Archival Report

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Lower Methylation of Glucocorticoid Receptor Gene Promoter 1_F in Peripheral Blood of Veterans with Posttraumatic Stress Disorder

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ABSTRACT

BACKGROUND: Enhanced glucocorticoid receptor (GR) sensitivity is present in people with posttraumatic stress disorder (PTSD), but the molecular mechanisms of GR sensitivity are not understood. Epigenetic factors have emerged as one potential mechanism that account for how trauma exposure leads to sustained PTSD symptoms given that PTSD develops in only a subset of trauma survivors.

METHODS: Cytosine methylation of a relevant promoter of the GR gene (*NR3C1*-1_F promoter) and three functional neuroendocrine markers of hypothalamic-pituitary-adrenal axis function were examined in a sample of 122 combat veterans.

RESULTS: Lower *NR3C1*-1_F promoter methylation in peripheral blood mononuclear cells (PBMCs) was observed in combat veterans with PTSD compared with combat-exposed veterans who did not develop PTSD. *NR3C1*-1_F promoter methylation was also associated with three functional measures of glucocorticoid activity that have been associated with PTSD in combat veterans: PBMCs' lysozyme inhibition on the lysozyme suppression test, plasma cortisol decline on the low-dose (.50 mg) dexamethasone suppression test, and 24-hour urinary cortisol excretion. Finally, *NR3C1*-1_F promoter methylation was inversely correlated with clinical markers and symptoms associated with PTSD.

CONCLUSIONS: Alterations in *NR3C1*-1_F promoter methylation may reflect enduring changes resulting from combat exposure that lead to functional neuroendocrine alterations. Because epigenetic measures are thought to reflect enduring effects of environmental exposures, they may be useful in distinguishing combat-exposed veterans who do or do not develop PTSD.

Keywords: Biomarkers, Combat, Cortisol, Glucocorticoid receptor, HPA axis, Methylation, *NR3C1* gene, *NR3C1*-1_F promoter, PTSD

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Cytosine methylation is an epigenetic mechanism that can mediate the sustained effects of environmental conditions on genomic transcription, moderating genetic predispositions (1). In rodents (2) and humans (3), early environmental experience is associated with hippocampal methylation of the glucocorticoid receptor (GR) gene (*NR3C1*) exon 1_7 promoter (rat) and its human ortholog, the exon 1_F promoter, and with regulation of the hypothalamic-pituitary-adrenal (HPA) axis. These findings are of interest for the study of posttraumatic stress disorder (PTSD), which has been related to enhanced GR sensitivity, low glucocorticoid levels, and a history of childhood abuse and neglect. Epigenetic signals associated with early life experiences offer a potential explanation for why stress responses do not abate in certain individuals after the stressor is removed and for the fact that not all people exposed to trauma develop PTSD (4). In Dutch soldiers who served in Afghanistan, higher GR levels in peripheral blood mononuclear cells (PBMCs) before deployment predicted PTSD (5).

The goal of the current study was to examine methylation of the *NR3C1*-1_F promoter in combat veterans with or without PTSD who served in Operation Enduring Freedom or Operation Iraqi Freedom or both. We hypothesized that *NR3C1*-1_F promoter methylation would be associated with functional markers of glucocorticoid sensitivity. Because PTSD is associated with enhanced GR sensitivity, greater feedback regulation, and lower basal HPA axis tone (e.g., lower ambient cortisol levels), we predicted lower GR promoter methylation in combat veterans with PTSD (PTSD+) compared to veterans with similar combat exposure without PTSD (PTSD−). Three functional and relatively stable neuroendocrine measures previously demonstrated to vary in association with PTSD—the lysozyme suppression test (an in vitro measure of GR sensitivity; see Methods), cortisol decline in response to a

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low-dose (.50 mg) dexamethasone (DEX) suppression test, and 24-hour urinary cortisol excretion—were examined to determine their association with PTSD and *NR3C1*-1_F promoter methylation. We hypothesized lower lysozyme $IC_{50\text{-}DEX}$ and a greater cortisol decline after DEX administration, both reflecting increased GR sensitivity, would occur in combat veterans with PTSD. *NR3C1*-1_F promoter methylation regulates transcriptional capacity in response to transcription factor binding (3). We hypothesized that *NR3C1*-1_F promoter methylation would be inversely associated with *NR3C1*-1_F expression and GR functional outcomes such that lower methylation would be associated with indices reflecting enhanced GR sensitivity. Additionally, because higher GR sensitivity is thought to underpin lower ambient 24-hour cortisol excretion, frequently observed in individuals with combat-related PTSD, we further hypothesized an association between *NR3C1*-1_F promoter methylation and basal urinary cortisol.

METHODS AND MATERIALS

Participants and Clinical Assessment

This article reports on data from 122 participants (61 with PTSD, 61 without PTSD) in whom *NR3C1*-1_F promoter methylation and gene expression was determined in PBMCs, in addition to other neuroendocrine biomarkers. Participants were recruited as part of a larger study that was designed as an initial investigation of male combat veterans, to be followed by a replication and longitudinal validation study in men and women. Combat veterans were recruited at two sites, the James J. Peters Veterans Affairs Medical Center affiliated with Icahn School of Medicine at Mount Sinai and New York University Langone Medical Center affiliated with New York University School of Medicine through advertising in the clinic (Veterans Affairs Medical Center) and community (New York University). The study was approved by the institutional review boards of the James J. Peters Veterans Affairs Medical Center, Icahn School of Medicine at Mount Sinai, New York University Langone Medical Center, and New York University School of Medicine; all participants provided written, informed consent.

The presence versus absence of a PTSD diagnosis was determined by doctoral-level psychologists using the Clinician Administered PTSD Scale (CAPS) (6). Because it was of interest to evaluate biomarkers that distinguished effects of PTSD from effects of exposure, all participants had exposure to a combat-related criterion A trauma. Participants in the group with war zone exposure and no PTSD diagnosis had current CAPS scores $\leq$20 and had never met criteria for PTSD. Participants in the PTSD group were required to have a CAPS score $\geq$40 and to meet full DSM-IV criteria for war zone–related PTSD; combat veterans with CAPS scores between 20 and 40 were not included in the study. The Structured Clinical Interview for DSM-IV (7) was used by the same clinician to determine other DSM-IV diagnoses including substance use disorders. All participants remained eligible if they met criteria for a current or lifetime mood or anxiety disorder other than PTSD; all subjects with lifetime history of any psychiatric disorder with psychotic features, bipolar disorder, or obsessive-compulsive disorder; prominent suicidal or homicidal ideation; or a suicide attempt in the past year were excluded. Participants with current alcohol dependence or a current drug abuse or dependence diagnosis were also excluded from participation. Diagnostic information was reviewed in a weekly teleconference including all diagnostic interviewers, which ensured a similar use of diagnostic measures and convergent application of inclusion and exclusion criteria between the two sites. Medical exclusions included neurologic disorder, loss of consciousness >10 min, or other systemic illness affecting central nervous system function. Relevant self-reported clinical assessments were obtained to determine severity of PTSD [with the Posttraumatic Stress Checklist (8) and depressive symptoms [with the Beck Depression Inventory (9)]. The Early Trauma Inventory (10) was used to assess childhood trauma exposure. Other measures included the Pittsburgh Sleep Quality Index (11), the Peritraumatic Dissociative Experiences Questionnaire (to assess the extent of dissociation at the time of the focal deployment trauma) (12), and the Symptom Checklist-90-R (13).

Biological Methods

Blood was drawn before and after .5 mg DEX ingestion between 8:00 AM and 8:30 AM. An aliquot of whole blood was reserved from the day 1 blood sample for measurement of leukocyte count and cell type distribution. Plasma samples were collected into ethylenediamine tetraacetate–containing tubes, and an aliquot was frozen for subsequent hormone analysis. Using ACCUSPIN tubes (Sigma-Aldrich, St. Louis, Missouri), PBMCs were purified by Ficoll-Paque (Amersham, Piscataway, New Jersey). After two washes in Hanks' Balanced Salt Solution (Life Technologies, Grand Island, New York), PBMCs were counted with a hemocytometer. The purified PBMCs were divided into aliquots for DNA extraction and cell culture for determination of the $IC_{50\text{-}DEX}$ of lysozyme inhibition. Assessment of the distribution of white blood cell populations was performed in a clinical certified (Clinical Laboratories Improvement Act) laboratory. Because cell type composition in PBMC samples covaries with DNA methylation (14), the PBMC ratio (lymphocyte-to-monocyte ratio), a proxy of PBMC type, was used as a covariate in DNA methylation analyses.

Participants were also instructed to collect 24-hour urine samples (15), and completeness of collections was determined by participants' report and assessment of creatinine levels. Urine volumes <500 mL were deemed incomplete, and results were not used in subsequent analyses.

Five main variables were measured: 1) *NR3C1*-1_F promoter methylation; 2) *NR3C1*-1_F expression; 3) in vitro sensitivity to DEX of lysozyme, a GR-regulated enzyme, assessed in cultured PBMCs; 4) cortisol decline in response to .50 mg DEX; and 5) 24-hour urinary cortisol excretion.

DNA Cytosine Methylation of *NR3C1*-1_F Promoter. Genomic DNA was extracted from the frozen PBMC pellets following the FlexiGene DNA Kit protocol (Qiagen, Valencia, California). Methylation mapping of the 39 C-phosphate-G (CpG) sites in the *NR3C1*-1_F promoter was performed as previously described (3,16) using 30 clones per sample.