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## **Childhood maltreatment and HPA axis gene expression in bipolar disorders: a gene network analysis**

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## **Abstract**

**Introduction:** Bipolar disorder (BD) is highly associated with childhood maltreatment (CM), the exposure to such early adversity being suggested to disrupt the expression of several biological pathways. This study aims at exploring associations between the mRNA levels of 9 HPA axis genes in lymphoblastoid cell lines from patients with BD according to their self-reported exposure to CM.

**Methods:** The sample consisted of 33 Caucasian patients with a diagnosis of BD type 1, assessed for the exposure to CM with the Childhood Trauma Questionnaire (CTQ). Quantitative RT-PCR was performed on 9 transcripts of the HPA axis genes: *DGKH*, *FKBP5*, *NR3C1*, *SGK1*, *SGK2*, *SGK3*, *SKA2*, *STAT5A* and *UCN*. RT-qPCR data were analyzed using the method of disjoint gene networks with SARP.compo package for R.

**Results:** We found no associations between CTQ total score and the amount of HPA axis transcripts neither in univariate analyses, nor with network analyses. Emotional abuse (EA) was associated with a significant decreased expression of two transcripts, *DGKH* ( $p = 0.009$ ) and *NR3C1* ( $p = 0.04$ ). This was confirmed by the disjoint network analysis, which showed that *NR3C1* and *DGKH* were expressed differently from the rest of the HPA axis network in presence of emotional abuse.

**Discussion:** This study described the expression levels of a comprehensive set of HPA axis genes according to childhood maltreatment in a sample of patients with BD type 1 and suggested that emotional abuse decreased the expression of *NR3C1* and *DGKH*. Our results require further replication in independent larger samples.

**Key words:** bipolar disorder, childhood maltreatment, stress, gene expression, HPA axis, NR3C1, DGKH

## 1. Introduction

Bipolar disorder (BD) is a chronic and burdensome psychiatric condition that results from interactions between genetic and environmental risk factors. In this context, childhood maltreatment (CM) has been suggested as a major risk factor for developing BD (Aas et al. 2016). All subtypes of CM (neglect or abuse) were more frequent and severe in patients with BD as compared to healthy controls, with a predominant role of emotional abuse (EA) (Palmier-Clauss 2016). Two thirds of patients with BD reported multiple types of CM (Etain et al. 2010).

CM has been suggested to modify the functioning of multiple biological pathways (hypothalamic–pituitary–adrenal (HPA) axis, neurotransmission, immuno-inflammation, or neuroplasticity), but the associated biological disturbances remain poorly described. A meta-analysis of 41 studies focusing on the HPA axis alterations reported an association between BD and significantly increased levels of cortisol and HPA axis dysregulation. This has been suggested to be related to the exposure to environmental risk factors such as CM (Belvederi Murri et al. 2016). Nevertheless, findings on the association between CM and HPA axis disturbances remain relatively conflicting since the exposure to CM has been associated to both exaggerated and attenuated HPA axis activity. This heterogeneity might be explained by differences in the severity of CM and their unique or repetitive nature (Tyrka et al. 2016).

Among genes involved in the HPA axis, *NR3C1*, encoding for the glucocorticoid receptor (GR), has been the most widely studied in association with CM. Indeed, CM has been associated with a lower expression of *NR3C1* in leukocytes (Bustamante et al. 2016), these results being consistent with a decreased mRNA levels of *NR3C1* in the hippocampus of suicide victims who were exposed to childhood abuse (McGowan et al. 2009). Furthermore, some studies have suggested that the level of transcripts of *NR3C1* were modified in patients with BD as compared with controls, however without further investigations of the influence of childhood maltreatment in these samples (Sinclair et al. 2012; Sinclair et al. 2012B).

We hypothesized that the level of CM in patients with BD was associated with differences in expression of genes involved in the HPA axis functioning. For this purpose, we measured the mRNA levels of nine HPA axis genes in lymphoblastoid cell lines from patients with BD according to their self-reported exposure to CM and using disjoint graph analyses.

## 2. Material and Methods

2.1 Study sample: The sample consisted of 33 Caucasian euthymic patients with a diagnosis of BD type 1 according to DSM-IV criteria, from a multicentric cohort (Clinical Trials Number NCT02627404) that investigated the genetic and environmental factors of vulnerability in BD. The ethical committee (Comité de Protection des Personnes - La Pitié-Salpêtrière hospital – Paris - France - reference: P111002-IDRCB2008-AO1465-50) approved the study. All participants provided written informed consent prior to inclusion. Details on inclusion and exclusion criteria have been described previously (Etain et al. 2010).

The exposure to CM was assessed with the Childhood Trauma Questionnaire (CTQ) that characterizes the presence and severity of five subtypes of CM: emotional abuse (EA), emotional neglect (EN), physical abuse (PA), physical neglect (PN) and sexual abuse (SA) (Bernstein et al. 1994). The CTQ also generates a total score that is recommended to be used as a continuous variable corresponding to the global exposure to CM (range 25-125).

2.2 Biological sample preparation: Lymphoblastoid cell lines (LCLs) were cultured in RPMI-1640 medium containing 2 mM of L-glutamine, 10 % fetal bovine serum and 1 % penicillin/streptomycin (Life Technologies, France) in a 5 % CO<sub>2</sub> humidified incubator at 37 °C. LCLs were seeded at 2×10<sup>5</sup> cells/ml. After 4 days, cells were harvested for RNA isolation. Total RNA was extracted from 5×10<sup>6</sup> cells pellets using the miRNeasy Mini Kit according to the manufacturer's protocol (QIAGEN, France) and quantified with a NanoDrop One spectrophotometer (ThermoFisher Scientific, France). Total RNAs were stored at -80 °C until processing.

### 2.3. Quantitative RT-PCR:

1 µg of total RNA was reverse transcribed, in a final volume of 25 µl, using the iScript™ Reverse Transcription Supermix following the manufacturer's protocol (Bio-Rad laboratories, France). After reverse transcription, cDNAs were stored at -20 °C. Custom designed 384 wells Prime PCR plates were used. Plates were pre-plated with 16 genes of the HPA pathways and 5 reference genes (Bio-Rad laboratories, France; supplementary Table A).

SsoAdvanced Universal SYBR Green Supermix (Bio-Rad laboratories) was used for amplification following the manufacturer's instructions. The amplification was performed on a 7900HT instrument (Thermofischer). The specificity of PCR products was verified using a melting curve analysis step. Assays were carried out in duplicate. *GAPDH*, *SDHA* and *HPRT1* were selected as reference genes using built-in GeNorm analysis of the CFX Maestro software (Biorad). Baseline correction and threshold setting were performed using automatic calculation. Expression levels of 7 genes (*CRH*, *CRHBP*, *CRHR1*, *CRHR2*, *UCN2*,

*UCN3*, and *NR3C2*) were too low to be analyzed. We studied the expression of the 9 following genes: *DGKH*, *FKBP5*, *NR3C1*, *SGK1*, *SGK2*, *SGK3*, *SKA2*, *STAT5A* and *UCN*.

#### 2.4. Statistical analyses:

All analyses were performed using R version 3.5.1. RT-qPCR data were analyzed using the method of disjoint graphs gene networks, described in (Curis et al. 2019), as implemented in the SARP.compo package version 0.9.0 for R. Briefly, all pairwise gene expression ratios are compared between two conditions, using a Student test on log-transformed ratios. A graph is built using these tests results: each node is a gene, and two nodes are linked if the corresponding ratio does not change significantly between the two conditions. Difference in gene expression is then defined as disjoint sets of genes. To ensure a Type I error of incorrectly observing a disconnected graph less than 5 %, the individual cut-off for a given ratio was set to 0.23 based on 5 000 simulations (14 nodes, for two groups of 16 and 17 subjects respectively; simulation results: 95 % confidence interval [0.2395; 0.2610]). Here the conditions being studied in association with gene expression were: 1) the global level of CM measured by the quantitative total score of the CTQ and 2) the presence of CM subtypes based on the binary classification of severity, using the cut-offs for “absent or low CM” versus “moderate or severe CM” as described in (Bernstein et al. 1994).

### **3. Results**

All patients (n=33) presented with a BD type 1 diagnosis, with a mean age of onset of BD around 28 years, and a mean duration of illness around 19 years. They were mainly women (57.6%). The mean CTQ total score was 40,33 +/- 12,04 (range: 25-83). When considering the presence of CM subtypes (defined by a moderate or severe levels), we observed the following frequencies: EN= 24 %, EA= 15 %, PN= 27 %, PA= 9 % and SA= 9 %. Further details are given in supplementary Table B.

We first explored the association between the global level of childhood maltreatment and levels of transcripts. We found no correlations between CTQ total score and amount of HPA axis transcripts (see correlogram in Supplementary Figure A). This was then confirmed by the network analysis using SARP.compo (with the CTQ total score as a continuous variable) that did not show any association between the CTQ total score and any of the expression level of the genes (Figure A.1).

We then explored the five trauma subtypes. Since only 3 patients (9 %) had a “moderate or severe” SA or PA, analyses using these two scores were considered as unreliable. Hence, PA and SA were not further investigated. Using Wilcoxon rank tests, we found no differences of transcripts levels according to the presence/absence of EN or PN (data available on request). For EA, the univariate analyses using Wilcoxon rank tests (see Table A) identified a decreased expression of two transcripts, *DGKH* ( $p = 0.009$ ) and *NR3C1* ( $p = 0.04$ ), in presence of a moderate or severe score of EA.

Finally, for EA (used as dichotomous variable), we confirmed the results of the univariable analysis by a disjoint graphs network analysis that showed two groups of genes being differently co-expressed in presence of EA (Figure A.2). The smallest group consisted of *NR3C1* and *DGKH* genes that, in presence of EA, were not co-expressed anymore (i.e. excluded from the network) with the other HPA axis genes. Dendograms are shown on supplementary Figure B.

No further covariate (listed in Supplementary Table C) was included in the graph analysis since we identified no association between EA and these potential confounders.

#### **4. Discussion**

This study investigated the association between CM, and more specifically emotional abuse, and the levels of expression of nine genes of the HPA axis in lymphoblastoid cell lines of euthymic patients with BD type 1. The gene expression covariation network analysis identified, in presence of EA, a modified co-expression of *DGKH* and *NR3C1*, as compared to the other genes of the HPA axis. According to the univariate analyses, this modification corresponds to a decreased co-expression of these two transcripts in presence of emotional abuse.

Several previous studies have addressed the issue of interactions between early life adversities and comprehensive sets of HPA axis genes in BD (or related phenotypes), mainly using genetic variants (e.g. (Segura et al. 2019; Spijker et al. 2011)). Hence, this study reinforces the existing literature by further suggesting an association between CM and alterations of HPA axis gene expression. One strength of this study is to use disjoint graph analysis of a comprehensive set of HPA axis genes, and not only focusing on a single gene. Interestingly, these results are consistent with the literature investigating methylation of HPA axis genes in association with CM. Indeed, several independent studies have reported

differential methylation of *NR3C1* (mostly in the sense of hypermethylation) in presence of CM leading to transcriptional silencing (Watkeys et al. 2018). Moreover, *NR3C1* has been described as one of the 9 genes whose expression were similarly modulated in blood and brain of “PTSD-like” rats (Daskalakis et al. 2014). According to this latter study, *NR3C1* could be one of the best candidates for the study of the effects of CM on gene expression level in blood since it could be representative of the alterations in the brain.

Another strength of this study is to provide a disentanglement of CM, this being feasible thanks to the use of the CTQ. Indeed, a finding of our study is that the total score of CM did not seem to influence HPA genes expression, whereas EA did. This observation deserves several comments. First, a global measure of CM may not be specific or precise enough, or too noisy, to identify the biological correlates. Second, emotional abuse, even if considered as a possible “low-grade” CM (as compared to PA and SA) may be enough to alter biological systems. This result is parallel to those concluding that the effect of EA in BD was particularly robust (Palmier-Clauss 2016). Nevertheless, we cannot exclude also possible contributions of SA and PA but these were not investigated here due to the small number of patients having been exposed. Therefore, further studies are required to extend this disentanglement.

This study has several limitations. First, we included a small number of participants, leading to potential false negative results. The small sample size also implies that we have not been able to use other subtypes of CM in the analyses since the number of individuals with moderate to severe sexual or physical abuse was too small. Second, the assessment of CM has been made using a self-report questionnaire. Although validated in clinical and non-clinical samples, the CTQ may lead to biases in under or over-reporting CM. Nevertheless, it has been reported that the risk of minimization is higher in controls as compared to individuals with psychiatric disorders (with BD, recurrent depression or schizophrenia) (Church et al. 2017). In a sample of individuals with first-episode psychosis and controls, it has been suggested no strong evidence that the validity of the two used measures (CTQ and a comprehensive interview about childhood trauma) differed between cases and controls (Gayer-Anderson et al. 2020). Furthermore, the CTQ does not provide the chronology and repetitive nature of the different CM subtypes an individual has been exposed to. Third, RT-qPCR was performed on lymphoblastoid cell lines, which might not correspond to the mechanisms at stake in the brain. Nevertheless, given the difficulties to access to brain samples of patients, lymphoblastoid cell lines have been proposed as a good model for the identification of biomarkers of brain diseases (Wheeler et al. 2012). Finally, our results were based on association tests that cannot lead to infer causality.



In conclusion, the joint graph analysis of a set of nine genes involved in the HPA axis functioning suggested a decreased co-expression of *DGKH* and *NR3C1* in association with emotional abuse in euthymic patients with BD. Further studies would be required to elucidate the causal relationships between the exposure to CM and HPA axis dysregulation in BD.

#### CRediT authorship contribution statement

D. Grillault Laroche: Formal analysis, Writing - original draft  
E. Curis: Formal analysis,  
F. Bellivier: Writing -review & editing  
C. Nepost: Methodology  
C. Courtin: Methodology  
B. Etain: Conceptualization, Funding acquisition, Writing -review & editing  
C. Marie-Claire: Conceptualization, Writing -review & editing

#### Declaration of Competing Interest

The authors report no financial affiliation or other relationship relevant to the subject matter of this article.

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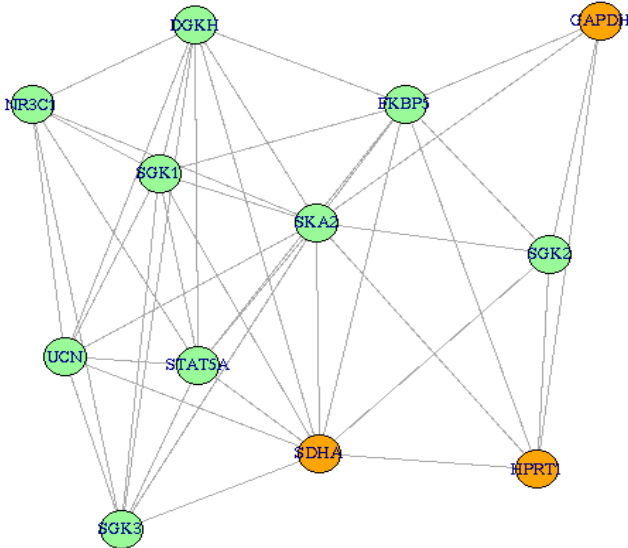
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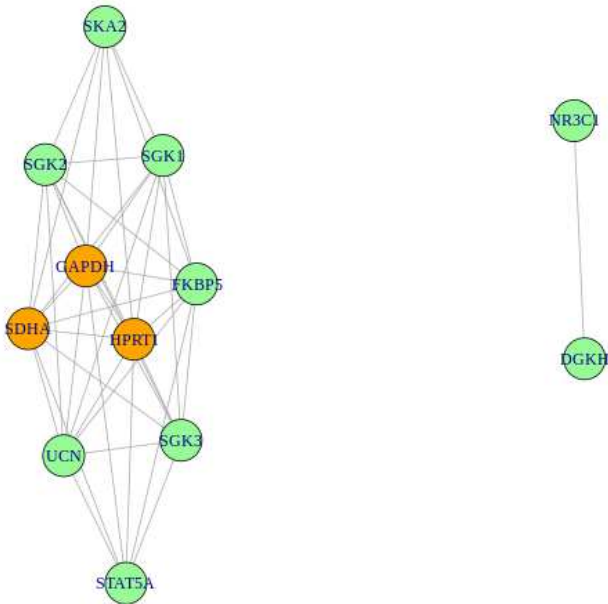
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Figure A: Concordance graph made from co-expression data of HPA axis genes relative expression, showing changes with childhood trauma (A1: quantitative CTQ Total score and A2: Emotional Abuse in 2 classes of severity).



A.1



A.2

Table A: Associations between emotional abuse and the level of transcripts of HPA axis genes

Gene	Mean $\Delta$ Cq EA- (+/-SD)	Mean $\Delta$ Cq EA+ (+/-SD)	P *
DGKH	1.20 (+/- 1.39)	3.52 (+/- 2.89)	<b>0.009</b>
FKBP5	0.34 (+/- 0.20)	0.38 (+/- 0.17)	0.44
NR3C1	3.71 (+/- 11.44)	8.47 (+/- 9.09)	<b>0.04</b>
SGK1	0.65 (+/- 0.39)	0.55 (+/- 0.38)	0.71
SGK2	0.79 (+/- 0.64)	1.23 (+/- 0.79)	0.30
SGK3	0.61 (+/- 0.30)	0.57 (+/- 0.47)	0.51
SKA2	1.08 (+/- 0.62)	1.96 (+/- 1.06)	0.12
STAT5A	0.84 (+/- 0.24)	0.97 (+/- 0.26)	0.15
UCN	0.85 (+/- 0.25)	0.75 (+/- 0.12)	0.30

\*Wilcoxon rank tests