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Altered Levels of Basal Cortisol in Healthy Subjects with a 118G Allele in Exon 1 of the Mu Opioid Receptor Gene

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The mu opioid receptor is centrally involved in the development of the addictive diseases. It also modulates the stress responsive hypothalamic–pituitary–adrenal axis. Receptors encoded by the variant 118G polymorphism in exon 1 of the mu opioid receptor gene have a threefold increase in beta-endorphin binding and beta-endorphin is three times more potent in receptor-mediated activation of G protein-coupled inwardly rectifying potassium channels. Humans with this variant have increased stress response following opioid antagonism. Here, we study basal levels of adrenocorticotropic hormone and cortisol in subjects with this variant. In all, 59 healthy adults were genotyped and had morning levels of adrenocorticotropic hormone and cortisol measured following intravenous administration of saline placebo. Subjects with a 118G allele had significantly greater levels of cortisol than subjects with the prototype gene. Groups did not differ in levels of adrenocorticotropic hormone. A planned comparison revealed significantly greater cortisol in females with at least one copy of the 118G allele compared to females with the prototype gene. There was no significant effect of gender alone, nor was there a significant interaction between gender and genotype, on ACTH or cortisol. Subjects with at least one copy of the 118G allele have increased basal levels of cortisol, which may influence the susceptibility to and treatment of the stress responsive dyscrasia.
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INTRODUCTION

The addictive diseases and several other psychiatric disorders are characterized by alterations in the stress responsive hypothalamic–pituitary–adrenal (HPA) axis (Ehlert *et al*, 2001; Kreek and Koob, 1998). In the case of the addictive diseases, these alterations are primarily due to the pharmacological effects of drugs of abuse but are also influenced by developmental, environmental, and genetic factors. As an example of the latter, nonalcoholic sons of alcoholic fathers have altered HPA responsiveness to alcohol and mu opioid receptor antagonism compared to sons of nonalcoholic fathers (King *et al*, 1998; Schuckit *et al*, 1987; Wand *et al*, 1998). In fact, up to 50% of inter-individual differences in basal and stress-induced cortisol levels in healthy human volunteers may be explained by genetic factors (Federenko *et al*, 2004; Linkowski *et al*, 1993). While this is likely due to the interaction of several genes, so too are the genetic determinants of drug addiction. Indeed, several genes have been associated with the vulnerability to develop an addictive disease (for a review, Kreek *et al*, 2005). Of particular interest are the genes of the endogenous opioid system whose expression and/or protein products are either directly or indirectly affected by each of the substances of abuse. Self-administration of opioids, cocaine, alcohol, and nicotine does not develop or is reduced in mu opioid receptor gene (*OPRM1*) knockout mice (Becker *et al*, 2000; Berrendero *et al*, 2002; Hall *et al*, 2001; Mathon *et al*, 2005; Roberts *et al*, 2000). We have shown that a functional single nucleotide polymorphism in the first exon of *OPRM1* accounts for up to 21% of the attributable risk for developing heroin addiction and 11% of the risk for developing alcoholism in cohorts from central Sweden (Bart *et al*, 2004, 2005).

This A118G polymorphism (rs1799971) results in an asparagine to aspartic acid substitution at amino-acid position 40 and leads to increased receptor-binding affinity for the endogenous opioid beta-endorphin and increased potency of ion channel activation following beta-endorphin binding (Bond *et al*, 1998). Two other studies, one in transiently-expressing COS cells (Befort *et al*, 2001), the other in stable HEK293 cell lines (Beyer *et al*, 2004), did not replicate these findings. In our prior study (Bond *et al*, 1998), the variant and prototype receptors were stably-transfected AV-12 cells, which confer *N*-glycosylation. These cells were chosen because, in the mu opioid receptor, amino-acid position 40 is a putative site of glycosylation. Further, a recent report on human receptors harvested from

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post-mortem brain tissue has found that some of the functional effects seen by Bond *et al* may be related to a decrease in mu opioid receptor mRNA expression in subjects with a variant 118G allele (Zhang *et al*, 2005). Other studies have also identified reduced receptor expression in transfected cell lines of receptors encoded by the 118G allele, a finding also noted in our ongoing studies (Beyer *et al*, 2004; Kroslak *et al*, 2003). The mu opioid receptor system is also involved, through tonic inhibition, in modulation of the stress responsive HPA axis and normal volunteers with a 118G allele have an increased HPA response (measured through levels of plasma cortisol) following antagonism of this inhibition with the opioid antagonist medication naloxone (Chong *et al*, 2006; Hernandez-Avila *et al*, 2003; Wand *et al*, 2002).

In this report, we tested the hypothesis that, while a 118G allele influences HPA responsivity to stress and pharmacological probes, it will not affect basal HPA activity measured as plasma levels of ACTH and cortisol.

PATIENTS AND METHODS

Subjects

In all, 59 healthy subjects (28 female) with no history of substance abuse or dependence (including nicotine) were recruited by newspaper advertisement and by word of mouth. All subjects provided written informed consent for participation in ongoing neuroendocrine studies as well as separate written consent for genetics research. The Rockefeller University Institutional Review Board approved the study protocols and consent forms.

Evaluation for medical and psychiatric inclusion and exclusion criteria were made by an internist or psychiatrist using clinical interview, physical exam, and review of laboratory and corroborative data. Subjects gave informed consent for HIV testing. Aliquots of urine were tested daily, both during the screening process and during the inpatient stay, for the presence of opioids, cocaine, cannabinoids, or benzodiazepines. Subjects included in the study were free of significant medical problems, HIV antibody negative, urine toxicology negative, were not pregnant (urine beta-hCG confirmation upon admission), and did not meet DSM-IV criteria for any axis I diagnosis including substance and/or alcohol abuse or dependence. Female subjects were not on hormone replacement therapy or using hormonal contraceptives. Subjects were not taking prescription medications, and were not regularly using over-the-counter medications or herbal preparations.

Procedure

Subjects were admitted to the stress-minimized inpatient unit at least one evening prior to the test date. Subjects participated in one of six related neuroendocrine protocols with similar methodology, however, for this report, placebo-day data were compiled from all six studies to achieve a large sample size. Subjects fasted at least 9 h prior to the beginning of testing (between 0930 and 1030 h) and were allowed to eat only after the first 2 h of testing had elapsed. Normal saline placebo (10 ml) was administered through, and blood was withdrawn from, an indwelling intravenous catheter (BD Angiocath Autoguard, Becton, Dickinson, Franklin Lakes, NJ), inserted at least an hour prior to the beginning of testing.

Plasma adrenocorticotropic hormone (ACTH) and cortisol levels were determined in blood samples drawn at sequential time points. Time points started 10 min prior to, immediately prior to, and then 10, 20, 30, 40, 50, 60, 75, and 90 min following placebo administration. Blood was drawn into sodium EDTA vacutainers, and immediately placed on ice. Samples were stored on ice for up to 40 min, and then centrifuged at 4°C at $3000 \times g$ for 5 min. Plasma was removed, aliquoted and stored at −40°C until assayed. Plasma ACTH and cortisol levels were determined in duplicate by radioimmunoassay procedures, with slight modifications (ACTH: Nichols Institute, San Juan Capistrano, CA; cortisol: Diagnostic Products, Los Angeles, CA). ACTH intra- and inter-assay coefficients of variation were 9.4 and 15.1%, respectively. Cortisol intra- and inter-assay coefficients of variation were 2.5 and 6.0%, respectively.

Genotyping

Genomic DNA was isolated from peripheral blood lymphocytes. Approximately 100 ng of genomic DNA was amplified by polymerase chain reaction (PCR) using primers designed to amplify the entire coding region of exon 1 of *OPRM1*. PCR products were electrophoresed on agarose gel to verify amplification size and were then purified and sequenced at The Rockefeller University. The amplification and sequencing primers have been described previously (Bart *et al*, 2004) and we have compared the accuracy of these primers with a TaqMan-based methodology and found the concordance of genotyping results between the two methods to be greater than 99% (Proudnikov *et al*, 2004). Electropherograms were analyzed by two independent readers who were blind to the neuroendocrine data.

Statistical Analysis

Area under the curve (AUC) from 0 to 90 min after placebo administration was calculated for each hormone (ACTH and cortisol) in each subject. A two-way analysis of variance (ANOVA), Condition by Genotype was used to evaluate the differences between each hormone and any possible effect of genotype. As there were only two subjects homozygous for the 118G allele, they were combined in the analyses with the 118G heterozygotes.

RESULTS

Subject demographics by A118G allele group are shown in Table 1. Gender distribution was similar for both groups. In accordance with previously documented differences in 118G allele frequency among ethnicities, there were more African Americans in the prototype group. However, there were more Hispanics in the 118G allele group than expected. Basal levels of ACTH and cortisol did not differ between genders ($F(1,55) = 1.82$, $p = 0.1832$ and $F(1,55) = 1.18$, $p = 0.2815$, respectively) (Figure 1). Area under the curve for ACTH did not differ significantly between subjects with a 118G allele and those without a 118G allele ($F(1,55) = 3.19$,

$p = 0.0794$) (Figure 2a). However, subjects with a 118G allele had significantly greater cortisol AUC than subjects without a 118G allele ($F(1,55) = 4.27$, $p < 0.05$) (Figure 2b). While there was no significant interaction between gender and genotype for either ACTH or cortisol ($F(1,55) < 1.0$ and $F(1,55) = 2.84$, $p = 0.0974$, respectively), a planned comparison of females with a 118G allele and those with the prototype gene showed significantly greater cortisol AUC in females with a 118G allele ($p < 0.02$) (Figure 2c).

DISCUSSION

In this report, we found a significant increase in basal levels of plasma cortisol in healthy subjects with a 118G allele in exon 1 of *OPRM1*. Previous reports have shown that subjects with a 118G allele have increased cortisol response following administration of the opioid antagonist naloxone (Hernandez-Avila *et al*, 2003; Wand *et al*, 2002; Chong *et al*, 2006) and, possibly, decreased response to a social stressor (Chong *et al*, 2006). These reports did not note an effect of the 118G allele on basal levels of either ACTH or cortisol. Interestingly, neither our study nor the other reports have noted an effect of the 118G allele on basal or stimulated levels of ACTH. While opioid peptide mRNA has been detected in the adrenal medulla, there is no known adrenal cortical expression of the mu opioid receptor and, therefore, it is unkown whether the effect of the 118G variant allele on cortisol in these studies is centrally or peripherally mediated.

A possible effect of gender must also be considered. Wand *et al* studied only men and Hernandez-Avila *et al* included only four women (three A118A and one 118G). While Chong *et al* studied a large cohort including at least 30 women (21 A118A and nine A118G), no basal difference in ACTH or cortisol was noted, although their baseline was determined using only two time points separated by fifteen minutes. Given positron-emission tomography (PET) imaging data indicating that mu opioid receptor-binding potential is higher in women (Zubieta *et al*, 1999), replication of our findings will be needed with a larger sample size and frequent sample measurements in order to determine if a gene–gender interaction contributes to the increase in basal levels of cortisol noted in 118G subjects. Also, given the difference in ethnic distribution between groups, the possibility of other, more ethnicity-specific polymorphisms that influence HPA axis function cannot be ruled out. The direct influence of ethnicity on basal and stress-induced cortisol levels has been studied with conflicting results, which indicates that ethnicity alone may not influence cortisol; however, disparities in socioeconomic factors, which are often along ethnic lines may explain these conflicting results (eg Bennett *et al*, 2004; Masi *et al*, 2004). This study was not large enough to control for demographic factors such as socioeconomic status and education.

In summary, this preliminary report shows that basal levels of serum cortisol are significantly greater in subjects with a 118G allele than in subjects with the prototypical A118A genotype. Further work is needed to address whether the differences in basal and stress-induced cortisol response in subjects with a 118G allele are due to altered receptor function and/or expression, and if these differences contribute to the vulnerability to develop specific addictions and other psychiatric disorders characterized by altered HPA axis responsivity. Whether the effectiveness of treatment agents for these disorders (such as increased treatment response to naltrexone in alcoholics with a 118G allele described by Oslin *et al*, 2003) may be influenced by the effects of a 118G allele on HPA axis responsiveness should also be investigated.

Table 1 Demographic Characteristics

Characteristic	A118A ($n = 40$)	A118G ($n = 19$) 1 or 2 copies
Male (*n*)	20	11
Race/ethnicity (*n*)		
White, non-Hispanic	20	9
African American	17	3
White, Hispanic	2	7
Other	1	0
Mean age in years (SD)	34.1 (10.7)	29.1 (4. 5)
Mean body mass index (SD)	25.3 (4.5)	27.1 (7.0)

Values represent absolute numbers or means. Group differences in ethnic distribution were evaluated with a χ^2 analysis. There was a significant difference ($\chi^2 = 10.9$; df = 2; $p < 0.01$) in the proportion of ethnicities between the prototype group and the group with a 118G allele.

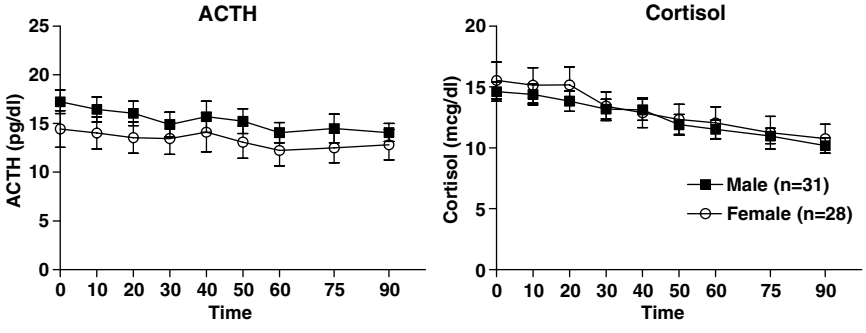

Figure 1 Plasma levels of adrenocorticotropic hormone and cortisol by gender. Plasma levels from just before to 90 min after injection of placebo are shown for adrenocorticotropic hormone (ACTH, on the left) and cortisol (right). Means and SE are displayed for men and women. There was no significant difference between genders for each hormone.

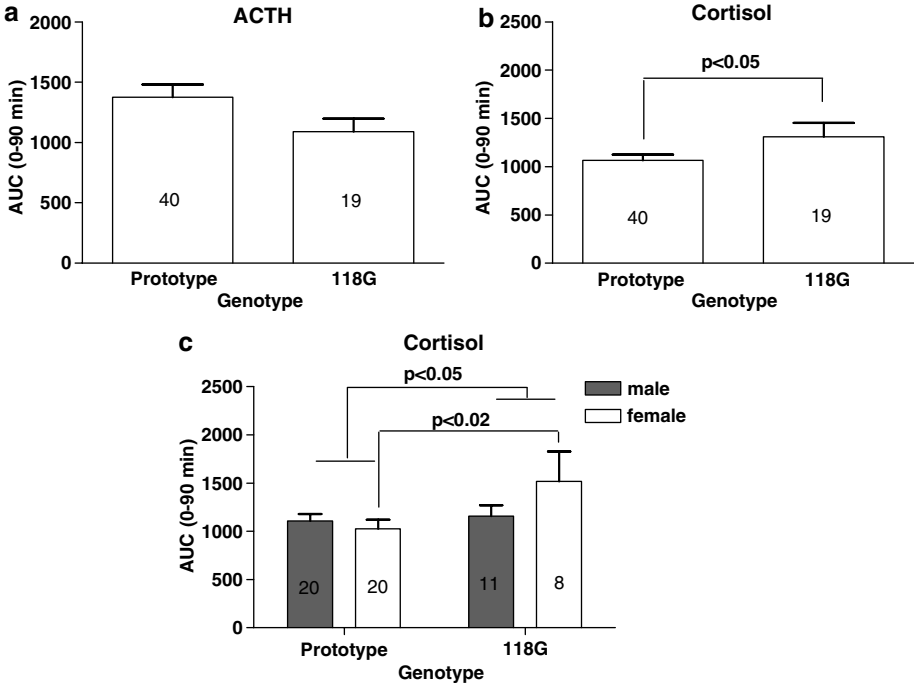

Figure 2 Area under the curve of adrenocorticotropic hormone and cortisol by genotype (prototype *vs* subjects with a 118G allele). (a) The 90-min area under the curve (AUC) for adrenocorticotropic hormone (ACTH) and (b) cortisol are shown for the genetic prototype group and the group with a 118G allele. Analysis of variance showed a significant effect of the 118G allele on cortisol ($p < 0.05$) but not ACTH. (c) AUC for cortisol by genotype and gender is shown, the significant group effect of the 118G allele on cortisol appears to be due to the women with at least one copy of the 118G allele who have significantly greater cortisol levels than women with the prototype gene ($p < 0.02$).

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