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Effects of the G(–656)A Variant on *CREB1* Promoter Activity in a Neuronal Cell Line: Interactions with Gonadal Steroids and Stress

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Abstract

Major depressive disorder (MDD) constitutes a major public health problem worldwide and affects women twice as frequently as men. Previous genetic studies have revealed significant evidence of linkage of the *CREB1* region (2q33–35) to Mood Disorders among women from families with recurrent, early-onset MDD (RE-MDD), a severe and familial subtype of MDD. A rare G to A transition at position –656 in the *CREB1* promoter cosegregates with Mood Disorders in women from these families, implicating *CREB1* as a sex-related susceptibility gene for unipolar Mood Disorders. In the current study, the functional significance of the *CREB1* promoter variant was determined using transfection experiments that employed plasmid constructs containing the wild-type or variant *CREB1* promoters coupled to a reporter gene. The results support the hypothesis that the A_{–656} allele contributes to the development of MDD in women through selective alteration of *CREB1* promoter activity by female gonadal steroids in noradrenergic neuronal cells. Furthermore, the exaggeration of these effects during a simulated stress condition may be relevant to reported gene-environment interactions that contribute to the emergence of MDD in clinical populations.

Keywords

CREB1; Genetics; Gonadal Steroids; Noradrenergic Neurons; Sex; Depression; Mood

INTRODUCTION

Major Depressive Disorder (MDD) constitutes a major public health problem worldwide and affects women twice as frequently as men.¹ Families identified by individuals with Recurrent, Early-Onset MDD (RE-MDD), a severe and strongly familial form of MDD, have provided an important resource in efforts to identify and characterize genes that contribute to the risk of developing MDD and related conditions.^{1,2}

Model-free linkage analysis of a region of chromosome 2q33–35, highlighted by previous case-control studies^{3,4} and supported by within-family analyses employing the transmission disequilibrium test,⁵ has revealed evidence of sex-specific linkage to unipolar Mood Disorders extending over 15 cM in our 81 RE-MDD families.^{6,7} Peak multipoint LOD scores of 6.33 and 6.87 occurred at D2S2321 and D2S2208, respectively. This finding resulted from linkage of the 2q33–35 region to unipolar Mood Disorders among the women in these 81 RE-MDD families; no evidence of linkage of the 2q33–35 region to Mood Disorders was detected among

the male family members (peak LOD score 0.00). The 451Kb region between the adjacent STRPs D2S2321 and D2S2208 includes an attractive candidate gene, *CREB1*(2q33.2-34), which encodes the cAMP response element binding protein.⁸⁻¹⁰

The *CREB1* gene encodes a 43 kDa cAMP-responsive element-binding protein (CREB) consisting of 341 amino acids that is a member of the basic leucine zipper family of transcription factors.¹¹ In addition to the full-length, 341 aa CREB protein (isoform B), additional isoforms are synthesized by alternative mRNA splicing that are tissue-specific and differentially-expressed during development. In response to elevated cAMP levels produced by the activation of g-protein coupled receptors, catalytic subunits of protein kinase A diffuse into the nucleus and induce cellular gene expression by phosphorylating CREB at Ser133. Phosphorylated CREB molecules induce the transcription of genes whose promoters include a cAMP-responsive element (CRE), a conserved palindromic 8-nucleotide sequence (TGACGTCA) or single CGTCA motif. In addition to the cAMP signaling system, several growth factor and stress signals stimulate CREB-mediated transcription by promoting the phosphorylation of CREB at Ser133 by various cellular kinases.¹¹ *CREB1* is ubiquitously expressed in human tissues and its target genes encode biosynthetic enzymes and receptors for neurotransmitters, neuropeptides, neuronal and non-neuronal growth factors; transcription factors and proteins that regulate the cell cycle/cell survival/DNA repair, reproduction/development, and intercellular signaling and transport; and a range of metabolic/catabolic enzymes and structural proteins. Promoter sequences in addition to CRE and the participation of co-activation factors impose specificity on the particular programs of gene expression that are initiated by CREB.

CREB1 is an excellent candidate for a susceptibility gene, alleles of which might alter *CREB1* gene expression or encode CREB protein variants that influence the risk of developing unipolar Mood Disorders. Alterations in *CREB1* gene expression and CREB phosphorylation have been reported in clinicopathologic studies of temporal cortex from patients with MDD, in the hippocampus and nucleus accumbens of animal models of MDD and related disorders, and in the brains of rodents exposed to chronic treatment with antidepressant drugs.⁸⁻¹⁰ CREB has also been implicated in neuronal plasticity, cognition, and long term memory,¹² abnormalities of which commonly occur in patients with MDD, may predispose patients to the onset or recurrence of MDD, and may be related to the eventual development of irreversible dementia in some patients.^{1,13} Finally, reports of synergistic interactions of CREB with nuclear estrogen receptors¹⁴⁻¹⁶ may provide a mechanism by which CREB facilitates sex-specific patterns of gene expression that manifest themselves in the sex-specific effects of risk alleles for unipolar Mood Disorders.

Sequence variants in the *CREB1* promoter have been detected that cosegregate with depressive disorders in women from RE-MDD families, providing support for *CREB1* as a sex-limited susceptibility gene for unipolar Mood Disorders and related conditions in these families.¹⁷ A rare G to A transition at position -656 in the *CREB1* promoter (A₋₆₅₆) appeared to confer unipolar Mood Disorders with high penetrance among women in two (2.5%) of the 81 RE-MDD families. By comparison, no A₋₆₅₆ carriers were identified among 149 unrelated women who had no history of psychiatric disorders (allele frequency < 0.003). Based on these observations, we hypothesized that the A variant at position -656 alters the activity of the *CREB1* promoter, an effect that is dependent upon or enhanced by the presence of female gonadal steroids.

We determined the effects of gonadal steroid hormones (17 β -estradiol, progesterone, testosterone) on the activity of the wild-type (wt) human *CREB1* promoter and assessed the functional significance of the *CREB1* promoter variant using transfection experiments that employed constructs containing the wt or variant *CREB1* promoters coupled to a reporter gene,

chloramphenicol acetyltransferase (CAT). Transfection was performed using noradrenergic CATH.a cells derived from the locus ceruleus because a substantial body of evidence has implicated alterations of noradrenergic neurons in the pathogenesis of mood disorders, as well as in the mechanism of action of antidepressant drugs.^{18,19} Expression was assessed in CATH.a cells grown in the absence and presence of physiologically-relevant concentrations (100nM) of gonadal steroid hormones, at baseline and during activation of the cyclic-adenosine monophosphate (cAMP) signaling pathway. The latter condition simulated stress-induced activation of G protein-coupled neurotransmitter/growth factor receptors on brain cells grown in the presence of different gonadal hormones. Since stressful life events have been reported to contribute to the emergence of major depressive episodes among individuals who carry risk alleles for MDD,²⁰ we hypothesized that the activation of the cAMP signaling pathway augments the effect of the A₋₆₅₆ sequence variant on *CREB1* promoter function.

MATERIALS AND METHODS

Source and Growth Conditions for CATH.a Cells

The CATH.a cell line was developed from a brain stem tumor of a transgenic mouse expressing the SV40 T antigen under the control of the tyrosine hydroxylase promoter, exhibits a neural, noradrenergic phenotype, and resembles locus ceruleus neurons in their signal transduction profile.^{21,22} This cell line was obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10 mM HEPES, 4.5 g/L glucose, 1 mM sodium pyruvate, 2 g/L sodium bicarbonate, 8% horse serum, and 4% fetal bovine serum, at 37°C, 5% CO₂, 100% humidity. Cells were subcultured at a ratio of 1:2 to 1:4.

Construction of Promoter - CAT Expression Plasmids

The 5' regulatory region of the *CREB1* gene has been intensively studied, and it exhibits high nucleotide sequence homology across mouse, rat, and man.²²⁻²⁸ The human *CREB1* promoter includes most of the untranslated exon 1 (bps 1 to 130) and extends 1080 bps from the major *transcriptional* start site in the 5' direction. This -1080 to 130 bp sequence is identical to the -1264 to -51 bp promoter region described by Meyer and coworkers that was originally numbered relative to the invariant *translational* start site of the cloned cDNA sequence.²³ This 5' regulatory region includes 1210 bps with restriction sites for *Sau* 3AI at both termini.

The wild-type *CREB1* promoter and variant promoter containing the G to A transition located at position -656 were cloned using genomic DNA prepared from an individual who was heterozygous for these alleles. To achieve this, a 1580 bp region containing the 1210 bp *Sau* 3AI fragment was amplified using the primers 5'-CCAGAATCGAACCTCTCTGCTTCC-3' and 5'-CCTCCTCTGCTCCTC TTACCG-3', and GeneAmp[®] High Fidelity Enzyme Mix (Applied Biosystems, Foster City, CA). The *Sau* 3AI fragment containing the *CREB1* promoter was excised from the PCR product, purified by phenol extraction and ethanol precipitation, and ligated into the *Bgl* II site of the pCAT[®]3-Basic Vector (Promega, Madison, WI). The cloning product was transformed into One Shot[®] TOP10 Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA) and plated on selective plates containing ampicillin. Colonies were selected and inoculated into LB medium containing ampicillin, and grown overnight. Plasmid DNA was isolated using the Wizard[®] Plus Minipreps DNA Purification System (Promega Corporation, Madison, WI). Plasmid insert orientation was determined by digestion with restriction endonuclease *Fsp* I, followed by agarose gel electrophoresis and staining with ethidium bromide. Distinguishing between wild-type or variant promoter inserts was accomplished by PCR and RFLP analysis that detected the presence/absence of an *Msp* I restriction site that was eliminated by the G to A transition in the variant promoter.¹⁷ Large scale preparation of the plasmids from cultures was performed using Wizard[®] Plus Maxipreps

DNA Purification System (Promega Corporation, Madison, WI). The base sequences of the cloned *CREB* promoters in the two final plasmid preparations to be used in the transfection experiments were confirmed in their entirety by automated DNA sequencing, to ensure that they differed from one another only by the SNP at position -656 and were devoid of PCR or cloning artifacts.

The female research subject with RE-MDD whose genomic DNA was used to clone the wt and variant *CREB1* promoters had experienced two major depressive episodes beginning at age 23. She provided written informed consent to participate in a research project on the molecular genetics of affective disorders that was approved by the Institutional Review Board of the University of Pittsburgh.

Transfection of CATH.a Cells

Approximately 18 hrs prior to transfection, CATH.a cells were seeded in 60 mm cell culture dishes (Corning Inc., Corning, NY) at a density of 0.8 to 1.0×10^6 cells/ dish using medium that lacked or contained physiologically-relevant concentrations (100nM) of a gonadal steroid hormone (17 β -estradiol, E; progesterone, P; or testosterone, T; Sigma, St Louis, MO). This concentration of gonadal steroids is in the midrange of those used in cell culture experiments reported in the literature. In addition, 100nM is in the midrange of circulating concentrations of progesterone achieved during the estrus cycle of the female rat, and is similar to the circulating testosterone levels reported for the male rat. While circulating levels of 17 β -estradiol in the female rat are lower, the synthesis of this hormone in brain is likely to produce substantially higher local concentrations of this gonadal steroid in brain regions. The 100nM concentration of 17 β -estradiol is sufficient to induce both slow, long-lasting genomic effects, as well as more rapid, transient actions through non-genomic mechanisms.²⁹

Cells were transfected with the *CREB1* promoter-CAT reporter constructs using methods employing Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) that were optimized for CATH.a cells. The transfection cocktail was formed by diluting 15 μ l of Lipofectamine™ 2000 reagent in 500 μ l of serum free culture medium, and then adding 3 μ g of plasmid DNA followed by gentle mixing. The 3 μ g of plasmid DNA included in each transfection cocktail consisted of an equimolar mixture of a *CREB1* promoter-CAT reporter construct and the pSV- β -Galactosidase Control Vector (Promega, Madison, WI), or 3 μ g of the native pCAT®3-Basic Vector that served as a sham control. The DNA/Lipofectamine™ reagent complex developed at room temperature for 20 min. The cocktail was then added dropwise to the cell culture dish, which was rocked gently to distribute the complex. Cells were grown overnight for approximately 20 hrs before further manipulation.

Activation of the cAMP Signaling Pathway

At 20 hrs (\pm 1 hr) post transfection, cAMP pathway activation was achieved within the weakly-adherent CATH.a cells by removing sufficient medium to leave 4.5 ml in the dish, and adding 0.5 ml of medium containing a 10x concentration of cAMP activators, such that the resulting culture medium contained a final concentration 10 μ M forskolin and 0.25 mM IBMX (Sigma, St. Louis, MO). Cells were then incubated from 0 (no replacement) to 48 hrs prior to harvest and assay. Maximal *CREB1* promoter activity occurred after 48 hrs of activation.

Chloramphenicol Acetyltransferase (CAT) Assay

CAT assays were performed using the CAT Enzyme Assay System With Reporter Lysis Buffer (Promega, Madison, WI). Aliquots of cleared cell lysate were assayed in 125 μ l reactions containing 40 μ M chloramphenicol with 0.20 μ Ci of ³H-chloramphenicol (PerkinElmer Life Sciences, Boston, MA) added as tracer and 25 μ g n-butyryl CoA, in 20 mM Tris, pH 8 (Sigma, St. Louis, MO). Following incubation at 37°C for 2 hours, the reactions were quenched by the

addition of 300 μ l of mixed xylenes (Sigma, St. Louis, MO), vortexed, and the phases clarified by centrifugation at maximum speed for 3 min at room temperature. The upper organic phase containing the reaction product (n-butyryl chloramphenicol) was back-extracted twice with 0.25 M Tris, pH 8 (Sigma, St. Louis, MO). A 100 μ l volume of xylene phase was combined in a 20 ml glass scintillation vial with 10 ml of Opti-Fluor[®] liquid scintillation cocktail (PerkinElmer, Boston, MA), and counted in a Beckman Instruments (Fullerton, CA) LS 1801 liquid scintillation counter. Enzyme specific activity was expressed as nmol of n-butyryl chloramphenicol produced/hr/mg lysate protein.

β -galactosidase Assay

β -galactosidase assays were performed using the β -Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega, Madison, WI). A 150 μ l volume of cell lysate was mixed with 150 μ l of Assay 2X Buffer, and incubated at 37°C for 3.5 hrs. The reaction was stopped by the addition of 500 μ l of 1M sodium carbonate. The hydrolysis of the chromogenic substrate ONPG (*o*-nitrophenyl- β -D-galactopyranoside) was determined by measuring the absorbance of the reaction product *o*-nitrophenol at 420 nm using a Beckman DU-640 spectrophotometer (Fullerton, CA). Enzyme specific activity was expressed as pmole of *o*-nitrophenol produced/min/ μ g of lysate protein.

Protein Assay

Protein concentrations of cell lysates were determined using the BCA[™] Protein Assay (Pierce, Rockford, IL), which is insensitive to the detergent included in the Lysis Buffer. Twenty-five μ l volumes of clarified cell lysate were combined with 75 μ l of 1 \times Reporter Lysis Buffer and 2 ml of prepared assay reagent. Samples were incubated at 37°C for 33 minutes, and then cooled to room temperature for 5 min prior to measuring absorbance at 562 nm. Protein concentrations were determined by comparison to bovine serum albumin, fraction V as a standard.

Statistical Analysis

Statistical analysis was performed using SPSS Version 10 (SPSS, Chicago, IL). Experimental results are presented as means \pm SD. The activity of the *CREB1* promoter in transfected cells was measured by the ratio of CAT/ β -galactosidase specific activity ($\times 1000$). The effects of gonadal steroid hormones and promoter genotype on *CREB1* promoter activity, during basal conditions or following activation of the cAMP pathway for 48 hrs, were determined using a two-way analysis of variance (ANOVA) with post hoc comparisons. When significant effects of hormone environment were detected by the two-way ANOVA, pairwise comparisons of mean *CREB1* promoter activity during different hormonal conditions were made using the Tukey HSD test. When significant effects of *CREB1* promoter genotype were detected by the two-way ANOVA, the mean activities of the wild type and variant promoters were compared within each hormone condition using a two-tailed t test. The relationship of basal *CREB1* promoter activity to maximal activation during stimulation of the cAMP pathway was explored using linear regression, along with parametric (Pearson *r*) and nonparametric (Spearman ρ) correlation coefficients.

RESULTS

Effects of gonadal steroid hormones on the basal activity of the wild-type and variant *CREB1* promoters in CATH.a cells

Cultures of CATH.a cells were grown in medium that lacked or contained physiological (100 nM) concentrations of 17 β -estradiol, progesterone, or testosterone. When the cultures reached a density of approximately 50% confluence, the cells were transfected with an equimolar

mixture of a CAT reporter construct containing (a) either the wild-type *CREB1* promoter or the G(-656)A variant *CREB1* promoter, and (b) a pSV- β -galactosidase control vector that constitutively expresses β -galactosidase and was included to adjust for potential differences in transfection efficiency across experiments. Sham transfections employing the native pCAT@3-basic vector were performed to control for any background level of reporter or β -galactosidase activity.

At 20 hrs (\pm 1 hr) post-transfection, cells were harvested, washed in PBS, lysed, and assayed for CAT, β -galactosidase activity, and protein concentration. Similar β -galactosidase specific activities were observed across experiments, reflecting the reproducible transfection efficiency of CATH.a cells under the conditions employed. Exposure of transfected cells to 100 nM concentrations of 17 β -estradiol, progesterone, or testosterone had negligible effects on β -galactosidase specific activity, confirming the appropriateness of the pSV- β -galactosidase control vector for use under these experimental conditions. Negligible CAT specific activity was found in cells that lacked the *CREB1* promoter-CAT reporter construct. *CREB1* promoter activity was expressed as the ratio of CAT/ β -galactosidase specific activity (\times 1000). Each experiment was performed six times and the results were calculated as mean \pm standard deviation (SD).

As shown in Figure 1, the hormonal environment had a significant effect on the basal activity of the wild-type and variant *CREB1* promoters (two-way ANOVA hormone effect; $F = 107.11$, $df = 3,40$, $p < 0.000001$). Progesterone was associated with a significant reduction in promoter activity ($p < 0.05$, post hoc Tukey HSD), while testosterone increased promoter activity compared to the no hormone condition ($p < 0.05$ for both comparisons, post hoc Tukey HSD). The G to A transition at position -656 also had a significant effect on basal *CREB1* promoter activity compared to the wild type promoter (two-way ANOVA genotype effect; $F = 11.21$, $df = 1,40$, $p < 0.002$). A significant hormone-genotype interaction ($F = 3.04$, $df = 3,40$, $p = 0.04$) reflected the observation that the effect of the G(-656)A polymorphism on *CREB1* promoter activity was hormone-dependent. Significant differences between the activity of the wild-type and variant *CREB1* promoters occurred in absence of gonadal steroids ($t = 3.14$, $df = 10$, $p = 0.01$), and in the presence of the female gonadal steroids 17 β -estradiol ($t = 2.76$, $df = 6.37$, $p = 0.03$) and progesterone ($t = 3.14$, $df = 10$, $p = 0.01$). In both the no hormone and 17 β -estradiol conditions, the activity of the variant promoter exceeded that of the wild-type promoter. In the presence of progesterone, the variant promoter exhibited a small reduction in activity compared to the wild-type promoter. The G(-656)A polymorphism had no effect on the activity of the *CREB1* promoter when CATH.a cells were grown in the presence of testosterone.

Effects of the cAMP signaling pathway on wild-type and variant *CREB1* promoter activity in CATH.a cells grown in the absence/presence of gonadal steroid hormones

CATH.a cells were grown in the absence or presence of gonadal steroids and transfected with equimolar amounts of either of the *CREB1* promoter-CAT reporter constructs and pSV- β -galactosidase control vector, as described in the previous section. At 20 hrs (\pm 1 hr) post transfection, cAMP pathway activation was achieved by removing sufficient medium to leave 4.5 ml in the dish, and adding 0.5 ml of medium (\pm steroids) containing a 10x concentration of cAMP activators, such that the resulting culture medium contained a final concentration 10 μ M forskolin and 0.25 mM IBMX. Forskolin increases intracellular cAMP levels by direct stimulation of adenylate cyclase, while IBMX inhibits the breakdown of cAMP by inhibition of phosphodiesterase. This condition simulates the activation of G protein-coupled neurotransmitter/growth factor receptors on brain cells grown in the presence of different gonadal hormones. Cells were harvested at intervals after activation, lysed, and assayed for CAT, β -galactosidase, and protein concentration. Each experiment was performed six times and the results expressed as mean \pm SD.

Maximal *CREB1* promoter activity occurred 48 hours after activation of the cAMP signaling pathway and the results at this time point are presented in Figure 2. As observed for the basal condition, both hormonal environment and promoter genotype had significant effects on maximal *CREB1* promoter activity (two-way ANOVA hormone effect; $F = 295.03$; $df = 3,40$; $p < 0.000001$ and genotype effect, $F = 35.40$; $df = 1,40$; $p = 0.000001$, respectively). A significant hormone-genotype interaction ($F = 7.69$, $df = 3,40$; $p = 0.0004$) reflected the observation that the effect of the G(-656)A polymorphism on *CREB1* promoter activity was hormone-dependent. Significant differences between the activity of the wild-type and variant *CREB1* promoters occurred in absence of gonadal steroids ($t = 12.24$, $df = 10$, $p < 0.001$), and in the presence of the female gonadal steroids 17 β -estradiol (3.43, $df = 10$, $p = 0.006$) and progesterone ($t = 3.74$, $df = 10$, $p = 0.004$). In both the no hormone and 17 β -estradiol conditions, the activity of the variant promoter exceeded that of the wild-type promoter. In the presence of progesterone, the variant promoter exhibited a small reduction in activity compared to the wild-type promoter. The G(-656)A polymorphism had no effect on the activity of the *CREB1* promoter when CATH.a cells were grown in the presence of testosterone.

As shown in Figure 2, stimulating the cAMP signaling pathway significantly increased the activity of the *CREB1* promoter in CATH.a cells in ways that were dependent on hormonal environment and promoter genotype. However, the *relative* effects of hormonal environment and genotype resembled those observed for the unstimulated, basal conditions described in Figure 1. The relationship between the basal and maximally-stimulated activity of the *CREB1* promoter in transfected CATH.a cells was evaluated using linear regression, which revealed a strong positive correlation between these variables (Figure 3; Pearson $r = 0.925$, $p = 0.001$; Spearman $\rho = 0.952$, $p = 0.0003$). Upon activation of the cAMP signaling pathway, the effects of the A₋₆₅₆ variant on *CREB1* promoter activity were enhanced compared to the unstimulated, basal conditions.

DISCUSSION

These findings revealed that the G to A transition at position -656 had functional consequences for *CREB1* promoter activity in CATH.a neuronal cells that were dependent on exposure to gonadal steroid hormones. The A₋₆₅₆ genotype produced an increase in basal promoter activity when cells were grown in the presence of 17 β -estradiol and a small, but statistically-significant, reduction in promoter activity when cells were grown in the presence of progesterone. Activation of the cAMP signaling pathway by forskolin/IBMX, which simulated stress-induced activation of G protein-coupled neurotransmitter/growth factor receptors, augmented the changes in *CREB1* promoter activity produced by the A₋₆₅₆ genotype in the presence of 17 β -estradiol and progesterone. In contrast, the A₋₆₅₆ genotype did not have a significant effect on the basal or stimulated activity of the *CREB1* promoter when CATH.a cells were grown in the presence of testosterone.

These findings support the hypothesis that the A₋₆₅₆ allele contributes to the development of MDD in women by selectively altering the activity of the *CREB1* promoter in noradrenergic neurons exposed to female gonadal steroids. The exaggeration of this functional consequence of the A₋₆₅₆ allele during a simulated stress condition may also provide a molecular model that is relevant to reported gene-environment interactions that contribute to the emergence of MDD in clinical populations. The mechanism and level of expression of *CREB1*, as well as the splicing of its transcript, are cell-specific characteristics.^{10,11} Therefore, the manifestation of the functional effects of this pathogenic allele in noradrenergic neurons is consistent with a role of this brain cell type in the pathogenesis of MDD and related disorders.^{13,19} Although the relationship of *CREB1* expression levels to behavior in animal models is complex and region-specific, elevated *CREB1* expression in neurons within the nucleus accumbens of rats produces multiple "depression-like" effects in behavioral tests of these rodents.¹⁰

In addition to alterations of *CREB1* promoter activity during exposure of neuronal cells to static physiological concentrations of female gonadal steroids, the results of these transfection experiments (Figure 1 and Figure 2) suggest that natural fluctuations between 17 β -estradiol and progesterone predominance in women may lead to substantial variations in *CREB1* promoter activity in neuronal (and potentially other brain) cells regardless of genotype. This dynamic phenomenon may contribute to the increased lifetime prevalence of MDD in women compared to men, and may be especially relevant to the development of depressive disorders in women at times of fluctuations in gonadal hormones that occur during menarche, menses, pregnancy/childbirth, and menopause. At such times, our findings suggest that the A₋₆₅₆ allele would augment the amplitude of the variations in *CREB1* promoter activity and may thereby enhance the risk of an emergent depressive disorder in female carriers. The experimental results also suggest that environmental stresses that impact the cAMP signaling pathway may further exaggerate swings in *CREB1* promoter activity along with the risk of developing a depressive disorder. This model is also consistent with the reduction in age-specific prevalence of MDD that occurs in late adulthood in both sexes as circulating levels of gonadal steroids wane.

A probable mechanism by which the SNP₋₆₅₆ influences the activity of the *CREB1* promoter is by affecting the biological activity of a transcription factor binding site at this location. Previous *in silico* analysis identified four putative binding sites whose corresponding transcription factors are expressed in human brain.³⁰ Of these four candidates, several lines of evidence suggest that the effects of the SNP₋₆₅₆ may be mediated by CP2 binding. The pathogenic A₋₆₅₆ allele creates a perfect match to the core of the CP2 binding site, reflecting a gain of function that is consistent with the dominant effect (penetrance $\geq 82\%$) of this variant on the development of depressive disorders among women who are heterozygous carriers.¹⁷ Among its target genes, CP2 appears to regulate the expression of glycogen synthase kinase 3 β ,³¹ which has been implicated in the pathophysiology of both mood disorders and AD.^{8, 32, 33} In addition, a non-coding polymorphism in the 3' untranslated region of the CP2 gene has been reported to affect the risk of MDD³⁴ and Alzheimer's disease,³⁵ both of which aggregate in RE-MDD families.¹ Nonetheless, a degree of caution is warranted in the interpretation of the *in silico* results, which are sensitive to the threshold settings employed to minimize false positive and false negative results, and which do not always reflect the biological activity of a putative binding site.

It is noteworthy that the *in silico* analysis did not identify an estrogen receptor binding site, even when relaxed similarity score thresholds were employed. This finding suggests that the influence of 17 β -estradiol on the activity of the wt *CREB1* promoter, and the interaction of this gonadal steroid with the SNP₋₆₅₆ genotype, were mediated through effects upstream of promoter binding. Potential examples include the involvement of estrogen-induced effects on the expression or kinase-activation of transcription factor(s) that bind to the *CREB1* promoter, or by the involvement of a co-transcription factor whose binding to the *CREB1* promoter is dependent on a physical interaction with an estrogen receptor.^{11, 15, 36}

CREB and other transcription factors appear to participate at the top level of the molecular and cellular cascade that controls aspects of neuronal plasticity that regulate mood, cognition, and related phenotypes. The interaction of sex with *CREB1* variants that influence the development of psychiatric syndromes, or their clinical features, seems likely to be complex and allele specific. As an example, Perlis and colleagues have recently described associations of non-coding SNPs in the *CREB1* region with expressed anger and treatment-emergent suicidal ideation among men with MDD that are less evident or absent among women with this disorder.^{37, 38} Whether these genotypes affect the risk of developing syndromic MDD among men or women cannot be determined from these studies. Since testosterone potentiates aggression/impulsivity, it is tempting to speculate that the association of these *CREB1* alleles with anger and suicidal ideation in men with MDD may be related to the effects of testosterone on CREB-

mediated gene expression. Molecular consequences of target genes and signaling pathways lower in this regulatory hierarchy may also contribute to sex-related differences in vulnerability to developing MDD and/or modify its clinical presentation.

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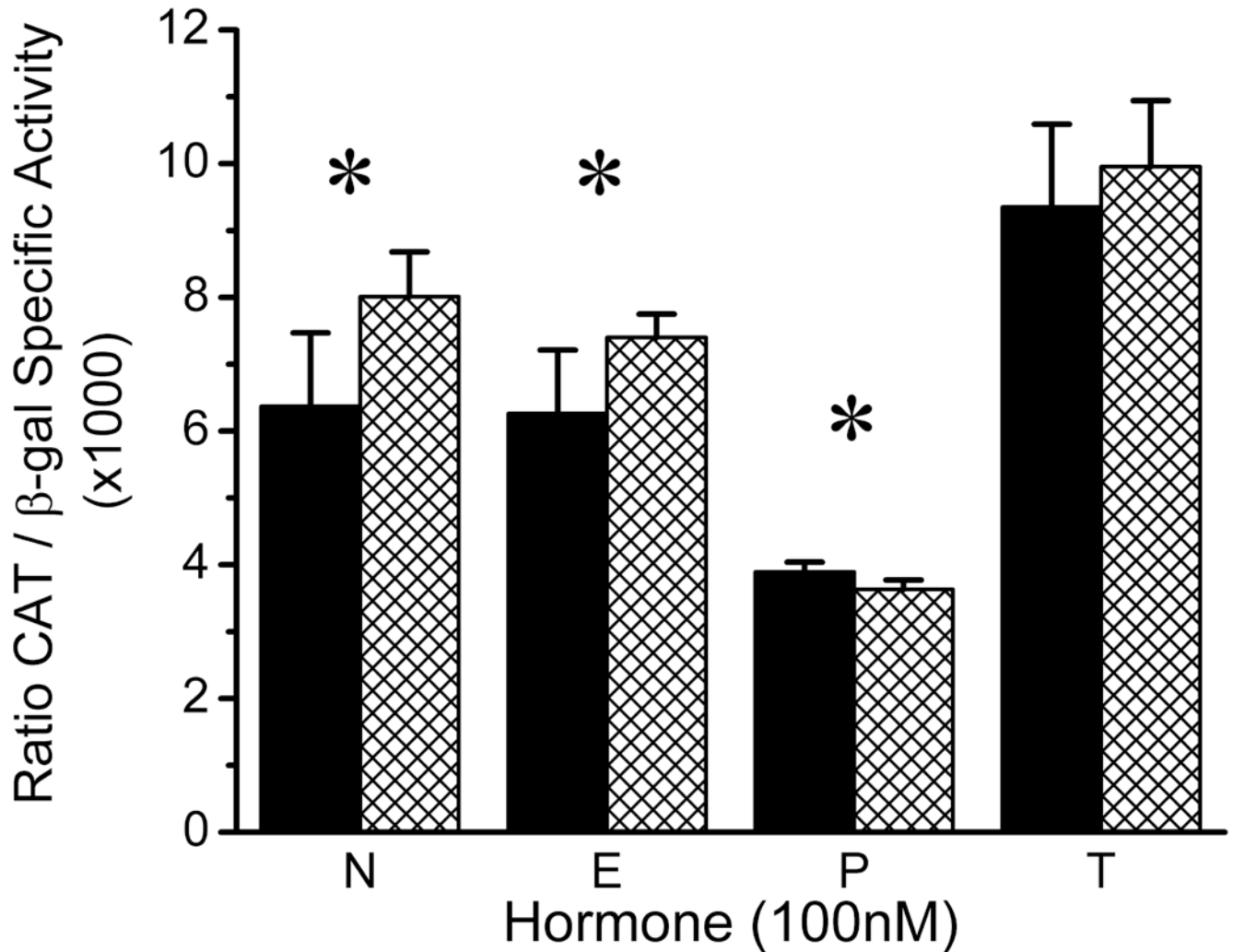


Figure 1. Effects of Gonadal Steroid Hormones on the Basal Activity of the Wild-Type and Variant *CREB1* Promoters in CATH.a Neuronal Cells. Wild-type promoter, solid bars. Variant promoter, hatched bars. Corresponding means (\pm SD) for wild-type and variant promoter activity were: No Hormone (N), 6.37 (1.10) and 8.01 (0.67); 17 β -Estradiol (E), 6.26 (0.95) and 7.40 (0.35); Progesterone (P), 3.89 (0.15) and 3.63 (0.14); and Testosterone (T), 9.35 (1.24) and 9.95 (0.99). Results of two-way ANOVA: Hormone effect, $F = 107.11$; $df = 3,40$; $p < 0.000001$; Genotype effect, $F = 11.21$; $df = 1,40$; $p < 0.002$; Hormone \times Genotype interaction, $F = 3.04$, $df = 3,40$; $p = 0.04$. All pairwise post hoc comparisons of hormone effects, $p < 0.05$, Tukey HSD, except for N vs. E ($p = 0.69$). Significant pairwise comparisons of G(-656)A genotypes within hormone conditions are indicated by an asterisk: N, $t = 3.14$, $df = 10$, $p = 0.01$; E, $t = 2.76$, $df = 6.37$, $p = 0.03$; P, $t = 3.14$, $df = 10$, $p = 0.01$.

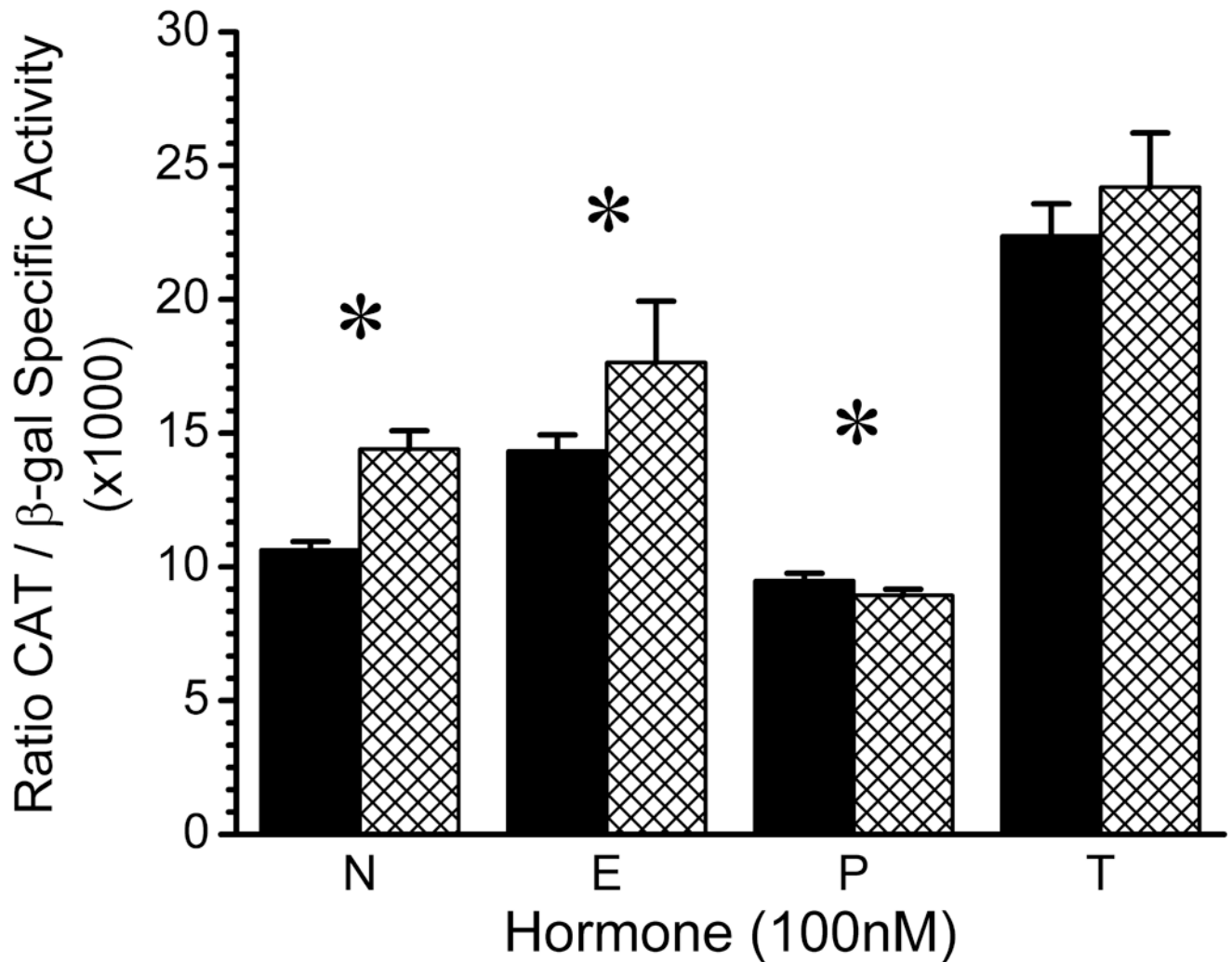


Figure 2.

Effects of the cAMP Signaling Pathway on Wild-Type and Variant *CREBI* Promoter Activity in CATH.a Neuronal Cells Grown in the Absence/ Presence of Gonadal Steroid Hormones. Wild-type promoter, solid bars. Variant promoter, hatched bars. Corresponding means (\pm SD) for wild-type and variant promoter activity were: No Hormone (N), 10.63 (0.31) and 14.40 (0.69); 17 β -Estradiol (E), 14.33 (0.60) and 17.64 (2.29); Progesterone (P), 9.48 (0.28) and 8.93 (0.23); and Testosterone (T), 22.37 (1.20) and 24.19 (2.03). Results of two-way ANOVA: Hormone effect, $F = 295.03$; $df = 3,40$; $p < 0.000001$; Genotype effect, $F = 35.40$; $df = 1,40$; $p = 0.000001$; Hormone \times Genotype interaction, $F = 7.69$, $df = 3,40$; $p = 0.0004$. All pairwise post hoc comparisons of hormone effects, $p < 0.05$, Tukey HSD. Significant pairwise comparisons of G(-656)A genotypes within hormone conditions are indicated by an asterisk: N, $t = 12.24$, $df = 10$, $p < 0.001$; E, $t = 3.43$, $df = 10$, $p = 0.006$; P, $t = 3.74$, $df = 10$, $p = 0.004$.

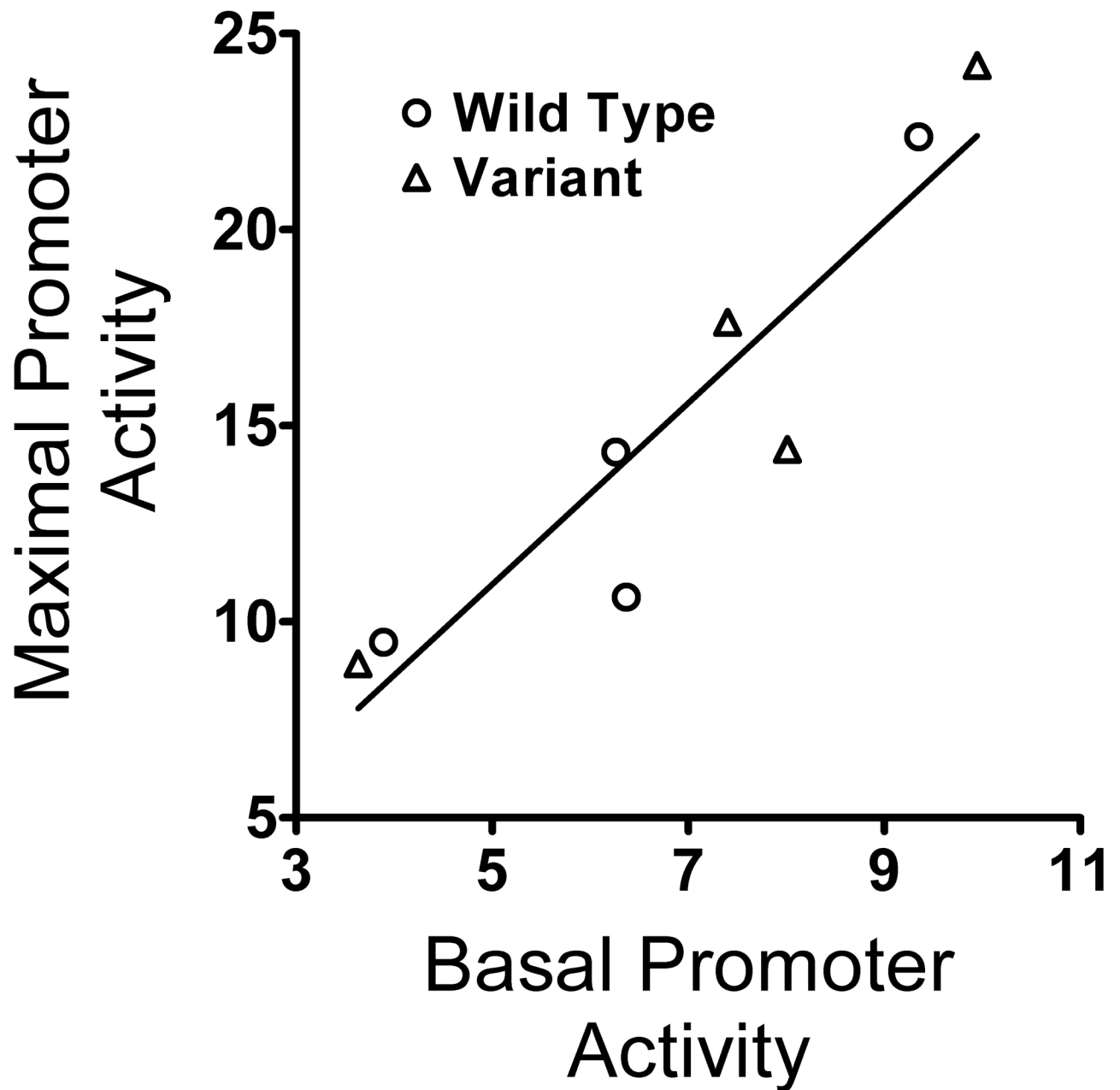


Figure 3. Relationship of Basal and Maximal *CREB1* Promoter Activity Following Activation of the cAMP Signaling Pathway in CATH.a Neuronal Cells. Wild-type promoter, circle. Variant promoter, triangle. Linear regression: slope = 2.31; y-intercept = -0.61; x-intercept = -0.26; Pearson $r = 0.925$, $p = 0.001$; Spearman $\rho = 0.952$, $p = 0.0003$.