PRELIMINARY ANALYSIS OF GENES INVOLVED IN INFLAMMATORY, OXIDATIVE PROCESSES AND CA2+SIGNALING IN BIPOLAR DISORDER AND COMORBIDITY FOR SUBSTANCE USE DISORDER

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Abstract

Objective: Genes involved in inflammation, oxidative stress and calcium signaling may be involved in Bipolar disorder (BD). Comorbidity for substance use disorders (SUD) is frequent in BD, and shared genetic mechanics may be hypothesized. In the present study we preliminarily investigated polymorphisms within Interleukin 1-beta (IL1b), neuronal Nitric oxide adaptor protein (NOS1AP) and Transient receptor potential cation channel 2 (TRPM2) in BD and comorbidity for SUD.

Method: One-hundred and thirty-one (131) BD patients (66 comorbid for SUD) and 64 healthy controls were genotyped for rs1143634, rs1143627, rs16944, rs1143623 (IL1b), rs12742393 (NOS1AP) and rs1556314 (TRPM2).

Results: Genetic variants were not found associated to BD, while rs1143627 and a haplotype in IL1b showed significant associations with SUD, both comparing SUD subjects with healthy controls and to non-comorbid BD patients.

Conclusions: The present study has several limitations, mainly linked to the small sample size and the naturalistic characterization of the study design. Taking into account these limitations and the preliminary nature of the study, present data do not support a role of IL1b, NOS1AP and TRPM2 in BD, though ILb1 may play a role in SUD.

Key words: bipolar depression, substance-related disorders, gene, IL1b, NOS1AP, TRPM2

Declaration of interest: none

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Introduction

The problem of comorbidity in Bipolar Disorders (BD) is undoubtedly one of the most important emerging challenges for research and clinical practice (Cassidy et al. 2001). The comorbidity of BD and SUD is frequently reported in literature (Cassidy et al. 2001), and people with both disorders usually show a more severe course of the illness (Frye and Salloum 2006) and poorer treatment outcome (Goldberg et al. 1999).

There is evidence that genetic factors play an important role in the risk to develop BD and genetic influence explains 60-85% of variance in risk (Smoller and Finn 2003). Similarly, evidence has been reported for a genetic liability for SUD (Muller et al. 2010). Despite a large number of studies performed so far, only few consistent data have been obtained and mostly conflicting results are reported in literature for both BD and SUD, though a number of genomic areas and some variations within specific genes have been repeatedly associated to BD (Serretti and Mandelli 2008, Muller et al. 2010). Several lines of evidence seem to suggest that the factors influencing variation in one set of symptoms and those affecting one or more disorders are observed to a marked extent, which ought to facilitate the search for susceptibility genes in comorbid brain disorders (Palomo et al. 2007). In comorbid BD and SUD disorders, polymorphisms in the dopamine and

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serotonin pathways have been mainly investigated (Gorwood et al. 2000, Szczepankiewicz et al. 2007, Yasseen et al. 2010). Results from genome wide association studies (GWAS) found a large number of genes contributing to both BD and SUD (Johnson et al. 2009), but most of them have been never investigated in association studies.

In recent years, basic research focused on inflammation, calcium (Ca2+) signalling, nitric oxide (NO) and oxidative stress as pathogenetic mechanisms in many psychiatric disorder (Antkiewicz-Michaluk 1999, Garcia-Bueno et al. 2008, Steckert et al. 2010, Warsh et al. 2004, Wood et al. 2009). These three domains are mutually interconnected: inflammation, via cytokines Interleukin 1 alpha and beta (IL-1a, IL-1b), modulates enzyme Nitric Oxide Synthase (NOS) to produce nitric oxide that, in large amounts, can produce oxidative stress damage. Further, NOS functioning depends on intracellular Ca2+ levels that are controlled with negative feedback by nitric oxide itself. In the present study we aimed to preliminarily investigate three genes related to inflammation processes (interleukin 1 beta - IL1b), oxidative stress (nitric oxide adaptor protein - NOS1AP) and Ca2+ signaling (transient receptor potential cation channel 2 - TRPM2).

IL1b encode for an important mediator of the inflammatory response, further involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. Specifically in BD, a recent post mortem frontal cortex study reported high IL1b protein and mRNA levels in subjects affected by BD (Rao et al. 2010). IL1b serum levels have also been reported high in depressed BD patients, with an inverse pattern in manic BD patients (Ortiz-Dominguez et al. 2007). Further, treatment with lithium has been shown to restore IL1b levels (Knijff et al. 2007). IL1 cluster has also been associated with gray matter deficits, ventricular enlargement and hypoactivity of prefrontal cortex in schizophrenia. A study by Papiol et al. (Papiol et al. 2008) on BD patients, reported a polymorphism in IL1b (rs16944) associated with whole-brain gray matter deficits and left dorsolateral prefrontal cortex deficits in BD patients. However, to our knowledge, no study has considered IL1b genetic variations in case-control studies with BD so far. The only study investigating ILb in SUD (alcohol dependence) that we found in literature was the one by Pastor et al (Pastor et al. 2005), reporting the rs16944 variant associated to alcohol dependence. Other studies have mainly focused on alcohol related features. Our group was unable to found rs3087258 in IL1b associated to a set of alcohol related features (Drago et al. 2009). Another study reported association with alcohol liver cirrhosis considering a diplotype composed by a variable number tandem repeat (VNTR) in interleukin 1 receptor antagonist (IL1RN) and IL1b rs1143627 (Petrasek et al. 2009), and the rs16944 was associated with alcoholic liver disease in Japanese subjects (Takamatsu et al. 2000).

The second gene here investigated, NOSAP1, encodes for a cytosolic protein that binds NOS and functions as an adapter protein linking NOS to specific targets (Jaffrey et al. 1998). The NOS gene has been implicated mostly in schizophrenia (Cui et al. 2010, Fallin et al. 2005, Silberberg et al. 2010, Shinkai et al. 2002, Tang et al. 2008) but some evidence has also been reported in BD (Fallin et al. 2005). Evidence for an involvement of nitric oxide pathway in BD has been reported (Hoekstra et al. 2006, Yanik et al. 2004) and increased expression of NOS in the hippocampal formation (Oliveira et al. 2008) as well as increased expression of NOSAP1 in the prefrontal cortex was observed in BD patients (Xu et al. 2005). Animal studies also suggested that nitric oxide availability may decrease after lithium administration (Sadeghipour et al. 2007). However, again, with the exception of the study of Fallin et al., focused on NOS1 (Fallin et al. 2005), no study has yet investigated NOS1AP in BD. In relation to SUD, early human post mortem studies reported increased NOS1 protein levels in the frontal cortex and the nucleus accumbens, brain regions which are suggested to be involved in the dopaminergic mesolimbic reward system (Gerlach et al. 2001). It has been therefore hypothesized that one of the mechanisms by which ethanol augments oxidative damage may include overproduction of nitric oxide (Jimenez-Ortega et al. 2007). Moreover, NOS1 seem to play an important role in the development of morphine-induced tolerance and dependence in rats (Herraez-Baranda et al. 2005) and that alcohol effect on nitric oxide and its synthesis results in the inhibition of new cell formation and memory impairment (Jang et al. 2002). Nevertheless, to our knowledge, no study investigated genetic variations in NOS1 and NOS1AP in case-control studies in SUD.

Finally, TRPM2 encodes for a Ca2+ permeable, non-selective cation channel involved in oxidative stress-induced cell death and inflammation processes (Du et al. 2009), but to our knowledge no study has focussed on SUD and related effects of substance use. At the opposite, as compared to the other genes considered in this study, TRPM2 is well established as a risk gene for BD (Kato 2007).

Since the evidence reported, in the present study we aimed to preliminarily investigate the association of these three candidate genes, IL1b (markers: rs1143634, rs1143627, rs16944, rs1143623), NOS1AP (rs12742393) and TRPM2 (rs1556314) in association to BD and SUD comorbidity, in a small but well clinically characterized sample of 131 BD patients and 64 healthy controls.

Methods

Sample and evaluations

The sample under investigation was composed by 131 European Caucasian BD patients (64 BP-I and 67 BP-II) and 64 healthy controls. Sixty-six BD patients (50.4%) met criteria for a comorbid SUD. Healthy controls were recruited among hospital staff, students and volunteers. Methods of recruitment and evaluation were previously described (Mazza et al. 2009). Briefly, patients were included if they met DSM-IV criteria for BD disorder (Bipolar Disorder I or Bipolar Disorder II) and were age 18-80. Exclusion criteria were considered: 1) a diagnosis of mental retardation or documented IQ<70; 2) unstable general medical conditions or clinically significant pre-study physical exam, electrocardiogram, laboratory or urinalysis

abnormalities indicating serious medical disease potentially impairing evaluations; 3) pregnant or breast-feeding women, to avoid remote but possible risks related to taking blood sample.

Diagnosis were made by the Semi-structured Interview for Axis I psychiatric disorders – SCID-I (First et al. 1995) and axis II personality disorder SCID-II (First et al. 1990). Healthy controls were evaluated by means of the same evaluation tools to exclude psychiatric diagnosis. All subjects were included after their acceptance of study procedures and sign of an informed consent. The study was approved by the local ethical committee.

Statistical analyses

The statistical software StatSoft (StatSoft 1995) was employed to perform statistical analyses. Simple linear associations were performed by the correlation analysis, t-Student, Chi-square tests and the one-way analysis of variance (ANOVA), depending on the nature of variables. Haplotypes analyses were performed by the R software (R-2.11.1 for Windows, http://www.r-project.org/) employing the haplo.stat package. For haplotype analysis, we considered only those SNPs that were in strong linkage disequilibrium (LD) (Haploview, (Barrett et al. 2005)). Control for multiple testing was performed by the False discovery rate (FDR) separately on BD and SUD (6 polimorphisms and 1 haplotype, lowest p-value =0.0071).

Genetic analyses

DNA was extracted from whole blood, using an Extragen 8C Automated DNA extractor. Genetic analysis were performed according previously reported procedures for IL1b (Misener et al. 2009, Yu et al. 2003), NOS1AP (Wratten et al. 2009) and TRPM2 (McQuillin et al. 2006). SNPs were selected according to these criteria: known functional polymorphisms, previously reported positive findings, reported association with the disease, known biological interactions.

Results

Genetic data

IL1b markers were all in Hardy Weinberg Equilibrium (HWE) (rs1143634 p=1.0, rs1143627 p=0.51, rs16944 p=0.08, rs1143623 p=0.58) and the last three markers were in strong LD (**figure 1**), with 4 haplotypes detected (frequencies: TCG: 0.58, CTC: 0.29, CTG 0.106, CCG: 0.02). Genotypes in NOS1AP (rs12742393) were in HWE (p=0.67) as well as genotypes in TRPM2 (rs1556314) (p=0.85).

Demographic and clinical variables

No differences were observed between BD patients and healthy individuals in terms of age (45.1±12.5), gender (females: 49.7%), educational level (12.6±3.7 years of school), marital (53.6% married) and employment status (40.6% permanently employed). Fifty patients satisfied criteria for an axis II personality disorder (38.2%) and 66 for a comorbid SUD (50.4%, 98.5% alcohol). Mean age of onset of BD was 29.7±7.1 and mean age of onset of SUD was 39.3±8.8.

Comparison between BD patients with and without comorbid SUD in terms of demographic and clinical variables has been previously analyzed (Mazza et al. 2009). Briefly, BD patients with SUD were more likely males, single or divorced, diagnosed for BP type 2 and for an axis II personality disorder.

Case-control associations

None of the genetic variants considered was found differentially distributed among BP patients and controls; IL1b haplotypes were not differentially distributed among BD patients and controls as well (**table 1**), while the IL1b rs1143627 variant showed an association with comorbidity for SUD and IL1b haplotypes were significantly associated to SUD (Global stat=15.73 df=4 p=0.0033) **(table 2).** The common TCG showed a protective effect against SUD while the rare CCG was associated to SUD. Comparing comorbid and non-comorbid BD, the C allele in rs16944 was more frequent in SUD patients (freq. 0.49 vs. 0.36, Chi-sq=4.86 p=0.032), while the CTG haplotype was again protective against SUD (Stat=2.56 p=0.010). Controlling for diagnosis subtype and axis II personality disorders, the effects remained significant (Global stat=12.49 p=0.014).

Figure 1. *Linkage disequilibrium map for Interleukin 1 beta (IL1b). genetic polymorphisms*

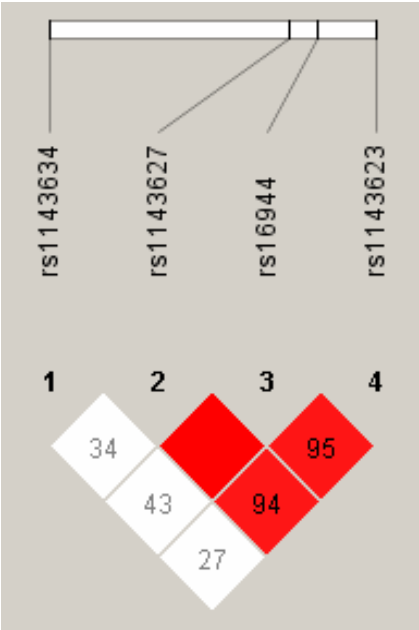

Table 1. *IL1b, NOS1AP and TRPM2 polymorphisms stratified in healthy controls and BD patients*

Polymorphyms / haplotypes		Heathy controls (n=64) *N (%)*	BD patients (n=131) *N (%)*	Statistics *Chi-sq, p*
IL1b				
rs1143634	CC	37 (32.7)	76 (67.3)	0.19, 0.91
	CT	24 (33.8)	47 (66.2)	
	TT	3 (27.3)	8 (72.7)	
rs1143627	TT	25 (38.5)	40 (61.5)	1.39, 0.50
	TC	30 (30.0)	70 (70.0)	
	CC	9 (30.0)	21 (70.0)	
rs16944	CC	25 (38.5)	40 (61.5)	2.15, 0.34
	CT	30 (28.3)	76 (71.7)	
	TT	9 (37.5)	15 (62.5)	
rs1143623	GG	35 (37.2)	59 (62.8)	1.64, 0.44
	GC	25 (29.1)	61 (70.9)	
	CC	4 (26.7)	11 (73.3)	
rs1143627- rs16944- rs1143623 haplotypes		*Freq.*	*Freq.*	*Chi-sq, p*
	TCG	0.56	0.62	1.45, 0.23
	CTC	0.30	0.26	0.94, 0.33
	CTG	0.10	0.12	0.29, 0.59
	CCG	0.02	<0.01	2.98, 0.09
		N (%)	*N (%)*	*Chi-sq, p*
NOS1AP				
rs12742393	AA	19 (35.2)	35 (64.8)	0.19, 0.91
	AC	32 (32.0)	68 (68)	
	CC	13 (31.7)	28 (68.3)	
TRPM2				
rs1556314	TT	47 (33.8)	92 (66.2)	2.71, 0.26
	TG	13 (26.5)	36 (73.5)	
	GG	4 (57.1)	3 (42.9)	

LEGEND: BD, Bipolar disorder; IL1b, Interleukin beta1; NOS1AP, nitric oxide synthase 1 adaptor protein; TRPM, transient receptor potential cation channel 2

Table 2. *IL1b markers and haplotypes in BD comorbid for SUD*

Marker	Minor Allele	SUD, Control ratios	Chi-sq	P
rs1143634	T	0.265, 0.225	0.78	0.38
rs1143627	C	0.492, 0.368	5.57	0.018
rs16944	T	0.455, 0.364	2.97	0.085
rs1143623	C	0.356, 0.267	3.28	0.070
Haplotype	Freq.	SUD, Control ratios	Stat.	P
T-C-G	0.581	0.484, 0.631	2.82	0.0048
C-T-C	0.289	0.332, 0.266	-1.44	0.15
C-T-G	0.106	0.122, 0.098	-0.75	0.45
C-C-G	0.015	0.038, 0.004	-2.60	0.0093

Discussion

To our knowledge, this is the first study investigating, though preliminarily, novel interesting candidate genes for BD in both the risk for disease and comorbidity for SUD in a naturalistic and well characterized sample of BD patients. According to our data, the genetic variants considered do not play a substantial role in the risk of developing BD, while a haplotype within IL1b showed an association with SUD.

IL1b has been poorly investigated in the risk to develop SUD in human. To our knowledge, only Pastor et al (Pastor et al. 2005) found an association between IL1b and alcohol dependence, though the association regarded the rs16944, while in our sample the polymorphism mostly contributing to SUD was rs1143627, not investigated by Pastor, but in full LD with rs16944. However, when comparing only BD patients with or without SUD, the rs16944 was associated to SUD, and in the same direction as reported by Pastor and colleagues. We did not restrict inclusion criteria to alcohol dependence as in Pastor et al., but the large part of comorbid SUD patients were alcohol abusers/dependent (80%); on the other hand, we investigated SUD in BD, but we found IL1b associated to SUD independently from BD. Therefore our data support an involvement of IL1B in SUD. In line with this data, previous studies suggested an involvement of IL1b on direct and resulting effects of substances. For example, it is known that exogenous opiate administration can alter immune responding (McCarthy et al. 2001) and a rodent study reported that centrally administered IL1b could attenuate naloxone-precipitated withdrawal in morphine-dependent mice (Katsumata et al. 1995). In humans, a study on injecting drug users reported increased levels of IL1b (Herold et al. 1994). Finally, the rs16944 has been associated with alcoholic liver disease in Japanese subjects (Takamatsu et al. 2000). Based on these studies, we may therefore hypothesize that some variations in IL1b may moderate the effects of the use of substances, therefore increasing/decreasing the risk of sustained use in some individuals. However, because of the paucity of addressed studies, the small size of our sample and all the limitations that we will discuss below, the reader should be aware of the very speculating nature of our hypothesis.

Despite previous suggesting evidence, NOS1AP and TRMP2 were found neither associated to BD nor to comorbidity for SUD. Nevertheless, limitations of the present study may have not allowed to detect smaller effects exerted by these genes on analyzed variables in this study. Moreover, single markers were considered within these genes, therefore incomplete gene coverage may have contributed to the negative findings.

A major limitation of our study was represented by its small sample size, which strongly reduced the possibility to detect small effects exerted by single polymorphisms. Indeed, considering an alpha value of 0.05, we had a sufficient power of 0.80 to detect medium effect sizes of w=0.223 in case control-association with BD and of w=0.273 with comorbid SUD. Considering that 6 polymorphisms in three different genes were analyzed in BD and comorbid SUD, which yet implied a high number of analyses, we could not perform further analyses on other variables such as age of onset, BD subtype, symptomathological features and so on. The limited sample size also prevented us to perform gene-gene interaction analyses. We also applied a correction for multiple testing according to the procedure of Benjamini et al (Benjamini et al. 2001), that further reduced the magnitude of the smallest alpha value detected, that had to be lower than 0.007, resulting in enlarged effect sizes detectable (w=0.276 in case-control association with BD and w=0.338 in case-control association with SUD). On the other hand, this strengthen the significance of the association detected on IL1b and SUD, increasing to some extent the reliability of the result obtained. Obtaining as much as possible reliable results was a priority in this study, since its preliminary nature and the high risk for stratification biases in a such small sample. However, stratification bias could not be still completely excluded.

The naturalistic approach that we employed for the recruitment of patients, though we opted intentionally for this approach in order to consider the effect of multiple variables, represents a further limitation of the study. Indeed, just few exclusion criteria were considered and both patients affected by BD type 1 and 2 were included, as well as patients with any axis II personality disorders. On the other hand, as stated above, many variables could not be taken into account for the present study.

In conclusion, despite many limitations characterize the present study, our data preliminarily support an involvement of IL1b in SUD, and independently from BD. The other genes investigated, NOS1AP and TRPM2, do not seem to play a critical role in BD and BD comorbid for SUD.

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