Association of the *Period3* clock gene length polymorphism with salivary cortisol secretion among police officers

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Abstract

OBJECTIVE: This study evaluated whether measures of waking or diurnal cortisol secretion, or self-reported psychological disturbances differed among police officers with a *Period3* (*PER3*) clock gene length polymorphism.
METHODS: The cortisol awakening response was characterized via the area under the salivary cortisol curve with respect to the increase (AUC_I) or total waking cortisol (AUC_G). Diurnal cortisol measures included the slope of diurnal cortisol and the diurnal AUC_G. Psychological disturbances were characterized using the Center for Epidemiologic Studies Depression Scale, Impact of Events Scale, and Life Events Scale.
RESULTS: Officers with a 4/5 or 5/5 genotype had higher awakening AUC_G and greater diurnal cortisol AUC_G levels compared to officers with the 4/4 genotype. Among those working more afternoon or night shifts, waking AUC_I and AUC_G were greater among officers with a 4/5 or 5/5 genotype compared to the 4/4 referents.
CONCLUSION: Cortisol secretion was modified among police officers with different *PER3* VNTR clock gene variants.

ORIGINAL ARTICLE

Abbreviations:

SCN	- suprachiasmatic nuclei
VNTR	- variable number tandem repeat
PER3	- Period 3 gene
HPA	- hypothalamic-pituitary-adrenal
ACTH	- adrenocorticotropic hormone
CVD	- cardiovascular disease
BCOPS	- Buffalo Cardio-Metabolic Occupational Police Stress
AUC_I	- area under the curve with respect to increase
AUC_G	- area under the curve with respect to ground
CES-D	- Center for Epidemiologic Studies Depression scale
IES	- Impact of Events Scale
LS	- least squares
CKIε	- casein kinase I epsilon
EEG	- electroencephalography
REM	- rapid eye movement
PTSD	- post-traumatic stress disorder

INTRODUCTION

Clock genes are part of the body's intrinsic timekeeping system. Their expression exhibits positive and negative transcription-translation feedback loops that help maintain tissue-specific circadian rhythmicity (Cermakian & Boivin 2009; Yu & Weaver 2011). Clock genes have been described in the body's central pacemaker, the suprachiasmatic nuclei (SCN) (Fu & Lee 2003; Okamura *et al.* 2010), and in most peripheral tissues and major organ systems (Hastings *et al.* 2007). Clock gene expression helps maintain ~24-hour cyclical variation in numerous physiological and cellular processes in a manner that is synchronized with ambient light-dark cycles, and dysregulation of clock genes can impact sleep-wake cycles, cardiovascular, digestive and endocrine systems, and mental state (Hastings *et al.* 2007; Kripke *et al.* 2009; Takeda & Maemura 2010; Landgraf *et al.* 2012). Clock genes also influence cell cycle control (e.g. cell proliferation, DNA repair, apoptosis) (Khapre *et al.* 2010), and disrupted clock gene expression may increase susceptibility to several chronic diseases including cancer (Huang *et al.* 2011).

A variable number tandem repeat (VNTR) sequence within the human *Period3* (*PER3*) clock gene (rs57875989) codes for 4 or 5 copies of a 54-base pair length polymorphism. Phenotypic differences in various physiological or psychological measures have been described among those with the 4/4 or 5/5 genotype, Some investigators have reported that the 4/4 genotype may be associated with evening diurnal preference (Archer *et al.* 2003; Ellis *et al.* 2009) and greater heroin dependence (Zou *et al.* 2008), whereas others suggest that the 5/5 or combined 4/5+5/5 genotypes are associated with morning preference (Archer *et al.* 2003; Ellis *et al.* 2009), poorer cognitive performance after short-term (40-hr) sleep deprivation (Groeger *et al.* 2008), higher circulating concentrations of insulin-like growth factor-1 or interleukin-6 (Chu *et al.* 2008; Guess *et al.* 2009), earlier age of onset of bipolar disorder (Benedetti *et al.* 2008), a tendency for depressive symptoms (Guess *et al.* 2009), or increased odds of breast cancer primarily among premenopausal women (Zhu *et al.* 2005). However, associations among those with different *PER3* VNTR genotypes have not been consistent. For example, several studies have found no association between *PER3* VNTR genotypes and diurnal preference or breast cancer (Dai *et al.* 2010; Barclay *et al.* 2011; Osland *et al.* 2011). Individuals with the 5/5 *PER3* VNTR had strong correlations between *PER3* expression and the timing of peak cortisol or melatonin levels in blood, whereas those with the 4/4 genotype did not (Boivin *et al.* 2003; Archer *et al.* 2008). Thus, the *PER*3 VNTR may serve as a marker of genetic susceptibility to the effects of sleep deprivation or circadian misalignment that can influence the timing of secretion of cortisol or melatonin, two hormones with typically robust circadian rhythms (Dijk & Archer 2010). However, the functional consequences of this polymorphism in terms of adaptation to work schedule or work-related stress are not completely understood, particularly among shiftworkers.

Cortisol is a well-described adrenal steroid "stress hormone" (Henry 1992). Salivary cortisol typically increases by 40–75% upon awakening (cortisol awakening response), and then decreases throughout the day (except for a common post-prandial spike after the mid-day meal) (Kudielka *et al.* 2006; Fries *et al.* 2009). Daily cortisol rhythms are controlled by the SCN through efferent connections with neurons of the paraventricular nucleus and through hypothalamic-pituitary-adrenal (HPA) axis-independent alterations in ACTH (adrenocorticotropic hormone) sensitivity in the adrenal cortex (Nader *et al.* 2010). Stressful circumstances stimulate an increase in cortisol secretion (Kudielka *et al.* 2006). If these stressful circumstances become chronic in nature, they may result in an inability of the HPA axis to self-regulate cortisol, referred to as the "exhaustion stage" of the general adaptation syndrome, or allostatic overload (Motzer & Hertig 2004). The extent to which diurnal cortisol secretion patterns are influenced by polymorphic variation in clock genes is not well understood (Hastings *et al.* 2007; Archer *et al.* 2008; Nader *et al.* 2010). However, cortisol dysregulation can perturb physiological processes controlling inflammation (Elenkov 2008), and possibly augment susceptibility to depression, post-traumatic stress disorder, cardiovascular disease (CVD), type II diabetes, or stroke (Zuzewicz *et al.* 2000; Neylan *et al.* 2005; Huber *et al.* 2006; Gidron & Ronson 2008; Scheer *et al.* 2008). An altered pattern of circadian cortisol secretion also is associated with poor cancer survival (Sephton *et al.* 2000; Sephton *et al.* 2012).

The Buffalo Cardio-Metabolic Occupational Police Stress (BCOPS) cohort study provides a prospective framework for examining biological processes through which stressors associated with police work may mediate adverse health outcomes. The protocol combines the characterization of stress biomarkers, subclinical CVD measures, psychosocial factors, and shiftwork to

examine their potential associations with psychological disturbances and chronic diseases afflicting police officers (Violanti *et al.* 2006; Violanti *et al.* 2009). Shiftwork can result in circadian rhythm dysregulation, sleep insufficiency, and chronic stress (Shields 2002; Burch *et al.* 2009), which could eventually lead to cortisol dysregulation and allostatic overload (McEwen & Stellar 1993). Previously, we found that recent night work (within 3–14 days), or a high number of cumulative shift changes over a period of years was associated with reductions in the salivary cortisol awakening response among officers in this cohort (Wirth *et al.* 2011). The possible implications for cortisol dysregulation, or changes in the human stress response due to *PER3* genetic variation among police or other workers in stressful occupations remains to be determined. Our objective in the present analysis was to test the hypotheses that police officers with a 4/5 or 5/5 *PER3* genotype have a more robust awakening (waking AUC_I or AUC_G) or diurnal cortisol rhythm (diurnal AUC_G, slope) compared to those with the 4/4 genotype, and whether this relationship may be modified by shiftwork. Potential differences in depressive or stress-related symptoms among those with different *PER3* VNTR genotypes were also examined.

METHODS

Study population

Police officers from the Buffalo, New York Police Department enrolled in the BCOPS cohort were selected using a computer-generated random sample (42 females, 58 males) (Violanti *et al.* 2006; Wirth *et al.* 2011). The study received Institutional Review Board approval and all subjects provided informed consent. Participants were examined at a health clinic on a scheduled training day or day off, and saliva collection occurred the day after the clinic visit at the officers' homes. Data collection included a peripheral white blood cell sample for DNA recovery, serial saliva collection over a single non-work day, long-term shiftwork history, basic demographic characteristics, and the completion of several validated instruments that ascertained stress related to traumatic events (Impact of Events Scale or IES) (Horowitz *et al.* 1979), significant life events (Life Events Scale) (Paykel *et al.* 1971), or depressive symptoms (Center for Epidemiologic Studies Depression scale or CES-D) (Radloff 1977). A majority of the participating officers (88%) were either working a day shift or had a day off prior to their clinic visit.

Shiftwork history

Daily work histories were obtained for each participant from 1994 or initiation of employment (if it occurred after 1994) to the date of study examination between 2001 and 2003 using electronic payroll records (Wirth *et al.* 2011). The typical work schedule after 1994 consisted of four work days, four days off, four work days, three days off, and then the cycle was repeated. Long-term shiftwork variables included the cumulative number of shift changes and a categorical shift status variable defined as the shift on which each participant spent the majority of her or his time working during the study period. Shifts were classified based on start times as day (between 04:00–11:59 h), afternoon (between 12:00–19:59 h), or night (between 20:00–03:59 h). Most subjects (>85%) spent a majority (≥70%) of their total work time on their designated shift. This process of classifying officers into a shift status has shown good consistency summarized over 30, 60, or 90 days, and after 5 years of employment (Violanti *et al.* 2009; Wirth *et al.* 2011). Those on night or afternoon shifts were combined for the stratified analyses. Although officers worked fixed shifts during the study period (i.e., day, afternoon, or night only), they occasionally worked for an absent colleague or were temporarily assigned to a different shift schedule. The frequency of shift changes was defined as the number of times a participant switched between any two shift types during the study period.

Salivary cortisol

Salivary cortisol measurements are noninvasive, thereby reducing participant burden and facilitating protocol compliance; and the biologically active hormone can be readily quantified via a sensitive and specific immunoassay (Violanti *et al.* 2009). Participants collected serial saliva samples: upon first awakening, then at 15-, 30-, and 45-minute intervals after waking and at 12:00h (before lunch), 17:00h (before dinner), and at bedtime (Wirth *et al.* 2011). Cortisol levels obtained based on collection from time of awakening have a higher test-retest stability compared to samples collected at specific clock times (Coste *et al.* 1994; Pruessner *et al.* 1997; Neylan *et al.* 2005). On the morning of participation, sample collection was achieved by placing a Salivette cotton roll (Sarstedt Aktiengesellschaft & Company, Numbrecht, Germany) into the mouth for three minutes to allow for saturation. The saturated roll was then refrigerated until delivery at the research laboratory for processing. Samples were shipped to the National Institute for Occupational Safety and Health (Toxicology and Molecular Biology Branch, Health Effects Laboratory, Morgantown, WV) where they were centrifuged and archived at –20 °C (Fekedulegn *et al.* 2007). Cortisol determinations were performed at the Technical University of Dresden, Dresden, Germany, using a chemiluminescence immunoassay (CLIA, IBL-Hamburg, Germany) (Fekedulegn *et al.* 2007; Violanti *et al.* 2009; Wirth *et al.* 2011). Quality control samples were quantified at low and high concentrations for each assay plate, and analyses were repeated if control samples were outside the range of the expected concentration. The intra- and inter-assay coefficients of variation were 8% or below for either the high (25 nmol/l) or low (3

nmol/l) control concentrations. Blind replicate samples of participants' salivary cortisol (10%) had a coefficient of variation of 15%.

Waking cortisol was summarized using the area under the curve with respect to increase above baseline (i.e. first waking sample), and AUC above the assay detection limit or ground concentration (Fekedulegn *et al.* 2007). The AUC_I represents the change in cortisol secretion after awakening, or its reactivity (Fekedulegn *et al.* 2007). If cortisol levels decrease relative to the first waking value, it is possible to obtain a negative AUC_I value. The AUC_G measures the total amount of cortisol secreted during the sampling period (Fekedulegn *et al.* 2007). The diurnal slope represents the change in cortisol secretion across the day, which was estimated by fitting the initial waking, noon, dinner, and bed time salivary cortisol sample concentrations to a linear equation and estimating the line of best fit (Kraemer *et al.* 2006; Heaney *et al.* 2012). All cortisol time points were required to calculate diurnal AUC_G measures, but this requirement was not true for diurnal slope. To maintain consistency, we restricted all main diurnal analyses only to participants with both diurnal AUC_G and diurnal cortisol slope measures.

Genotyping

Peripheral blood samples were collected on the day of the clinic visit, centrifuged using a Ficoll gradient to separate WBCs, then stored in capillary tubes at –80 °C for recovery of DNA. Genomic DNA was extracted using the DrGentle protocol (Takara, Japan) and DNA pellets (50–100 μg) were dissolved in 100–200 μL of TE buffer. About 200ng was subjected to polymerase chain reaction (PCR) using a Perkin Elmer GeneAmp System 9700 (Waltham, MA) according to the manufacturer's protocol. The *PER3* VNTR repeat polymorphism was amplified using the following two primers: (forward) 5'-CAAAATTTTATGACACTACCAGAATGGCTGAC-3' and (reverse) 5'-AACCTTGTACTTCCACATCAGTGCCTGG-3' (Zhu *et al.* 2005). The PCR was performed in a reaction mixture of 25 μl containing standard PCR buffer, 5% DMSO, 1.0 mM $MgCl_2$, 0.2 mM dNTP, 1 unit *Taq* polymerase (Gibco-Invitrogen), and 0.4 μM of each oligonucleotide primer. The reactions were heated to 94 °C for 2 minutes followed by 35 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 5 seconds. Reactions were extended for 7 minutes at 72 °C, and PCR products were then separated by electrophoresis on 3% agarose gel. Laboratory personnel were blinded to the identity and characteristics of the participants. Quality control re-analyses of 10% of the genotypes indicated 100% concordance.

Statistical analysis

Analyses were performed using SAS analytical software package (version 9.2, Cary, NC)®. Relationships between each dependent variable (waking AUC_I, waking AUC_G, diurnal AUC_G, diurnal slope, IES, CES-D, and Life Events Scale) and potential confounding factors (i.e. age, gender, race, education, marital status, rank, and years of police work) were evaluated univariately using the generalized linear models (PROC GLM) procedure in SAS. Variables were selected for further evaluation as potential confounders if their statistical significance was $p \leq 0.15$. A backward elimination procedure was then used to develop final models that included all variables that were statistically significant ($p \leq 0.05$) or, when removed from the model, changed the beta coefficient of the *PER3* VNTR genotype by at least 10%. One diurnal AUC_G observation was removed due to a studentized residual of 4.02 and a Cook's D of 0.48, which is greater than the suggested cut-point (4/sample size included in analysis [n=54] or 0.07). The GLM procedure in SAS was used to compute adjusted (least squares or LS) means of each dependant variable among those with different *PER3* VNTR genotypes, after adjustment for the selected covariates. A square root transformation was used to obtain normally distributed values and normalized model residuals of the Life Events and CES-D scores; the LS means were back-transformed for presentation in the tables. *A priori* comparisons included differences in mean dependent variables among the 4/5, 5/5 or combined 4/5+5/5 genotypes compared to the 4/4 genotype. In separate analyses, differences in LS mean cortisol measures among the *PER3* genotypes were stratified by shift status (night+afternoon vs. day shifts) or cumulative shift changes (a median split of <17 vs. >17). Individuals with 4/5 or 5/5 genotypes were similar with respect to all covariate and exposure variables, and were therefore combined in stratified analyses (Zhu *et al.* 2005; Dai *et al.* 2010). Ancillary logistic regression analyses indicated that participants with missing data did not differ with respect to the *PER3* VNTR, shiftwork, CES-D, IES, Life Events Scale, or any covariate data, and were thus considered missing at random. Additional adjustment for time of awakening (first saliva collection) did not alter the interpretation of the results presented below.

RESULTS

Complete waking cortisol data were available for 57 officers (32 missing waking cortisol samples, 11 missing *PER3* VNTR) and diurnal cortisol data were available for 54 participants (37 missing diurnal cortisol, 9 missing *PER3* VNTR). The distribution of *PER3* VNTR genotypes among participants was in Hardy-Weinberg Equilibrium ($\chi^2=0$, p-value=1.0). The average number of years of police work in this population (± standard deviation) was 14±9 years (range: 1–33 years). The mean age was 43±8 years (range: 29–63 years). Males comprised 60% of the study group and European Americans 75%. There were no statistically significant differences in age, gender, race, education, marital status, rank, years worked, or cumulative shift changes among the 4/5, 5/5, or 4/5+5/5 genotypes compared to the 4/4 group (Table 1). Similarly, there we no statistically

Tab. 1. Population Characteristics by *PER3* Variable Number Tandem Repeat, BCOPS Study, Buffalo, NY, USA, 2001–2003.

Characteristic	Total (n=57)	4/4 (n = 19)	4/5 (n = 28)	5/5 (n = 10)	4/5 + 5/5 (n = 38)
Age Group					
<40	20 (35%)	6 (32%)	12 (43%)	2 (20%)	14 (37%)
40–49	24 (42%)	7 (37%)	11 (39%)	6 (60%)	17 (45%)
>50	13 (23%)	6 (32%)	5 (18%)	2 (20%)	7 (18%)
Gender					
Male	34 (60%)	14 (74%)	14 (50%)	6 (60%)	20 (53%)
Female	23 (40%)	5 (26%)	14 (50%)	4 (40%)	18 (47%)
Race					
European American	43 (75%)	14 (74%)	22 (79%)	7 (70%)	29 (76%)
African American	14 (25%)	5 (26%)	6 (21%)	3 (30%)	9 (24%)
Education					
≤ High School	9 (16%)	3 (16%)	5 (18%)	1 (10%)	6 (16%)
College	18 (32%)	5 (26%)	9 (32%)	4 (40%)	13 (34%)
> College	30 (53%)	11 (58%)	14 (50%)	5 (50%)	19 (50%)
Marital Status					
Single	13 (23%)	4 (21%)	6 (21%)	3 (30%)	9 (24%)
Married	37 (65%)	13 (68%)	18 (64%)	6 (60%)	24 (63%)
Divorced	7 (12%)	2 (11%)	4 (14%)	1 (10%)	5 (13%)
Rank					
Police Officer	38 (67%)	13 (68%)	19 (68%)	6 (60%)	25 (66%)
Sergeant/Lieutenant	10 (18%)	2 (11%)	6 (21%)	2 (20%)	8 (21%)
Captain/Detective	9 (16%)	4 (21%)	3 (11%)	2 (20%)	5 (13%)
Years Worked					
1–5 (n=13)	13 (23%)	5 (26%)	6 (21%)	2 (20%)	8 (21%)
6–10 (n=7)	7 (12%)	0 (0%)	6 (21%)	1 (10%)	7 (18%)
11–15 (n=15)	15 (26%)	5 (26%)	7 (25%)	3 (30%)	10 (26%)
>15 (n=22)	22 (39%)	9 (47%)	9 (32%)	4 (40%)	13 (34%)
Shift Changes per Year					
Mean ± SD	35.6 ± 52.4	32.6 ± 45.9	38.5 ± 58.5	54.8 ± 72.8	42.7 ± 61.8

Abbreviations: SD – Standard deviation; Column percentages not totaling 100% are due to rounding.

significant differences in the mean time of first saliva sample collection among participants with the 4/4, 4/5 or 5/5 genotypes (08:16±112, 07:45±94, and 07:12±129 minutes, respectively) on the day of study participation.

Mean waking cortisol AUC_G levels were greater among those with the 4/5+5/5 genotype (775 vs. 448 nmol/L-minute, $p<0.01$) compared to the 4/4 group. The 4/5 and 5/5 groups showed a tendency for higher waking AUC_I values compared to the 4/4 group, although the differences were not statistically significant. The 4/5+5/5 group (7201 vs. 4996 nmol/L-minute, $p<0.01$) and those with the 5/5 genotype (7279 vs. 4996 nmol/L-minute, $p=0.02$) had greater mean diurnal AUC_G values compared to the 4/4 group (Table 2). Note that there were no differences between the 5/5 and 4/5 groups for any of the cortisol measures evaluated, which provides a reasonable rationale for combining 4/5 and 5/5 groups in the analysis. Only those with both a diurnal AUC_G value and a diurnal slope were included in the analyses. A post-hoc analysis of all individuals with a diurnal slope value (n=76) did not change the interpretation of the results.

Regardless of shiftwork status, officers with the 4/5 or 5/5 genotype had greater mean diurnal AUC_G values than officers with a 4/4 genotype (Table 3). After stratification by shift status, those participating in afternoon or night shiftwork who also possessed the 4/5+5/5 genotype had elevated mean waking

Tab. 2. Mean Waking and Diurnal Salivary Cortisol Levels (95% Confidence Intervals) by *PER3* Variable Number Tandem Repeat Genotype, BCOPS Study, Buffalo, NY, USA, 2001–2003

Dependent Variables	4/4 (n=19)	4/5 (n=28)	5/5 (n=10)	4/5+5/5 (n=38)	*p*-values			
					4/4 vs. 4/5	4/4 vs. 5/5	4/5 vs. 5/5	4/5+5/5 vs. 4/4
Waking AUC_I	63 (-45–172)	122 (36–209)	151 (6–296)	130 (56–203)	0.40	0.33	0.74	0.31
Waking AUC_G	448 (254–641)	826 (660–991)	646 (395–897)	775 (630–921)	<0.01	0.19	0.21	<0.01
Diurnal AUC_G	4,996 (3,833–6,162)	7,172 (6,215–8,130)	7,279 (5,768–8,789)	7,201 (6,368–8,033)	<0.01	0.02	0.90	<0.01
Diurnal Slope	-0.0026 (-0.0035–-0.0018)	-0.0030 (-0.0037–-0.0024)	-0.0020 (-0.0031–-0.0010)	-0.0028 (-0.0033–-0.0022)	0.47	0.38	0.13	0.84
CESD	5.9 (3.5–9.1)	5.8 (3.7–8.3)	6.1 (3.0–10.3)	5.9 (4.0–8.1)	0.91	0.95	0.88	0.95
IES	20.8 (13.0–28.5)	18.4 (11.8–25.0)	18.4 (8.3–28.4)	18.4 (12.6–24.1)	0.61	0.70	0.99	0.59
Life Events Scale	2.4 (1.3–3.7)	2.9 (1.8–4.2)	1.9 (0.7–3.7)	2.6 (1.7–3.7)	0.51	0.66	0.32	0.73

95% confidence intervals are in parentheses. Units for AUC_I and AUC_G are nmol/L-minutes. Abbreviations: AUC_G – Area Under the Curve with respect to ground; AUC_I – Area Under the Curve with respect to increase; CESD – Center for Epidemiologic Studies Depression scale; and IES – Impact of Events. Adjustments: Waking AUC_G, CESD, and IES adjusted for rank; Waking AUC_I adjusted for gender; Diurnal AUC_G adjusted for education and age; Diurnal Slope adjusted for gender and age group; Life Events Scale adjusted for race.

AUC_I (202 vs. −8 nmol/L-minute, respectively, p=0.03) and mean waking AUC_G values (791 vs. 361 nmol/L-minute, respectively, p=0.01) relative to shiftworkers with the 4/4 genotype (Table 3). However, these values did not differ by genotype among day workers. When cumulative shift changes were examined, officers with the 4/5 or 5/5 genotype tended to have greater mean waking AUC_I, waking AUC_G, or diurnal AUC_G values compared to those with the 4/4 genotype, regardless of whether they were above or below the median number of cumulative shift changes (Table 4). There were no statistically significant differences in the mean diurnal cortisol slopes (Tables 2–4) or mean scores for IES, Life Events Scale, or CES-D among those with different *PER3* VNTR genotypes (Table 2).

DISCUSSION

The role of the *PER3* length polymorphism in the regulation of sleep and circadian processes in human populations has not been fully elucidated. The extra copy of the 5-repeat *PER3* VNTR sequence contains several potential casein kinase Iε (CKIε) phosphorylation motifs (Archer *et al.* 2003). Phosphorylation of Period clock genes by CKIε is required for translocation of the period and cryptochrome protein complex into the cell nucleus so that it can exert its influence on the negative arm of the clock gene transcriptional-translational feedback loop. CKIε also facilitates metabolic degradation of this complex (Nader *et al.* 2010). In the *PERIOD* 2 clock gene, a CKIε binding site mutation has been associated with gene hypophosporylation and familial advanced sleep phase syndrome (Toh *et al.* 2001). Polymorphic variation in the *PER3* gene has been associated with differences in the homeostatic regulation of sleep and the timing of circadian hormone secretion (Archer *et al.* 2008; Dijk & Archer 2010). When participants with different *PER*3 VNTR genotypes were subjected to a 40-hour sleep deprivation protocol, those with the 5/5 genotype experienced greater changes in EEG activity and REM sleep, and increased sleep pressure compared to those with the 4/4 genotype (Cajochen *et al.* 1995; Dijk *et al.* 1997; Viola *et al.* 2007). Individuals with this genotype also performed poorly (relative to baseline) on a waking performance test after sleep deprivation, whereas impacts among those with the 4/4 genotype were much less apparent (Viola *et al.* 2007). Other studies suggest that *PER3* may only have a minimal role in regulating circadian processes (Shearman *et al.* 2000; Bae *et al.* 2001; Costa *et al.* 2011), and that the influence of the *PER*3 VNTR on sleep homeostasis may vary depending on the duration of sleep deprivation, or the strategy used to adjust to sleep loss (Goel *et al.* 2009; Gamble *et al.* 2011). Whether the *PER3* VNTR evokes differences in circadian endocrine secretion, for example among shift workers or those with altered sleep-wake timing, remains to be determined. Positive correlations between *PER3* expression and the timing of peak melatonin or cortisol secretion (but not amplitude) were previously observed among those with the 5/5 genotype, whereas these measures were not well correlated among subjects with the 4/4 genotype

Tab. 3. Mean Waking and Diurnal Cortisol (95% Confidence Intervals) by Shift Type and *PER3* VNTR Genotype, BCOPS Study, Buffalo, NY, USA, 2001–2003.

Risk Factor	4/4	4/5 + 5/5	*p*-value 4/4 vs. 4/5 + 5/5
	Waking AUC_I		
Shift Type			
Day	126 (-14–267) n=10	61 (-43–166) n=18	0.46
Night + Afternoon	-8 (-164–148) n=9	202 (93–311) n=17	0.03
p-value Day vs. Night + Afternoon	0.20	0.07	
	Waking AUC_G		
Shift Type			
Day	512 (261–762) n=10	736 (544–928) n=18	0.16
Night + Afternoon	361 (52–670) n=9	791 (573–1009) n=17	0.01
p-value Day vs. Night + Afternoon	0.44	0.69	
	Diurnal AUC_G		
Shift Type			
Day	4965 (3177–6753) n=9	7120 (5900–8340) n=17	0.04
Night + Afternoon	5003 (3222–6783) n=9	7347 (6078–8615) n=16	0.03
p-value Day vs. Night + Afternoon	0.98	0.79	
	Diurnal Slope		
Shift Type			
Day	-0.0033 (-0.0044–-0.0021) n=9	-0.0024 (-0.0032–-0.0015) n=17	0.22
Night + Afternoon	-0.0021 (-0.0034–-0.0007) n=9	-0.0032 (-0.0041–-0.0023) n=16	0.12
p-value Day vs. Night + Afternoon	0.21	0.19	

Units for AUC_I and AUC_G are nmol/L-minutes. Abbreviations: AUC_G – Area Under the Curve (Ground); AUC_I – Area Under the Curve (Increase). Adjustments: Waking AUC_G adjusted for rank; Waking AUC_I adjusted for gender; Diurnal AUC_G adjusted for education and age; Diurnal Slope adjusted for gender and age group.

(Archer *et al.* 2008) . Although no cause-effect relationship was established in that study, the results suggest that the 5/5 variant may facilitate coupling of cortisol secretion to the circadian system through *PER3* expression. In addition to its influence on the timing of cortisol secretion (Archer *et al.* 2008), the *PER3* VNTR has been associated with adverse health outcomes that might overlap with cortisol dysregulation, including delayed sleep phase syndrome (Ebisawa *et al.* 2001), bipolar disorder (Dallaspezia *et al.* 2011), and increased cancer risk (Zhu *et al.* 2009; Dai *et al.* 2010). However, the functional consequences of this relationship remain to be determined.

Salivary cortisol values among officers participating in the present study were generally representative of those observed in other populations, with higher levels in the morning and declining values throughout the day (Kudielka *et al.* 2007; Griefahn & Robens 2008). Consistent with the hypothesis that the 5/5 genotype may be linked with circadian cortisol secretion, officers with a 4/5 or 5/5 genotype had an adjusted mean waking AUC_I that was 106% greater, a waking mean AUC_G that was 73% greater, and mean cortisol output across the day that was 44% greater than those with a 4/4 genotype (Table 2). The reason that no difference in the mean diurnal cortisol slope was observed among those with different PER3 genotypes is uncertain, but suggests that the *PER*3 VNTR had a stronger influence on morning cortisol secretion than secretion occurring throughout the day. The primary mechanism for

Tab. 4. Mean Waking and Diurnal Cortisol (95% Confidence Intervals) by Shift changes and *PER3* VNTR Genotype, BCOPS Study, Buffalo, NY, USA, 2001–2003.

Risk Factor	4/4	4/5 + 5/5	*p*-value 4/4 vs. 4/5 + 5/5
	Waking AUC_I		
Cumulative Shift Changes			
<17	60 (-79–199) n=12	123 (2–244) n=15	0.50
≥17	68 (-110–247) n=7	134 (28–241) n=20	0.52
p-value <17 vs. ≥17	0.94	0.89	
	Waking AUC_G		
Cumulative Shift Changes			
<17	430 (192–667) n=12	877 (651–1104) n=15	<0.01
≥17	494 (189–800) n=7	708 (527–888) n=20	0.22
p-value <17 vs. ≥17	0.73	0.22	
	Diurnal AUC_G		
Cumulative Shift Changes			
<17	5,040 (3490–6590) n=11	7,290 (5975–8604) n=14	0.03
≥17	4,911 (3078–6745) n=7	7,181 (6027–8335) n=19	0.03
p-value <17 vs. ≥17	0.91	0.90	
	Diurnal Slope		
Cumulative Shift Changes			
<17	-0.0022 (-0.0033–-0.0011) n=11	-0.0031 (-0.0041–-0.0021) n=14	0.24
≥17	-0.0033 (-0.0046– -0.0019) n=7	-0.0026 (-0.0034–-0.0017) n=19	0.35
p-value <17 vs. ≥17	0.22	0.38	

Shift change (from 1994 or initiation of employment until clinic examination in 2001), categories were based on a median split. Units for AUC_I and AUC_G are nmol/L-minutes. Abbreviations: AUC_G – Area Under the Curve (Ground); AUC_I – Area Under the Curve (Increase). Adjustments: Waking AUC_G adjusted for rank; Waking AUC_i adjusted for gender; Diurnal AUC_G adjusted for education and age; Diurnal slope adjusted for gender and age group.

cortisol's robust circadian rhythm may be an increase in morning secretion due to enhanced light sensitivity occurring at that time of day (Clow *et al.* 2004). If so, then individuals with a morning circadian preference who typically exhibit earlier wake times would be expected to have an increased cortisol secretion due to an increased probability of elevated light exposure after awakening. This is consistent with studies that observed elevated cortisol levels among morning types compared to evening types (Bailey & Heitkemper 2001; Kudielka *et al.* 2006). Because the 5/5 *PER3* genotype tends to be more frequently associated with morningness (Archer *et al.* 2003; Ellis *et al.* 2009), our findings are consistent with these observations. However, diurnal preference has not always been associated with the *PER3* VNTR (Barclay *et al.* 2011; Osland *et al.* 2011), and chronotype was not characterized in this study. Nonetheless, we found that collection of the first saliva sample occurred about one hour earlier, on average, among those with the 5/5 genotype compared to the 4/4 genotype (08:16±112, 07:45±94, and 07:12±129 minutes for the 4/4, 4/5 and 5/5 genotypes, respectively). Although these differences were not statistically significant, it is possible that they contributed to the salivary cortisol measures that were observed.

The SCN synchronizes the circadian rhythms of ACTH and cortisol secretion, as well as clock gene expression within the pituitary gland and adrenal

cortex (Dickmeis 2009; Girotti *et al.* 2009; Nader *et al.* 2010; Tonsfeldt & Chappell 2012). There are currently several models describing pathways whereby light-entrained SCN activity, in conjunction with neural and endocrine effectors of the HPA axis, regulate diurnal cortisol secretion. Although, the role of *PER3* in these processes remains to be determined, one possibility is that *PER3* may influence sensitivity of the circadian system to ambient light exposure. A genetic predisposition to light-induced suppression of melatonin, another hormone with a robust circadian rhythm, was recently observed among individuals with the 5/5 *PER3* VNTR genotype, whereas 4/4 homozygotes were less responsive (Chellappa *et al.* 2012). Based on these observations, we speculate that light-induced morning cortisol secretion may occur in a similar, *PER3* genotype-dependent manner.

Examination of cortisol and psychometric measures in conjunction with the *PER3* VNTR among police officers in this study provided an opportunity to evaluate the influence of genotype and shiftwork on these parameters in a real-world setting. A strength of the BCOPS cohort is that long term shiftwork histories were quantified among participants via reconstruction of payroll records. We previously reported that officers working short-term night or afternoon shifts (3–14 days prior to saliva collection) had reduced waking cortisol AUC_I or AUC_G compared to day workers, consistent with several other studies among shiftworkers (Zuzewicz *et al.* 2000; Kudielka *et al.* 2007; Griefahn & Robens 2008). In addition, officers with more cumulative shift changes over periods of years also had reduced waking AUC_I values (Wirth *et al.* 2011). Thus, we hypothesized that shiftwork may modify the relationship between the *PER3* VNTR and cortisol secretion. When stratified by shift status, those with a 4/5 or 5/5 genotype who were working night or afternoon shifts had the highest waking AUC_I values, more than double what was observed among day workers with the 4/5 or 5/5 genotype, which suggests a possible gene-environment interaction. Caution in the interpretation of these findings is warranted given the relatively limited sample size among strata of shiftwork and genotype. Also, results obtained for waking or diurnal AUC_G indicated that, regardless of shift status, those with a 4/5 or 5/5 genotype had cortisol secretion patterns that were elevated compared to those with the 4/4 genotype. Overall, the results support the possibility that an extra *PER3* VNTR copy enhances cortisol secretion. If this effect is modified by shiftwork, it most likely influences the absolute increase in cortisol after awakening (AUC_I) rather than the total amount secreted after awakening (AUC_G), or cortisol secretion throughout the day.

In the present study, the *PER3* VNTR was not associated with stress-related psychological symptoms including depression or life events, in contrast with previous studies (Guess *et al.* 2009; Dallaspezia *et al.* 2011). Only about 6% of officers were depressed based on the CES-D definition (score ≥16), thus there may not have been enough variation in CES-D scores to determine differences between the *PER3* VNTR genotypes. Stress or depressive symptoms were also a potential source of bias related to cortisol in this study since these symptoms can be associated with both cortisol secretion and the *PER*3 genotype (Chida & Steptoe 2009). However, there was no change in the interpretation of the results when the cortisol analyses were adjusted for the effects of the Life Events Scale, CES-D, or IES. Although we adjusted for these and other important confounding factors, information on other covariates was unavailable, for example ambient light exposures (Sephton & Spiegel 2003; Clow *et al.* 2004). Similarly, poor sleep or diets high in fat and low in fruit and vegetable intake may be associated with disrupted or flattened diurnal cortisol slopes (Kumari *et al.* 2011; Heaney *et al.* 2012). Thus, the possibility of residual confounding by these or other factors cannot be entirely eliminated.

In conclusion, the cross-sectional nature of this study precludes the ability to infer causation, although the results suggest that the 5-repeat sequence of the *PER3* VNTR may facilitate increased cortisol secretion, particularly in the morning. Individuals with this genotype may be more susceptible to factors that can cause circadian rhythm disruption, such as shiftwork, poorly timed light exposures, or changes in sleep-wake timing. However, only modest evidence for a *PER3*-related influence of shiftwork on cortisol secretion was obtained in the present study. Cortisol dysregulation may have long-term health implications. Reduced cortisol secretion or a flattened slope has been associated with poor sleep quality (Backhaus *et al.* 2004), chronic fatigue syndrome and symptoms of burnout (Roberts *et al.* 2004), PTSD (Rohleder *et al.* 2004), depression (Stetler & Miller 2005), adverse cardiovascular health and mortality (Hurwitz Eller *et al.* 2001; Kumari *et al.* 2011), increased all-cause mortality (Kumari *et al.* 2011), and decreased breast or lung cancer survival (Sephton *et al.* 2000; Sephton *et al.* 2012). Although linkages between circadian clock gene expression and the HPA axis have been identified, the pathological implications of dysregulation of these processes await further characterization (Nader *et al.* 2010; Mavroudis *et al.* 2012).

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Declaration of Interest

The authors report no conflict of interest.

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