should be useful in developing more effective treatments for affective disorders.

Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; ARMS, amplification refractory mutation system; BPD, bipolar affective disorder; cAMP, cyclic adenosine monophosphate; MSBE, multiple single base extension approach; PCR-ASO, PCR allele specific oligonucleotide; SNPs, single nucleotide polymorphisms

To investigate the role of HTRs in the aetiology of BPD in Taiwan, single nucleotide polymorphisms (SNPs) located in the coding or regulatory regions of six genes, *HTR1A*, *HTR2A*, *HTR2C*, *HTR5A*, *HTR6*, and *HTR7*, were examined in 100 BPD patients and 106 normal controls. Significant differences in both the genotypic and allelic distribution of *HTR2C* and *HTR7* were found between the case and control groups. In addition, a logistic regression model including *HTR2C*, *HTR7*, *HTR2A*, and *HTR6* was found to be the best model for predicting disease status. Our results support the idea that *HTR2C* and *HTR7* play important pathophysiological roles in predisposition to BPD, and suggest that the *HTR2C* C12G and *HTR7* G3217A polymorphisms may be valuable as both functional and genetic markers, the products of which affect signalling networks, owing to their abnormal interaction with serotonin.

METHODS

Sample collection and DNA preparation

Starting in 1998, probands were recruited from bipolar outpatients in the Chung Shan Medical University Hospital and the Taichung Rehabilitation Hospital in Taiwan. Controls were local volunteer blood donors, with no family or personal history of major affective disorder, who were matched to cases based on ethnic or geographical origin, sex, and age. Patients and controls were interviewed by experienced psychiatrists after the study procedure had been fully explained, and information on general demographic data, such as age, sex, and ethnicity, was obtained. Patients were assessment solely by direct clinical interview by the treating clinician, according the procedure described in the DSM-*VI Diagnoses of lifetime major depressive disorder and bipolar I*. In addition, information required to reach diagnosis was also obtained from all clinical and hospital records where available. This study was approved by the university ethics committee, and written informed consent was obtained from all participants.

We recruited 100 unrelated BPD subjects and 106 normal control subjects. All participants were ethnic Chinese from Taiwan. Genomic DNA was prepared from peripheral blood by standard salt precipitation methods¹⁹ or using InstaGene Whole Blood kits (BioRad, Hercules, CA, USA) according to the manufacturer's protocol.

Selection of candidate genes and markers

The candidate gene approach focuses on genes, the products of which are likely to be of functional significance in the disease. To examine whether specific HTRs were involved in the pathology of BPD, we selected those that are abundantly expressed in the limbic system and have a high affinity for antipsychotic drugs. The candidate *HTRs* selected in this study were *HTR1A*, *HTR2A*, *HTR2C*, *HTR5A*, *HTR6*, and *HTR7*.^{10–20}

Information on SNPs in each candidate gene was obtained from the dbSNP database (<http://www.ncbi.nlm.nih.gov/snp>) or from the literature.²¹ All selected SNPs were located in the coding or regulatory region of the candidate *HTR*. The details of these selected markers are given in table 1.

Genotyping procedures and primer design

All primers used for genotyping were designed using OLIGO 6 Primer Analysis Software (Molecular Biology Insights, Inc., Cascade, CO, USA). Except for *HTR2C* and *HTR7*, in which SNPs were genotyped using the multiple single base extension (MSBE) approach, SNP markers were genotyped using the PCR allele specific oligonucleotide (PCR-ASO) method and the amplification refractory mutation system (ARMS).

The PCR-ASO method was modified from the original ASO hybridisation assay²² by the use of fluorescent primers. Two forward primers for the alternative alleles were labelled with different fluorescent markers and amplified together with one common anti-sense primer, the different fluorescent colours indicating the presence of the alternative alleles. In the ARMS approach, two pairs of primers were designed, each one of the coupled primers having an allele specific oligonucleotide at the 3'-end that targets the polymorphic base for amplification of different products. The two forward primers for the alternative alleles were labelled with the same fluorescent marker and amplified with the paired reverse primer. PCR products of different sizes indicated the presence of the two alleles of a given polymorphism.

MSBE was carried out using an ABI PRISM® SNaPshot™ Kit (Applied Biosystems, Foster City, CA, USA). Paired primers were designed for amplification of the flanking sequence of the SNP sites, and detection was based on the dideoxy single base extension of an unlabelled oligonucleotide primer. Each primer binds to a complementary template in the presence of fluorescent labelled ddNTPs, and the polymerase extends the primer by one nucleotide, adding a single ddNTP to its 3' end. Four different fluorescent dyes were used to label the different ddNTPs. Extension primers with sizes ranging from 18–60 bp were designed to separate the different products and detect multiple SNPs in a single reaction. Using a fluorescence detection system (ABI 310 Genetic Analyzer; Applied Biosystems), extended products with different sizes and colours were detected automatically.

PCR reaction

PCR for ASO and ARMS analyses was performed in a volume of 15 µl containing 50 ng of genomic DNA, 0.2 µmol/l primer, 200 µmol/l dNTP, 1 × buffer, 1.5 mmol/l MgCl₂ and 0.5 U of *TaqGold* DNA polymerase (Applied Biosystems). The PCR program started with 10 min at 95°C, followed by 43 cycles of 94°C for 30 s and 50–62°C for 60–90 s, and a final extension at 60–72°C for 10 min. In the MSBE system, PCR was performed using 0.5 U of *Taq* polymerase (ABgene, Epsom, Surrey, UK) and the PCR program was 5 min at 95°C, followed by 30 cycles of 94°C for 30 s and 50–55°C for 30–60 s, and a final extension at 60–72°C for 5 min.

Genotype analysis

The PCR and extension products were analysed using an ABI310 Genetic Analyzer. Capillary electrophoresis was performed by mixing 1 µl of the PCR product mix with 11.5 µl of formamide and 0.5 µl of size markers, heating the mixture at 95°C for 5 min, then quickly cooling it on ice. Onto a capillary polymer, 14 µl of each sample was loaded and run for 30 min. Products of different size were separated by polymer capillary electrophoresis, and the length of each PCR product determined from the size markers. Data were collected and analysed using ABI GeneScan software (Applied Biosystems). To avoid genotyping errors, all marker genotypes were scored independently by two investigators and checked for confirmation. In addition, discrepant genotypes were retyped to ensure the correct genotypes for final data analysis.

Statistical methods

Differences in genotype and allele distributions between patients and normal controls were analysed using Fisher's exact test and χ^2 test implemented by SAS (version 8; SAS Cary, NC, USA). The Hardy-Weinberg equilibrium in control groups was tested using Haldane's exact test²³ because of small sample size. As markers might show violation of the Hardy-Weinberg equilibrium, a complementary test for association was also conducted. This complementary test, a

Table 1 Sequence of primers and probes for the SNPs used in this study						
Gene	SNP location	Amino acid alteration	Primers and probe‡			dbSNP ID
<i>HTR1A</i> ‡	Exon1/G402A	Arg134Arg	WF1 WR1 MF MR WF2 WR2 P	FAM TET FAM TET	5'-CGCCATCGCGCTGGACACG-3' 5'-CCCCGACTCTCCATTACAC-3' 5'-CGCCATCGCGCTGGACACA-3' 5'-TGAGCAGCAGCGGGATGTAG-3' 5'-CGCTGTATCAGGTGCTCAAC-3' 5'-CCATGATCCTTGCTAATGGTG-3' 5'-CTTGACCTGTGCGCCATCGCGCTGGACAG-3' 5'-TCTACAGTAATGACTTTAACTGC-3' 5'-TCTACAGTAATGACTTTAACTGT-3' 5'-CAGCTATGGCAAGTGAC-3' 5'-TCTCTCCCAGAAAGGATGA-3' 5'-TTGAGCAAGCTACCAAGCAA-3' 5'-CTGAAGCAATCATGGTGAAC-3' 5'-TGACCCAGAGATGGATTACAA-3' 5'-GCCCAGCAAGGTGAGAATAA-3' 5'-TGACCCAGAGATGGATTACAT-3' 5'-CATGCTGAACGCGCTGTGC-3' 5'-CATGCTGAACGCGCTGTGT-3' 5'-CGTCTCCGAGGCCTGACT-3' 5'-GGATATTTTAAAGGCTAATGG-3' 5'-GGATATTTTAAAGGCTAATGA-3' 5'-GGGTGTGTTTCTCTG-3' 5'-AGGGGCTGCCAGTTGTATT-3' 5'-CAAACATTCAGGCCATAGCA-3' 5'-TAATTACAGAATATCTACTTCATTAAGGA-3'	-§ <

combination of Armitage’s test²⁴ and Monte Carlo simulation, is robust to the Hardy–Weinberg equilibrium, and can provide a precise statistical inference. In addition, estimation of the relative risk of BPD associated with a particular allele and power analyses were performed using, respectively, the RelRisk program²⁵ and the Power Analysis and Sample Size (PASS) 2002 software (NCSS, Kaysville, UT, USA).

An additional regression model was used to consider the relative importance of candidate *HTR* genes to the disease. Because the disease status was dichotomous, we used the SAS program Logistic to carry out the logistic regression analysis. For model selection, three effect selection methods, forward, backward, and stepwise selection, were used to consider models including all possible combinations of different numbers of the variables (the *HTR* types). Two other values, the $-2log_L$ and Akaike’s information criterion (*AIC*), which help in determining goodness of fit, were also used. In general, smaller values of $-2log_L$ and *AIC* are preferred, and a significant difference in the $-2log_L$ between two models of different complexity indicates that the more complex model is better. In addition, the Hosmer and Lemeshow test for goodness of fit was used to confirm the validity of the selected model. A significant *p* value means that the model fails the goodness of fit test.

RESULTS

Six polymorphisms of the targeted *HTRs* were genotyped to examine the association with BPD in Taiwanese. As the subjects were recruited from multiple centres, DNA quality was difficult to control, thus it varied significantly. The overall genotyping rate was 83.2% and the missing rates of genotyping for the six receptors ranged from 3% to 26% and from 9% to 13% in the patient and control groups, respectively. The frequencies of the alleles and genotypes for the selected markers in the BPD and control groups are shown in table 2. Significant differences in both the genotypic and allelic distribution for *HTR1A*, *HTR2A*, *HTR2C*, and *HTR7* were seen between cases and controls (table 2); in the case of *HTR2C* and *HTR7*, the differences were still

significant after Bonferroni correction. As few markers in the control groups exhibited significant deviations from the Hardy–Weinberg equilibrium (data not shown), a complementary Armitage’s test for association was also conducted. Results from Armitage’s test were consistent with the data obtained from the χ^2 test (table 2). No association was seen between *HTR5A* and *HTR6* gene polymorphisms and BPD.

Logistic regression analyses were used to consider several markers simultaneously to investigate any multiple gene effect. According to all three selection criteria, the chosen model contains markers *HTR2C* and *HTR7* (model 1 in table 3). Under hierarchical modelling, all complex models based on model 1 were examined and three were judged as candidate models (model 2, 3 and 4 in table 3). All *p* values of likelihood ratio tests for four candidate models showed the significance of gene effects. On the basis of their $-2log_L$ and *AIC* values, models 2, 3, and 4 were, respectively, the best three, four, or five variable models. The $-2log_L$ value decreased considerably from model 1 to model 2 (31.983) and from model 2 to model 3 (20.14), indicating a significant improvement in model fitting. However, the difference in $-2log_L$ between models 3 and 4 was small (6.04) indicating modest improvement. Based on a conservative viewpoint, the results of the logistic regression analyses suggest that model 3, which included *HTR2A*, *HTR2C*, *HTR6*, and *HTR7*, was the best model under the selection from multiple gene effects. A *p* value of 0.8 in the goodness of fit test demonstrated that the model was valid.

DISCUSSION

BPD is a common and genetically heterogeneous disease. Many genes involved in serotonin transmission, such as the tryptophan hydroxylase gene coding for the rate limiting enzyme in serotonin biosynthesis,²⁶ have been studied for an association with BPD. However, the results from previous studies have not been consistent and sometimes even controversial.⁵ These findings indicate that BPD is a complex disease involving multiple genetic factors.^{5 27}

Table 2 Genotype and allele frequencies for each SNP marker analysed in this study

Locus symbol (alternative alleles)	Group	n	Allele†		p value		Genotype			p value (Fisher's exact test)
			1	2	χ^2 test	Armitage's test	1/1	1/2	2/2	
HTR1A (G/A)	Patient	82	139	25	0.0117	0.0183	61	17	4	0.011
	Control	85	125	45			45	35	5	
HTR2A (C/T)	Patient	84	88	80	0.0421	0.0137	15	58	11	0.044
	Control	79	65	93			7	51	21	
HTR2C (C/G)	Patient	85	86	44	0.0005‡§	0.0060†	52	11	22	0.007‡
	Control	79	99	20			60	13	6	
HTR5A (A/T)	Patient	91	121	61	0.0574	0.1082	44	33	14	0.153
	Control	103	155	51			64	27	12	
HTR6 (C/T)	Patient	87	148	26	0.0961	0.1321	63	22	2	0.261
	Control	82	128	36			51	26	5	
HTR7 (G/A)	Patient	88	117	59	< 0.0001‡¶	0.0005‡	46	25	17	0.0025‡
	Control	84	143	25			64	15	5	

†The number 1 and 2 indicate, respectively, the major and minor allele.

‡Significant difference in the distribution between the case and control groups after Bonferroni correction.

§The relative risk is 0.39 (95% confidence interval (CI) 0.22 to 0.72) and power for the allele distribution of HTR2C is approximately 87% with $\alpha = 0.05$.

¶The relative risk is 0.35 (95% CI 0.20 to 0.59) and power for the allele distribution of HTR7 is about 98% with $\alpha = 0.05$.

To characterise the gene effect of *HTRs* on BPD aetiology, we examined the genetic association between SNPs of several *HTRs* and BPD in Taiwanese bipolar I disorder patients. The significant allelic and genotypic differences between patients and normal controls for the *HTR2C* and *HTR7* genes (table 2) suggest that genetic variation at these two loci may play important roles in the development of BPD. Although the newly characterised *HTR7* has not yet been associated with any diseases, multiple polymorphisms in *HTR2C* are known to be associated with BPD in various populations.^{28–29} Moreover, the chromosomal regions of *HTR2C* (Xq24) and *HTR7* (10q21–24) have been linked to BPD.^{27–30–31} The consistent results from different mapping studies in various populations suggest that dysfunction of *HTR2C* and *HTR7* is related to the pathogenesis of BPD.

HTR1A and HTR2A have been implicated in several psychiatric conditions, including BPD.^{12–32} The HTR2A antagonist, clozapine, and the HTR1A partial agonist, buspirone, are used as alternative drugs for the treatment of many psychiatric diseases. Moreover, *HTR1A* knockout mice exhibit increased anxiety behaviour and decreased aggressive behaviour.^{13–33} Although the two receptors seem important in regulating behavioural traits, previous association studies of *HTR2A* using the same polymorphism and *HTR1A* using a microsatellite marker with BPD have given negative results.^{12–34–35} In our study, statistical analysis showed significantly genotypic and allelic differences in the frequencies of *HTR1A* and *HTR2A* gene polymorphisms between case and control samples before the Bonferroni correction (table 2); however, these associations were weak and were lost after the Bonferroni correction, suggesting that the gene effects of *HTR1A* and *HTR2A* are minor. Much less

attention has been paid to *HTR5A* since it was cloned³⁶ and mapped to human chromosome 7q36.1.³⁷ No disease association has been studied, and no association was found between *HTR5A* gene polymorphism and BPD in our report. This suggests that, although the *HTR5A* gene appears to be expressed uniquely in the central nervous system,³⁶ the polymorphism probably does not represent a major genetic risk factor of BPD. *HTR6* gene polymorphism is not associated with tardive dyskinesia,³⁸ mood disorders,³⁹ or sporadic Alzheimer's disease.⁴⁰ As *HTR6* was not associated with BPD in our study using the χ^2 test, the effect seen in the logistic regression analysis (model 3) might imply that any role of *HTR6* in the pathogenesis of BPD involves an interaction with other factors in the regression model. However, models for testing interactions between genes did not fit well due to the small sample size in this study. More samples will be required to study the epistatic effects among these candidate genes.

In genetically complex (polygenic) diseases, susceptibility or resistance variants are expected to be few at each locus, relatively common in the human population, and enriched in the coding and regulatory sequence of genes.^{41–42} Moreover, these susceptibility or resistance loci are rarely functionally independent, but work together to trigger the disease. Several methodologies and statistical methods have been successfully applied to analyse the effect of several genes on one phenotype.^{43–44} In this study, we used a logistic regression model to investigate the multiple gene effects of *HTRs* on the aetiology of BPD in Taiwan. Results from regression analysis showed the best model for predicting disease status was that using *HTR2A*, *HTR2C*, *HTR6*, and *HTR7*, based on our data.

Table 3 Statistical summary for the different logistic regression models†

Model	Variables	Likelihood ratio test‡ (p value)	–2log _L	AIC	Goodness of fit (p value)	Number of cases/ controls
1	<i>HTR2C</i> + <i>HTR7</i>	24.9578 (<0.0001)	192.378	202.378	5.4955 (0.3584)	82/75
2	<i>HTR2A</i> + <i>HTR2C</i> + <i>HTR7</i>	23.9032 (0.0005)	160.395	174.395	5.2578 (0.5112)	74/60
3	<i>HTR2A</i> + <i>HTR2C</i> + <i>HTR6</i> + <i>HTR7</i>	20.9882 (0.0072)	140.255	158.255	3.8390 (0.7981)	70/49
4	<i>HTR2A</i> + <i>HTR2C</i> + <i>HTR5A</i> + <i>HTR6</i> + <i>HTR7</i>	23.7910 (0.0082)	134.215	156.215	7.0784 (0.4208)	67/49

†All possible combinations were tested; the four models shown were the best candidates.

‡The test statistic is for testing the difference between the fitted model and the model without gene effects.

Of those SNPs associated with BPD pathophysiology, *HTR2C* C12G involves a missense amino acid change (Leu4Val), and the *HTR7* G3165A polymorphism involves a sequence change in the 5' regulatory region. The *HTR2C* missense mutation may contribute to disease by changing the encoded amino acid, thus altering its properties. The substituted site is in the first extracellular domain of *HTR2C* and, although valine and leucine are both hydrophobic amino acids with similar biochemical properties, valine is smaller and more hydrophilic, which may influence the binding affinity of serotonin for *HTR2C*. A functional mutagenesis study is in progress to test the biophysiological effect of this missense mutation. The *HTR7* G3165A polymorphism is in the 5'UTR in the consensus region of the autonomously replicating sequence binding factor 1 (ABF1) binding site. ABF1 is a multifunctional, site specific DNA binding protein that is essential for cell viability in *Saccharomyces cerevisiae*.⁴⁵ The human *ABF1* gene has been cloned and found to be a member of the basic helix-loop-helix family of transcription factors.⁴⁶ An analysis of the transcription factor binding prediction using the MatInspector program (<http://www.gsf.de/biodiv/matinspector.html>) indicated that the base change from G to A alters the consensus sequence and abolishes ABF1 binding, thus could cause down-regulation of *HTR7* gene expression.

There have been reports that abnormalities in various second messenger systems (that is signal transduction pathways) could be involved in the pathophysiology of affective disorders.^{47–51} These signalling networks rely on membrane receptors to communicate information from the extracellular environment to the interior of the cell through binding to G-proteins. Inside the cell, the information induces changes in gene and protein expression. Of these pathways, the adenylate cyclase (cyclic adenosine monophosphate; cAMP) and phosphoinositide (protein kinase C) signalling pathways have been extensively investigated and alterations in the levels and function of G-proteins have been demonstrated in peripheral cells and the postmortem brains of patients with affective disorders.^{50–54} It is interesting to note that the model suggested by regression analysis in this study fits well with the biological roles of the involved receptors. Of the 15 mammalian HTRs so far cloned, all except *HTR3* are members of the G-protein coupled receptor superfamily. Of these, stimulation of *HTR4*, *HTR6*, and *HTR7* causes an increase in cAMP levels via G(s) coupling. The *HTR2C* receptor is a G-protein coupled receptor that stimulates phospholipase C catalysed hydrolysis of phosphatidylinositol biphosphate, leading to the mobilisation of intracellular calcium and activation of protein kinase C. It is possible that receptors with altered affinity or requirements for binding serotonin will affect signal transmission through these two major second messenger systems, leading to altered gene expression patterns and induction of BPD.

Although some important findings from statistical association analyses have inspired us, some cautionary remarks should be addressed, for example, effects on population stratification and type I and type II errors. Some auxiliary variables (gender, medical history, family register) were analysed to detect the confounding effect and population stratification (data not shown). The results indicate that these factors are not confounders misleading the positive associations obtained in this study. Moreover, the Bonferroni correction and logistic regression partially keep the false positive rate under control. However, the false positive event may still occur due to some intangible factors even after careful statistical treatments. On the other hand, results from the present study cannot exclude completely the non-associated receptors, such as *HTR6*. These receptors may still contribute minor effects to BPD but it is insufficient to be

supported in our study due to the modest sample size. Expanding the sample size and more efforts will be required to reproduce the results of this study in future works.

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