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%.[1,6] However, the lifetime prevalence for PTSD is 7–12%.[1,7]

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.[8] Indeed, changes in the activity of the hypothalamic-pituitary-adrenal (HPA) axis, the main stress regulatory system, are induced by exposure to severe stressors in animals and humans[9,10] 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,[11,12] lower plasma and serum morning cortisol release,[13] higher glucocorticoid receptor (GR) expression in lymphocytes[14] and greater GR sensitivity.[15] 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,[16] including GR expression.[17]

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.[20-23] Both rodent and human GR levels are regulated almost exclusively at the transcriptional level[24,25] and each non-coding exon 1 variant has its own promoter.[22,26-30] These multiple independent promoter regions contribute to the tissue specificity of GR expression.[25] DNA methylation is one of the epigenetic mechanisms controlling the expression of genes and has been shown to be modified by environmental stress.[31] For instance, in rats, maternal behavior regulates hippocampal GR1 expression in offspring through changes in GR1 promoter methylation.[17] Similarly, decreased hippocampal total GR, 1B, 1C, 1F (rat homolog for human GR1F) and 1H expression associates with respective GR exon 1 variant promoter methylation alterations in suicide completers who were abused in childhood.[18,19] 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 (1B, 1C, 1F and 1H), and their association with variations in exon 1 variant promoter DNA methylation in T lymphocytes. We...
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, 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\(^7\) and further corroborated by a self-report symptom measure.\(^3,4\) 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.\(^3,7\) 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\(^3,6\) including any form of substance abuse and living outside of the greater Montreal metropolitan area. Table 1 lists sociodemographic and clinical sample 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.\(^3,32\) 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 using an epityper (Sequenom, San Diego, CA, USA) was performed on the regions of interest \( \beta\text{-Actin}, \text{GAPDH} \) and \( 18S \) and averaged together. Expression data are expressed as ratios of average mean quantities.

### 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) |
| Type of trauma |               |          |
| 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 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 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 \( \mu l \) reaction as followed: 0.6 \( \mu l \) of GR primers/probes, 3.1 \( \mu l \) of water, 6.0 \( \mu l \) of Taqman Master Mix and 2.0 \( \mu l \) of cDNA (1 ng \( \mu l \) ). Thermal cycling conditions were: 50 °C for 2 min, 95 °C for 10 min and 40 cycles, each of 95 °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 (\( \beta\text{-Actin}, \text{GAPDH} \) 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 Biotech, 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 (\( \text{hGR} \)) 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 ± 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 \( (F(3,405) = 151.1, \)
Whole blood human glucocorticoid receptor expression levels

The relative expression of hGRtotal as well as the first non-coding exons 1B, 1C, 1F and 1H was compared across the PTSD and control groups in peripheral blood samples. Results showed a significantly higher expression of hGRtotal (F(1,43) = 5.3; \(P < 0.05\); Figure 2a), 1B (F(1,43) = 12.1; \(P < 0.005\); Figure 2b) and 1C (F(1,43) = 4.2; \(P < 0.05\); Figure 2c) in the PTSD group compared with controls. In contrast, hGR 1H relative expression was not significantly different between the groups (Figure 2d). In addition, consistent with previous findings showing that hGR 1c expression is mainly restricted to the brain, we were unable to quantify its expression in peripheral blood.

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 1B and 1C) that showed higher expression in the PTSD group compared with controls (Supplementary Figure S3). Methylation levels were analyzed across a region of 479 bp composed of 29 CpGs located upstream hGR 1B (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 of hGR 1B promoter was significantly lower in PTSD compared with controls (Figure 4a). Post hoc tests showed site-specific
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 1c methylation levels were analyzed across a 364 bp region composed of 54 CpG upstream hGR 1C (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 1c promoter was significantly lower in PTSD compared with the controls (Figure 3d). 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 1c methylation levels between groups. Our analyses showed no correlation between total methylation in 1c promoter and relative hGR 1c 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 1B, 1C, 1F and 1H, which have been previously studied in the brain of depressed subjects and abused suicide completers. 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, 3, 13, 15, 41, 42 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. 53 Under normal conditions, unbound cortisol increases by about 50% within the first 30 min after awakening. 44 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. For instance, the expression of the isoforms on cortisol release. More work is required to clarify the roles of GR protein in inhibition of the HPA-axis activity and subsequently decreasing morning cortisol levels. 43 Under normal conditions, unbound cortisol levels. 43 In agreement with previous findings showing that hGR variants 1B and 1C expression. On the other hand, hGR 1H expression was stable between groups and hGR 1F could not be detected. This is in accordance with the previous results showing that hGR 1α and 1β are highly expressed in peripheral blood whereas hGR 1γ is expressed almost exclusively in the brain. Similarly, one may assume that, under normal conditions, the expression of GR non-coding exon 1 variants has been associated with different GR protein isoforms. 45 These proteins include GRα, GRβ and GR-P. GRα acts as the main functionally active isoform while GRβ and GR-P inhibits and enhances GRα and β activity. 50–54 Interestingly, GRα and GR-P have been associated with hGR 1C and 1α expression, respectively. 49 Thus, our results suggest that both GRα 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 1α expression and cortisol levels at awakening, 30 and 45 min after awakening. This suggests that hGR 1α variant might 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 1α 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. 10 Our results show a significant overall and site-specific hypomethylation in the promoters of hGR 1B and 1C in the PTSD group compared to controls. In addition, hGR total and hGR 1B expression was inversely correlated with overall and site-specific methylation levels in hGR 1B promoter. This suggest that lower levels of methylation in hGR 1B promoter may allow the transcriptional machinery to bind within this region and potentiate expression as it has been suggested for multiple hGR variants’ promoters. 55

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. 56 In humans, Uddin et al. 57 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. 17–19, 48 Expression associated with promoter hypermethylation has been reported in the HPC of low licking and grooming mothers’ offspring. 17 Similarly, lower hGR 1α, 1γ and 1F expression has been associated with promoter hypermethylation in the HPC of abused suicide completers 18, 19 while hGR 1B, 1C, 1D, 1F, 1α, 1H and 1B expression patterns have been shown to vary in the brain of depressed individuals with no differences in promoter methylation levels. 40 However, our results suggest that trauma exposure together with a lifetime diagnosis of PTSD is associated with hypomethylation of hGR 1α and 1C 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. 69–71 In contrast, our results, as well as previous findings, 13, 15, 41, 42 show that the HPA axis in PTSD patients is hypoaactive and matches with the overexpression of hGR 1α and 1γ 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 corticosteroids to mineralocorticoid receptors. Indeed, the GR-induced inhibition is an inhibitory feedback on the activity of the axis. 71, 72 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. 14, 48, 49 Individual differences in lymphocyte GR responsiveness have been associated with changes in the HPA-axis activity 72 and with PTSD symptoms. 73 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.

© 2014 Macmillan Publishers Limited

Translational Psychiatry (2014), 1 – 7
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.\(^6\) 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 suggesting that this may not be the case for the regulation of the HPA-axis.\(^1\)

Translational Psychiatry (2014), 1

From the Canadian Institutes of Health Research (CIHR). BL is supported by a Frederick Banting and Charles Best doctoral fellowship (FRQ-S). The authors declare no conflict of interest.

To summarize, our results suggest that the expression of hGR\(_{1B}\) and hGR\(_{1C}\) 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.

ACKNOWLEDGMENTS

GT and AB obtained a salary award from the Fonds de Recherche du Québec en santé (FRQ-S). BL is supported by a Frederick Banting and Charles Best doctoral fellowship from the Canadian Institutes of Health Research (CIHR).

REFERENCES

1 Kessler RC, Sonnega A, Bromet E, Hughes M, Nelson CB. Posttraumatic stress disorder in the National Comorbidity Survey. Arch Gen Psychiatry 1995; 52: 1048–1060.

2 Breslau N, Kessler RC, Chilcoat HD, Schultz LR, Davis GC, Andreason P. Trauma and posttraumatic stress disorder in the community: the 1996 Detroit Area Survey of Trauma. Arch Gen Psychiatry 1998; 55: 626–632.

3 Van Ameringen M, Mancini C, Patterson B, Boyle MH. Post-traumatic stress disorder in Canada. CNS Neurosci Ther. 2008. Fall 14: 171–181.

4 Stein MB, Walker JR, Hazen AL, Forde DR. Full and partial posttraumatic stress disorder: findings from a community survey. Am J Psychiatry 1997; 154: 1114–1119.

5 Resnick HS, Kilpatrick DG, Dansky BS, Saunders BE, Best CL. Prevalence of civilian sexual assault and rape victimization and the relationship to PTSD and depression. J Consult Clin Psychol 1993; 61: 984–991.

6 Frans Q, Rimm MA, Aberg L, Fredriksson M. Trauma exposure and post-traumatic stress disorder in a representative national sample of women. J Consult Clin Psychol 1993; 61: 984–991.

7 Birnbaum L. The epidemiology of posttraumatic stress disorder: what is the extent of the problem? J Clin Psychiatry 2001; 62: 16–22.

8 Mehta D, Binder EB. Gene x environment vulnerability factors for PTSD: The HPA-axis. Neuropharmacology 2012; 62: 654–662.

9 Heim C, Newport DJ, Mietlozi T, Miller AH, Nemeroff CB. The link between childhood trauma and depression: insights from HPA axis studies in humans. Psychoneuroendocrinology 2008; 33: 693–710.
47 Pruessner JC, Gaab J, Hellhammer DH, Lintz D, Schommer N, Kirschbaum C.
42 Wessa M, Rohleder N, Kirschbaum C, Flor H. Altered cortisol awakening response
43 Umeda T, Hiramatsu R, Iwaoka T, Shimada T, Miura F, Sato T. Use of saliva for
44 Pruessner JC, Wolf OT, Hellhammer DH, Buske-Kirschbaum A, von Auer K, Jobst S
50 Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R
54 de Lange P, Segeren CM, Koper JW, Wiemer E, Sonneveld P, Brinkmann AO
53 Krett NL, Pillay S, Moalli PA, Greipp PR, Rosen ST. A variant glucocorticoid receptor
56 Roth TL, Zoladz PR, Sweatt JD, Diamond DM. Epigenetic modi
55 Cao-Lei L, Leija SC, Kumsta R, Wust S, Meyer J, Turner JD
© 2014 Macmillan Publishers Limited

Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)