

## ORIGINAL ARTICLE

## Epigenetic modulation of glucocorticoid receptors in posttraumatic stress disorder

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Some individuals suffering from posttraumatic stress disorder (PTSD) exhibit lower basal salivary cortisol and higher glucocorticoid receptor (GR) sensitivity. Recent studies suggest that epigenetic mechanisms regulate the activity of cortisol and GR. As a means to combine and cross-validate those findings, we compared cortisol, GR expression and promoter methylation levels in peripheral T lymphocytes of healthy controls versus individuals endorsing a diagnosis of lifetime PTSD. Thirty subjects with lifetime (current or remitted) PTSD and 16 subjects never exposed to trauma were recruited. Salivary cortisol was collected at six time points over the course of a single weekday and analyzed utilizing a time-resolved fluorescence immunoassay. GR expression (GR<sub>total</sub>, 1<sub>B</sub>, 1<sub>C</sub>, 1<sub>F</sub> and 1<sub>H</sub>) was measured by quantitative RT-PCR. DNA methylation levels in human glucocorticoid receptor (hGR) 1<sub>B</sub> and 1<sub>C</sub> variant's promoter were quantified by epityper in T lymphocytes isolated by magnetic-assisted cell sorting. Individuals with lifetime PTSD have lower morning cortisol release, higher mRNA expression of hGR<sub>total</sub>, 1<sub>B</sub>, and 1<sub>C</sub> and lower overall methylation levels in hGR 1<sub>B</sub> and 1<sub>C</sub> promoters. Cortisol levels were inversely correlated with hGR 1<sub>B</sub> mRNA expression. Moreover, overall and CpG site-specific methylation levels were inversely correlated with hGR<sub>total</sub> and 1<sub>B</sub> mRNA expression. There was no difference between current and remitted PTSD across cortisol, GR expression mRNA and DNA methylation data. Traumatic events induce DNA methylation alterations in distinct promoters of hGR with transcriptional modifications that associate with hypoactive hypothalamus-pituitary-adrenal axis in individuals with PTSD. Our results also point toward an important role of hGR 1<sub>B</sub> variant in PTSD.

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## INTRODUCTION

Posttraumatic stress disorder (PTSD) is characterized by symptoms of intrusions, avoidance and hyperarousal following exposure to a traumatic event. Lifetime trauma exposure has been estimated between 40–90%.<sup>1–6</sup> However, the lifetime prevalence for PTSD is 7–12%.<sup>1,7</sup>

If remission is considered the normative response to trauma exposure, a substantial proportion of individuals go on developing long-lasting intractable PTSD. The clinical manifestations of PTSD may reflect enduring changes in the stress-related neurobiological structures and systems.<sup>8</sup> Indeed, changes in the activity of the hypothalamus-pituitary-adrenal (HPA) axis, the main stress regulatory system, are induced by exposure to severe stressors in animals and humans<sup>9,10</sup> and dysfunction of the HPA axis activity is a well-characterized feature in PTSD. For instance, such patients exhibit higher corticotropin releasing factor in the cerebrospinal fluid,<sup>11,12</sup> lower plasma and serum morning cortisol release,<sup>13</sup> higher glucocorticoid receptor (GR) expression in lymphocytes<sup>14</sup> and greater GR sensitivity.<sup>15</sup> In addition, recent studies have shown that life-events, at least early in life, can modify the chemical structure surrounding the genome and influence gene expression,<sup>16</sup> including GR expression.<sup>17–19</sup>

The human GR gene contains eight coding exons (exons 2–9) and nine 5' non-coding first exons (exons A, B, C, D, E, F, H, I, J) spanned through a region of more than 80 kb within chromosome

5q31-q32.<sup>20–23</sup> Both rodent and human GR levels are regulated almost exclusively at the transcriptional level<sup>24,25</sup> and each non-coding exon 1 variant has its own promoter.<sup>22,26–30</sup> These multiple independent promoter regions contribute to the tissue specificity of GR expression.<sup>23</sup> DNA methylation is one of the epigenetic mechanisms controlling the expression of genes and has been shown to be modified by environmental stress.<sup>31</sup> For instance, in rats, maternal behavior regulates hippocampal GR1<sub>7</sub> expression in offspring through changes in GR1<sub>7</sub> promoter methylation.<sup>17</sup> Similarly, decreased hippocampal total GR, 1<sub>B</sub>, 1<sub>C</sub>, 1<sub>F</sub> (rat homolog for human GR1<sub>F</sub>) and 1<sub>H</sub> expression associates with respective GR exon 1 variant promoter methylation alterations in suicide completers who were abused in childhood.<sup>18,19</sup> These results suggest that methylation changes in GR promoters modify GR expression in individuals who experienced early-life adversity. It remains unknown if epigenetic regulation also explains HPA axis activity in other conditions associated with adversity such as PTSD. However, by definition, PTSD is etiologically related to environmental stress and, as such, it is likely to involve an important epigenetic component.

To address this question, we examined HPA axis functioning in individuals endorsing a diagnosis of lifetime PTSD by investigating salivary cortisol measures, the expression of four variants of GR (1<sub>B</sub>, 1<sub>C</sub>, 1<sub>F</sub> and 1<sub>H</sub>), and their association with variations in exon 1 variant promoter DNA methylation in T lymphocytes. We

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investigated T lymphocytes because these cells have an important role in cell-mediated immunity, and as such, are depositaries of cellular memory to environmental factors. In addition, DNA methylation patterns vary between different cell fractions,<sup>32</sup> thus it is preferable to investigate DNA extracted from individual cell populations rather than from homogenates. We hypothesized that the HPA axis alterations in PTSD are associated with variations in cortisol levels due to changes in the expression of the GR gene. We further hypothesized that modifications in DNA methylation levels within the promoter region of the GR gene are associated with changes in GR expression in PTSD.

## MATERIALS AND METHODS

### Subjects and procedure

Twenty seven individuals meeting DSM-IV criteria for lifetime PTSD (Current PTSD:  $N=15$ ; Past PTSD:  $N=12$ ) were recruited. Diagnosis was established with a semi-structured interview<sup>33</sup> and further corroborated by a self-report symptom measure.<sup>34</sup> Sixteen individuals with a negative trauma history were also recruited. Current comorbid depressive symptoms were assessed with a subscale of the symptom check list-90 using a cutoff of 70 for clinically relevant symptoms of depression.<sup>35</sup> The index traumatic events were as follows: motor vehicle accident ( $n=19$ ), participation in a peacekeeping mission ( $n=1$ ), assault with a weapon ( $n=2$ ), physical and/or sexual abuse in adulthood ( $n=14$ ), physical and/or sexual abuse during childhood ( $n=13$ ) or other ( $n=8$ ). All events met the DSM-IV A1 (life threat) and A2 (fear, helplessness or horror) criteria for trauma exposure. Exclusion criteria for controls were a lifetime trauma exposure, a current DSM-IV axis I psychiatric diagnosis<sup>36</sup> including any form of substance abuse and living outside of the greater Montreal metropolitan area. Table 1 lists sociodemographic and clinical sample's information. The Research Ethics Board at the Douglas University Mental Health Institute approved the project. Signed informed consent was obtained from every participant.

### Salivary cortisol

Basal salivary cortisol (unbound free cortisol) was sampled following previously described methods.<sup>37,38</sup> Participants were given six saliva collection tubes (Sarstedt, Montreal, QC, Canada) to use at home over the course of a single weekday at the following times: awakening, awakening +30 min, awakening+45 min, awakening+60 min, awakening+8 h and bedtime. The saliva samples were stored at  $-80^{\circ}\text{C}$ . Samples were analyzed in duplicate all at once using a time-resolved fluorescence immunoassay.<sup>38</sup> The average coefficient of variation between duplicates was 5.2%. Cortisol data were plotted on a graph and the area under the curve was measured for each time interval according to previously described methods.<sup>39</sup>

**Table 1.** Demographic and samples' clinical information

	Lifetime PTSD	Controls
Male:Female	15:15	8:8
Age (s.d.)	43.4 (12.1)	36.9 (11.2)
<i>Type of trauma</i>		
Motor vehicle accident	19	—
War	1	—
Armed assault	2	—
Adulthood physical/sexual abuse	14	—
Childhood physical/sexual abuse	13	—
Other	8	—
Probable depression	36%	0%
Current psychotropic medication use	40%	0%
RNA integrity number (s.d.)	7.1 (1.3)	7.2 (0.6)

Abbreviation: PTSD, posttraumatic stress disorder. PTSD symptom score is based on the self-report IES-R that ranges from 0 (no symptom) to 88. Probable depression was determined by a score  $>70$  on a subscale of the SCL-90.

### Peripheral blood RNA extraction

Blood samples drawn for the RNA extraction (12 ml) by a registered nurse were immediately put on Leucolock (total RNA isolation system, Ambion, CA, USA) and frozen at  $-80^{\circ}\text{C}$  until RNA extraction the next day. As opposed to DNA (see next section), RNA was extracted from whole blood. Once extracted, concentrations and quality were measured using a 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). Samples with a RNA Integrity Number greater than 5.5 on a scale of 1 (lowest) to 10 (highest) were used for further analysis.

### Human glucocorticoid receptor expression analysis

RNA was converted to cDNA using oligo dT primers according to manufacturer's recommendations (Invitrogen, Burlington, ON, Canada) and expression was analyzed using a real time polymerase chain reaction (RT-PCR) in an ABI 7900HT (Applied Biosystems, Foster City, CA, USA). Amplification of cDNA was performed using custom-designed taqman probes used in a previous study (Supplementary Table S1). Primers and taqman probes were obtained from Integrated DNA Technology (San Diego, CA, USA; Supplementary Table S1). Quantification was performed with five replicates each in a 12  $\mu\text{l}$  reaction as followed: 0.6  $\mu\text{l}$  of GR primers/probes, 3.1  $\mu\text{l}$  of water, 6.0  $\mu\text{l}$  of Taqman Master Mix and 2  $\mu\text{l}$  of cDNA (1 ng  $\mu\text{l}^{-1}$ ). Thermal cycling conditions were:  $50^{\circ}\text{C}$  for 2 min,  $95^{\circ}\text{C}$  for 10 min and 40 cycles, each of  $95^{\circ}\text{C}$  for 15 s and specific melting temperature for 60 s (Supplementary Table S1). The cycle threshold values for all replicates were pooled to obtain a mean value. Replicates with a standard deviation greater than 0.3 were excluded from the analysis. For each replicate, a quantity was extrapolated from a standard curve including six dots, each of which was five times diluted (5:1). A standard curve was composed of a mix cDNA from all subjects. Mean quantities from all sample replicates were normalized to each of the reference genes ( $\beta$ -Actin, GAPDH and 18S) and averaged together. Expression data are expressed as ratios of average mean quantities.

### T-lymphocyte isolation, DNA extraction and methylation quantification

Blood samples drawn for the T-lymphocyte isolation and DNA extraction (12 ml) were immediately put on ice before being processed on the same day. T lymphocytes were isolated following the Automacs Pro protocol using CD3 whole blood microbeads according to manufacturer's recommendations (Miltenyi Biotec, Boston, MA, USA). T-lymphocyte DNA was extracted using the DNA micro kit (Qiagen, Toronto, ON, Canada). DNA was diluted to 500 ng and sodium bisulfite conversion was performed using the EpiTect Bisulfite kit (Qiagen) according to the manufacturer's instructions. Bisulfite-treated DNA was then sent to the Innovation Center of Genome Quebec (Montreal, Quebec, Canada) where epityper (Sequenom, San Diego, CA, USA) was performed on the regions of human glucocorticoid receptor (hGR) promoters defined in Supplementary Figure S3. Methylation data are expressed as mean percentage of overall methylation (global methylation) and methylation at each CpG site.

### Statistical analyses

Analyses were conducted using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Analyses were adjusted for age and sex. Gene expression values were normalized and compared using univariate analysis of variance (ANOVA). Cortisol and methylation levels were analyzed using a mixed model analysis with group (PTSD and controls) as a fixed factor and cortisol sampling time (six samples) or CpG sites as a repeated measure, followed by LSD *post hoc* tests. For each analysis, outliers within groups were excluded when their value differed by more than two SDs from the group mean ( $Z$ -test). Correlations between cortisol, expression and methylation levels were analyzed with Pearson's correlation. Statistical significance was fixed at  $P < 0.05$ . Data are presented as mean  $\pm$  standard error of the mean (s.e.m.).

## RESULTS

### Salivary basal cortisol levels

A mixed two-way ANOVA performed on overall cortisol levels (area under the curve) revealed a significant main effect of group ( $F_{(1,87)} = 15.2$ ,  $P < 0.0005$ ), with cortisol levels being lower in the PTSD group compared with controls (Figure 1). The analysis also revealed a main effect of cortisol sampling time ( $F_{(4,40)} = 151.1$ ,

$P < 0.0001$ ) and a group by time interaction ( $F_{(4,40)} = 4.6$ ,  $P < 0.005$ ). *Post hoc* tests indicated that cortisol levels were significantly lower in the PTSD group compared with the controls for the time interval between 30 and 45 min post awakening ( $P < 0.01$ ) and between 45–60 min post awakening ( $P < 0.05$ ; Figure 1). In addition, a between-groups trend was found for the time interval between awakening and 30 min post awakening ( $P = 0.058$ ). An analysis of covariance (ANCOVA) revealed no between-group effect of age, sex and depression on cortisol levels.

We also compared cortisol levels in current and past PTSD patients. Interestingly, we found no significant difference in the cortisol levels between current and past PTSD ( $F_{(4,23)} = 0.106$ ,  $P = 0.98$ ) suggesting that changes in the cortisol levels are maintained after remission from PTSD symptoms (Supplementary Figure S1A). These effects were also independent of age and sex.

#### Whole blood human glucocorticoid receptor expression levels

The relative expression of hGR<sub>total</sub> as well as the first non-coding exons 1<sub>B</sub>, 1<sub>C</sub>, 1<sub>F</sub> and 1<sub>H</sub> was compared across the PTSD and control groups in peripheral blood samples. Results showed a significantly higher expression of hGR<sub>total</sub> ( $F_{(1,43)} = 5.3$ ;  $P < 0.05$ ; Figure 2a), 1<sub>B</sub> ( $F_{(1,43)} = 12.1$ ;  $P < 0.005$ ; Figure 2b) and 1<sub>C</sub> ( $F_{(1,42)} = 4.2$ ;  $P < 0.05$ ; Figure 2c) in the PTSD group compared with controls. In contrast,

hGR 1<sub>H</sub> relative expression was not significantly different between the groups (Figure 2d). In addition, consistent with previous findings showing that hGR 1<sub>F</sub> expression is mainly restricted to the brain,<sup>23</sup> we were unable to quantify its expression in peripheral blood. ANCOVA revealed no effect of age or sex on hGR<sub>total</sub>, 1<sub>B</sub> and 1<sub>C</sub> expression levels between groups although we found a significant effect of age on hGR 1<sub>H</sub> expression.

Interestingly, we found a significant negative correlation between hGR 1<sub>B</sub> relative expression and cortisol levels for the time intervals between awakening and 30 min post awakening ( $r = -0.34$ ,  $P < 0.05$ ; Supplementary Figure S2A), 30–45 min post awakening ( $r = -0.39$ ,  $P < 0.05$ ; Figure 3a) and 45–60 min post awakening ( $r = -0.37$ ,  $P < 0.05$  Supplementary Figure S2B). There was no correlation between hGR<sub>total</sub> or hGR 1<sub>C</sub> relative expression and cortisol levels.

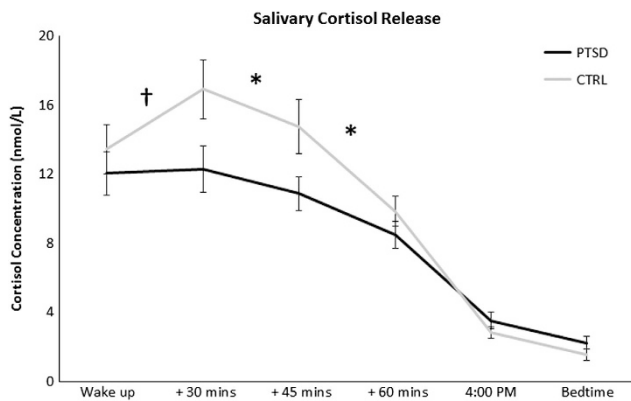
We next assessed whether hGR non-coding exons' expression differs in current and past PTSD individuals. Our analyses revealed no significant difference in the expression of hGR<sub>total</sub> ( $F_{(1,26)} = 2.277$ ,  $P = 0.145$ ), 1<sub>B</sub> ( $F_{(1,26)} = 1.611$ ,  $P = 0.217$ ), 1<sub>C</sub> ( $F_{(1,25)} = 0.102$ ,  $P = 0.752$ ), and 1<sub>H</sub> ( $F_{(1,26)} = 1.171$ ,  $P = 0.290$ ) between current and past PTSD (Supplementary Figure S1B). These effects were also independent of age and sex.

#### T-lymphocyte methylation levels

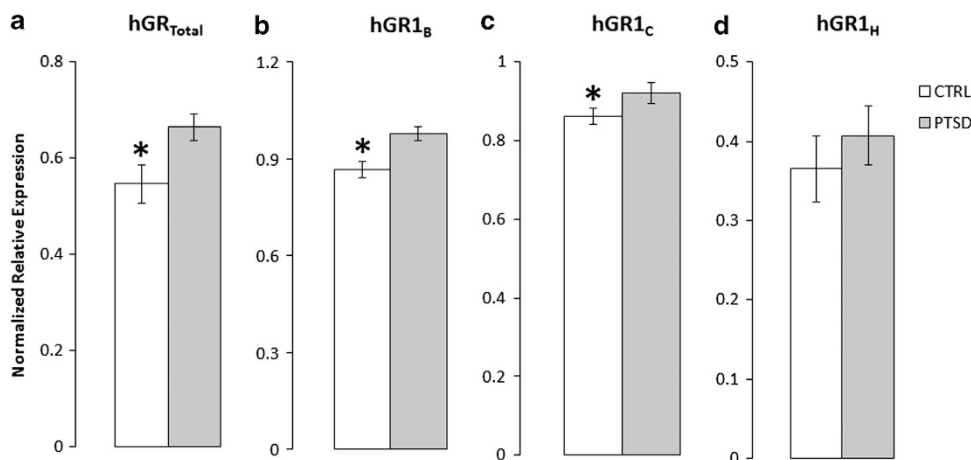
We sought to determine whether alterations in DNA methylation patterns within hGR variants' promoter could explain the expression changes observed between the PTSD group and controls. We focused on the two variants (hGR 1<sub>B</sub> and 1<sub>C</sub>) that showed higher expression in the PTSD group compared with controls (Supplementary Figure S3).

#### Human glucocorticoid receptor 1<sub>B</sub>

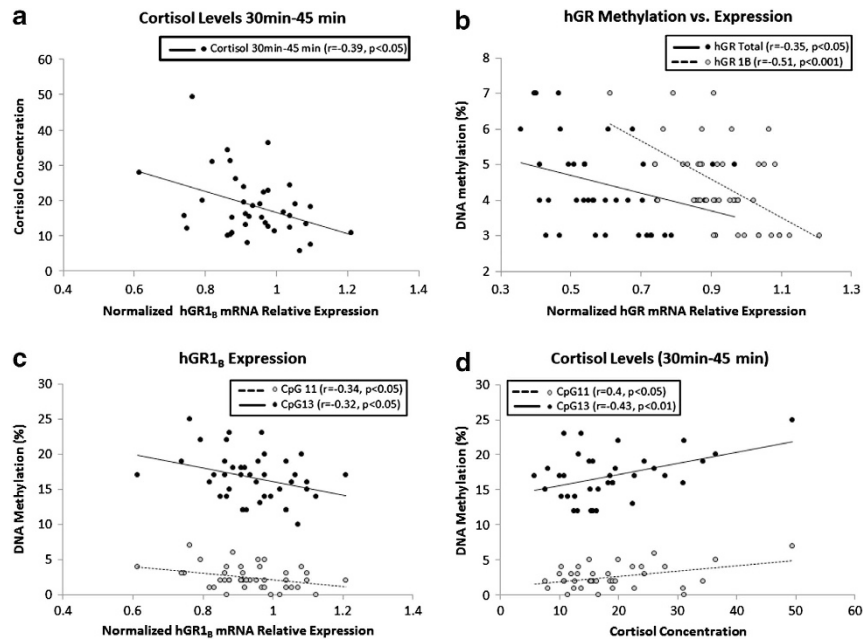
Methylation levels were analyzed across a region of 479 bp composed of 29 CpGs located upstream hGR 1<sub>B</sub> (Supplementary Figure S3). Mixed two-way ANOVA revealed a significant main effect of group on percentage of methylation ( $F_{(1,182)} = 28.7$ ,  $P < 0.0001$ ), CpG site ( $F_{(15,96)} = 307.8$ ,  $P < 0.0001$ ), and a group by CpG site interaction ( $F_{(15,96)} = 2.8$ ,  $P < 0.005$ ). Our analysis indicated a significant effect of age ( $F_{(1,52)} = 9.5$ ,  $P < 0.005$ ) on methylation levels although the main effects of group, CpG site, and group by CpG site interaction remained significant after correction for age. In accordance with the expression data, overall methylation level in hGR 1<sub>B</sub> promoter was significantly lower in PTSD compared with control (Figure 4a). *Post hoc* tests showed site-specific



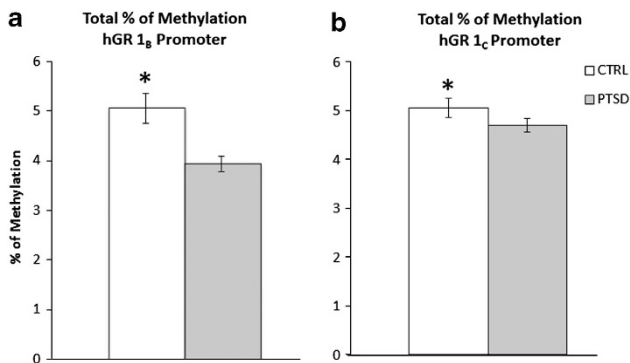
**Figure 1.** Diurnal variations in basal salivary cortisol levels (mean  $\pm$  s.e.m.) at awakening, +30 min, +45 min, +60 min, 4pm and bedtime as a function of group status. Note that time intervals are plotted evenly. Lifetime PTSD:  $n = 22$ , Controls:  $n = 15$ . \* $P < 0.05$ ; † $P = 0.058$ . CTRL, control; PTSD, posttraumatic stress disorder.



**Figure 2.** Glucocorticoid receptor mRNA expression levels (average relative mean quantity  $\pm$  s.e.m.) of (a) hGR<sub>total</sub>, (b) hGR 1<sub>B</sub>, (c) hGR 1<sub>C</sub>, and (d) hGR 1<sub>H</sub> as a function of group status. PTSD:  $n = 27$ , Controls:  $n = 16$ . \* $P < 0.05$ . CTRL, control; hGR, human glucocorticoid receptor; PTSD, posttraumatic stress disorder.



**Figure 3.** (a) Correlation between hGR 1<sub>B</sub> mRNA relative expression and salivary cortisol levels for the time interval between 30–45 min after awakening. (b) Correlations between total DNA methylation level in GR1<sub>B</sub> promoter and hGR 1<sub>B</sub> (gray dots) and hGR<sub>total</sub> mRNA relative expression (black dots). (c) Correlations between hGR 1<sub>B</sub> mRNA relative expression and DNA methylation level in hGR1B promoter at CpG sites 11 (gray dots) and 13 (black dots). (d) Correlation between cortisol levels for the time interval between 30–45 min after awakening and hGR1B promoter DNA methylation levels at CpG sites 11 (gray dots) and 13 (black dots). hGR, human glucocorticoid receptor.



**Figure 4.** Overall percentage of methylation (mean  $\pm$  s.e.m.) in (a) hGR 1<sub>B</sub>, and (b) hGR 1<sub>C</sub> promoters as a function of group status. PTSD:  $n = 30$ , Controls:  $n = 16$ . \* $P < 0.05$ . CTRL, control; hGR, human glucocorticoid receptor; PTSD, posttraumatic stress disorder.

hypomethylation at CpG sites 2–4 ( $P < 0.005$ ), 11 ( $P < 0.05$ ), 13 ( $P < 0.01$ ) and 14 ( $P = 0.05$ ) in individuals suffering from lifetime PTSD compared with controls (Supplementary Figure S4A).

Our results show a negative correlation between overall methylation levels in the hGR 1<sub>B</sub> promoter and hGR<sub>total</sub> ( $r = -0.35, P < 0.05$ ) and 1<sub>B</sub> ( $r = -0.51, P < 0.001$ ) relative expression (Figure 3b). GR<sub>total</sub> and 1<sub>B</sub> relative expression was also negatively correlated with methylation levels at CpG sites 2–4 (hGR<sub>total</sub>:  $r = -0.37, P < 0.05$ ; hGR 1<sub>B</sub>:  $r = -0.55, P < 0.005$ ; Supplementary Figure S2C), 11 (hGR 1<sub>B</sub>:  $r = -0.34, P < 0.05$ ), and 13 (hGR 1<sub>B</sub>:  $r = -0.32, P < 0.05$ ; Figure 3c). In addition, methylation levels at CpG sites 11 and 13 were positively correlated with cortisol levels obtained between awakening and 30 min post awakening (CpG site 11:  $r = 0.4, P = 0.05$ ; CpG site 13:  $r = 0.44, P < 0.01$ ; Supplementary Figure S2D), and +30 min and +45 min (CpG site 11:  $r = 0.4, P < 0.05$ ; CpG site 13:  $r = 0.43, P < 0.01$ ; Figure 3d).

We assessed DNA methylation levels in current and past PTSD samples and found no significant group difference (Supplementary Figure S1C). These effects were also independent of age and sex.

#### Human glucocorticoid receptor 1<sub>C</sub>

Methylation levels were analyzed across a 364 bp region composed of 54 CpG upstream hGR 1<sub>C</sub> (Supplementary Figure S3). Mixed two-way ANOVA revealed a significant main effect of group on percentage of methylation ( $F_{(1,354)} = 6.5, P < 0.05$ ), CpG site ( $F_{(21,698)} = 245.6, P < 0.0001$ ), but no group by CpG site interaction. In accordance with the expression data, total methylation level in hGR 1<sub>C</sub> promoter was significantly lower in PTSD compared with the controls (Figure 4b). *Post hoc* analysis showed significant hypomethylation in PTSD patients at CpG sites 40–41 ( $P < 0.05$ ). We also found one CpG site (CpG 51) to be significantly hypermethylated ( $P < 0.05$ ; Supplementary Figure S4B). An ANCOVA revealed no effect of age and sex on hGR 1<sub>C</sub> methylation levels between groups.

Our analyses showed no correlation between total methylation in 1<sub>C</sub> promoter and relative hGR 1<sub>C</sub> expression or cortisol levels.

We also compared DNA methylation levels in current and past PTSD samples. Our analysis revealed no difference in DNA methylation levels between current and past PTSD (Supplementary Figure S1D). These effects were also independent of age and sex.

## DISCUSSION

In this study, we investigated the HPA axis profile of individuals suffering from lifetime PTSD and healthy controls by measuring basal salivary cortisol levels, hGR peripheral expression and CpG methylation states in T lymphocytes. We focused our investigation on the non-coding exons 1<sub>B</sub>, 1<sub>C</sub>, 1<sub>F</sub> and 1<sub>H</sub>, which have been previously studied in the brain of depressed subjects<sup>40</sup> and abused suicide completers.<sup>18,19</sup> By combining these three molecular measures, we sought to obtain a comprehensive view of the HPA axis profile and to investigate whether epigenetic mechanisms, in response to traumatic events, modify GR expression and cortisol release in individuals with a diagnosis of lifetime PTSD.



In agreement with the previous findings,<sup>13,15,41,42</sup> our results suggest a dysregulation of the HPA axis activity in individuals with PTSD that was independent of current versus past PTSD status. Accordingly, the rise in morning cortisol release, up to 60 min after awakening observed in the control group was lower in the PTSD group. Importantly, the measure of cortisol in this study was obtained from saliva, which has been highly correlated with serum unbound cortisol levels.<sup>43</sup> Under normal conditions, unbound cortisol increases by about 50% within the first 30 min after awakening.<sup>44</sup> Once released in the blood, 90–95% of cortisol binds corticosteroid binding globulin proteins whereas the remaining fraction enters the cells and activate both the glucocorticoid and mineralocorticoid receptors.<sup>45,46</sup> The lower levels of morning salivary cortisol found in this study may indicate that the HPA axis in individuals with lifetime PTSD may be hypoactive. One may assume that this hypoactivity is the consequence of GR hypersensitivity.<sup>47</sup>

Accordingly, our data showed that hGR expression levels are increased in lifetime PTSD independently of current PTSD status. Although similar results have previously been reported,<sup>48</sup> it is the first time that individual hGR variants are investigated in PTSD, and our results suggest that the higher hGR expression may be attributed to the cumulative elevations in hGR 1<sub>B</sub> and 1<sub>C</sub> expression. On the other hand, hGR 1<sub>H</sub> expression was stable between groups and hGR 1<sub>F</sub> could not be detected. This is in agreement with previous findings showing that hGR variants 1<sub>B</sub>, 1<sub>C</sub> and 1<sub>H</sub> are highly expressed in peripheral blood whereas hGR 1<sub>F</sub> is expressed almost exclusively in the brain,<sup>23</sup> and suggests that modifications in peripheral hGR 1<sub>B</sub> and 1<sub>C</sub> expression may be associated with the pathophysiology of PTSD. It is still unclear whether these changes in hGR expression reported in the blood might also be found in the brain. For instance, similar transcriptional differences in the hippocampus (HPC) may alter the inhibitory feedback exerted on the HPA axis activity that would consequently associate with the hypoactivity of the axis reported in this study. More work is required to define whether changes occurring in the peripheral tissue may also be found in the brain.

The expression of specific hGR non-coding exon 1 variants has been associated with different GR protein isoforms.<sup>49</sup> These proteins include GR $\alpha$ , GR $\beta$  and GR-P. GR $\alpha$  acts as the main functionally active isoform while GR $\beta$  and GR-P inhibits and enhances GR $\alpha$  and  $\beta$  activity.<sup>50–54</sup> Interestingly, GR $\alpha$  and GR-P have been associated with hGR 1<sub>C</sub> and 1<sub>B</sub> expression, respectively.<sup>49</sup> Thus, our results suggest that both GR $\alpha$  and GR-P may be up-regulated in individuals with lifetime PTSD providing a potential explanation for the hypoactivity of the HPA axis found in these patients. In addition, we found a correlation between hGR 1<sub>B</sub> expression and cortisol levels at awakening, 30 and 45 min after awakening. This suggests that hGR 1<sub>B</sub> variant may be involved more specifically in the morning increase in cortisol release. Indeed, one may assume that, under normal conditions, the enhancing effect of GR-P may be attenuated to allow more cortisol to be released. However, the overexpression of hGR 1<sub>B</sub> in PTSD, leading to higher levels of GR-P, may counter this attenuation by potentiating the GR-induced inhibition of the HPA-axis activity and subsequently decreasing morning cortisol release. More work is required to clarify the roles of GR protein isoforms on cortisol release.

Our findings suggest that hGR expression may be regulated by changes in DNA methylation. DNA methylation is an epigenetic mark associated with transcriptional repression that acts by interfering with the binding of transcription factors and with the recruitment of the transcriptional machinery to the DNA.<sup>16</sup> Our results show a significant overall and site-specific hypomethylation in the promoters of hGR 1<sub>B</sub> and 1<sub>C</sub> in the PTSD group compared to controls. In addition, hGR total and hGR 1<sub>B</sub> expression was inversely correlated with overall and site-specific methylation levels in hGR 1<sub>B</sub> promoter. This suggest that lower

levels of methylation in hGR 1<sub>B</sub> promoter may allow the transcriptional machinery to bind within this region and potentiate expression as it has been suggested for multiple hGR variants' promoters.<sup>55</sup>

Previous studies suggested that epigenetic mechanisms may be involved in the pathophysiology of PTSD. For instance, DNA methylation alterations in the promoter of the brain-derived neurotrophic factor gene associated with changes in gene expression, have been found within the HPC in a rat model of PTSD.<sup>56</sup> In humans, Uddin *et al.*<sup>57</sup> used a genome-wide approach and reported associations between PTSD and methylation patterns in peripheral blood noting that differentially methylated CpG sites were overrepresented in genes related to immune function and inflammation. This was associated with overall DNA methylation levels being inversely correlated with gene expression levels. Collectively, these results suggest that traumatic events may induce alterations in DNA methylation patterns, which may change gene expression patterns and subsequently induce behavioral modifications in PTSD. It should be mentioned that we did not measure GR protein levels in this study and, although mRNA levels are consistently correlated with DNA methylation levels, future studies should consider measuring protein levels to strengthen the functional impact of DNA methylation on biological functions.

Early-life stress has been shown to interfere with gene expression by altering DNA methylation patterns at multiple genes including GR.<sup>17–19,56,58–68</sup> For instance, decreased GR 1<sub>7</sub> expression associated with promoter hypermethylation has been reported in the HPC of low licking and grooming mothers' offspring.<sup>17</sup> Similarly, lower hGR 1<sub>B</sub>, 1<sub>C</sub> and 1<sub>F</sub> expression has been associated with promoter hypermethylation in the HPC of abused suicide completers<sup>18,19</sup> while hGR 1<sub>B</sub>, 1<sub>C</sub>, 1<sub>D</sub>, 1<sub>F</sub>, 1<sub>H</sub> and 1<sub>J</sub> expression patterns have been shown to vary in the brain of depressed individuals with no differences in promoter methylation levels.<sup>40</sup> However, our results suggest that trauma exposure together with a lifetime diagnosis of PTSD is associated with hypomethylation of hGR 1<sub>B</sub> and 1<sub>C</sub> promoter. Although contrasting, these results are in agreement with the variations in the activity of the HPA axis found in victims of child abuse and depressed individuals compared with lifetime PTSDs. Indeed, the HPA axis in victims of child abuse, as well as depressed patients, is hyperactive.<sup>69–71</sup> In contrast, our results, as well as previous findings,<sup>13,15,41,42</sup> show that the HPA axis in PTSD patients is hypoactive and matches with the overexpression of hGR 1<sub>B</sub> and 1<sub>C</sub> associated with promoter hypomethylation found in PTSDs. Consequently, this suggests that the consequences of stress on the epigenetic regulation of hGR may differ according to type and timing of stressful events and psychiatric condition. Unfortunately, the design of our study did not allow to adequately control for depression. As such, it is unclear whether or not depressive-like symptoms may partly account for our results. More work will be needed to shed light on the important contribution that depression has on the molecular characterization of PTSD symptoms.

The regulation of the HPA axis takes place mainly in the HPC via the binding of GR by glucocorticoids inducing an inhibitory feedback on the activity of the axis.<sup>9,71</sup> However, given that the participants for this study are alive, our experiments were performed in peripheral blood samples, which have been frequently used in studies of PTSD.<sup>14,48,57</sup> Individual differences in lymphocyte GR responsiveness have been associated with changes in the HPA-axis activity<sup>72</sup> and with PTSD symptoms.<sup>73</sup> In addition, T lymphocytes have a major role in cell-mediated immunity and it is believed that traumatic events may induce long lasting epigenetic marks conferring this cell type a memory of past adverse life events. Consequently, we believe that these cells may represent a proxy to the molecular alterations that occur in the brain. More work is required to determine whether similar alterations can be observed in the blood and the brain.

It is important to mention that our analyses revealed no change between current and lifetime PTSD. This may be owing to the small sample size of this exploratory study. Alternately, it is possible that these epigenetic changes, which may be induced by traumatic events, are stable in time and may not be affected by treatment. These changes may also increase vulnerability to stress in individuals with a past PTSD. Epigenetic mechanisms and gene expression in the brain and in peripheral tissue can be affected by antidepressant treatment.<sup>65,66,74</sup> However, our results suggest that this may not be the case for the regulation of GR in PTSD. This is also consistent with our previous results obtained from the brain tissue of abused suicide completers.<sup>19</sup> Thus, although we did not correct for medication in this study, our analysis comparing current and past PTSD suggest that medication may not have an effect on cortisol levels, GR expression and DNA methylation. Nevertheless, more work is required to study the effect of treatment on epigenetic mechanisms and on the epigenetic control of GR expression in the blood.

To summarize, our results suggest that the expression of hGR 1<sub>B</sub> and 1<sub>C</sub> is increased in peripheral blood samples from individuals with lifetime PTSD. These differences in expression were associated with overall and site-specific DNA hypomethylation in T lymphocytes and with lower release of morning salivary cortisol in PTSD. Thus, our results suggest that traumatic events in PTSD induce DNA methylation alterations that modify gene expression and the activity of the HPA axis. However, these alterations differ from the ones described in depressed and abused suicide completers suggesting that different epigenetic mechanisms are involved in the regulation of GR in PTSD individuals. More work is required to better understand the epigenetic mechanisms regulating the expression of GR and the activity of the HPA axis in PTSD.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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