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Effects of the A(-115)G Variant on *CREB1* Promoter Activity in Two Brain Cell Lines: Interactions with Gonadal Steroids

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Abstract

Major depressive disorder (MDD) is a leading contributor to disease burden worldwide. Previous genetic studies have revealed significant evidence of linkage of the *CREB1* region to Mood Disorders among women from families with recurrent, early-onset MDD (RE-MDD), a severe and familial subtype of MDD. Systematic resequencing of the *CREB1* gene in affected members of these families has identified rare sequence variants at positions -656 and -115 that appear to cosegregate with unipolar Mood Disorders in two large multigenerational families and three small nuclear families, respectively. Results from previous transfection experiments that employed constructs containing the wildtype or variant *CREB1* promoters coupled to a reporter gene support the hypothesis that the A₋₆₅₆ allele contributes to the development of MDD in women by selectively increasing the activity of the *CREB1* promoter in brain cell lines exposed to 17 β -estradiol. Analogous transfection experiments described in the current study revealed that the G₋₁₁₅ promoter variant reduced promoter activity in CATH.a neuronal cells regardless of the hormonal environment, consistent with the observation that increased risk for unipolar Mood Disorders conferred by this allele was not limited by sex. The effects of *CREB1* promoter variants on promoter activity, their influence on the development of Mood Disorders and related clinical features, and the interaction of their phenotypic expression with sex, seem likely to be complex and allele-specific rather than a general property of the *CREB1* locus.

Keywords

Genetics; C6 Glial and CATH.a neuronal cell lines; Sex; Depression; Mood

Introduction

Major Depressive Disorder (MDD) is a leading contributor to disease burden worldwide and affects women twice as frequently as men (Zubenko et al., 2001). 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 (Zubenko et al., 2001; Maher et al., 2002).

Model-free linkage analysis of a region of chromosome 2q33-35, highlighted by previous case-control studies (Philibert et al., 2003; Zubenko et al., 2002c) and supported by within-

family analyses employing the transmission disequilibrium test (Zubenko et al., 2002b), has revealed evidence of sex-specific linkage to unipolar Mood Disorders extending over 15 cM in our 81 RE-MDD families (Zubenko et al., 2002a; Zubenko et al., 2003a; Maher et al., 2009). 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. The 451Kb region between the adjacent STRPs D2S2321 and D2S2208 includes *CREB1*, which encodes the cAMP response element binding protein (CREB) (Mayr and Montminy, 2001).

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 (Carlezon et al., 2005; Manji et al., 2001; Nestler et al., 2002). CREB has also been implicated in neuronal plasticity, cognition, and long term memory (Weeber and Sweatt, 2002), 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 (Zubenko, 2000; Zubenko et al., 2001). Finally, reports of synergistic interactions of CREB with nuclear estrogen receptors (Lazennec et al., 2001; McEwen, 2001; Tremblay and Giguere, 2001) 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 *CREB1* cosegregate with depressive disorders in women from these families, providing support for *CREB1* as a sex-limited susceptibility risk gene for unipolar Mood Disorders and related conditions in RE-MDD families (Zubenko et al., 2003a). A rare G to A transition at position -656 in the *CREB1* promoter appeared to confer unipolar Mood Disorders with high penetrance among women in two (2.5%) of our 81 RE-MDD families. Transfection experiments employing plasmids containing the wildtype (wt) or variant *CREB1* promoter coupled to a reporter gene further 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 brain cell lines during exposure to physiologically-relevant concentrations of 17 β -estradiol (Zubenko and Hughes, 2008, 2009).

Systematic resequencing of the *CREB1* promoter, nine exons, and exon-flanking regions in the remaining RE-MDD families revealed an additional rare A to G transition at position -115 that was segregating with unipolar Mood Disorders in 3 (3.7%) of our 81 RE-MDD families. By comparison, no G₋₁₁₅ carriers were identified among 146 unrelated women who had no history of psychiatric disorders (allele frequency < 0.004). Of the seven heterozygotes in these families, five (71.4%) met criteria for unipolar mood disorders (3F, 2M). These findings suggest that the G₋₁₁₅ allele of the *CREB1* promoter manifests high penetrance, similar to that previously reported for the A₋₆₅₆ risk allele, but with less dependence (if any) on sex for the risk it confers for unipolar Mood Disorders.

In the current study, we determined the functional significance of this novel *CREB1* promoter variant using transfection experiments that employed constructs containing the wt or variant *CREB1* promoters coupled to a reporter gene, chloramphenicol acetyltransferase (CAT). Expression was also assessed in transfected cells grown in the presence or absence of physiological concentrations of gonadal steroid hormones (estradiol, progesterone,

testosterone) to evaluate potential genotype-sex interactions. Transfection experiments were performed using two brain cell lines, noradrenergic CATH.a neuronal cells derived from the locus ceruleus and C6 glial cells. CATH.a cells were employed 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 (Ressler and Nemeroff, 1999; Harro and Orelund, 2001). C6 glioma cells were also included because pathological changes in glial cells have been reported in the brains of patients who suffer from Mood Disorders (Öngür et al., 1998; Rajkowska et al., 1999).

Materials and Methods

Source and Growth Conditions for Brain Cell Lines

The rodent derived CATH.a neuronal cell line and glioma C6 cell line were acquired from the American Type Culture Collection (Manassas, VA). 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 (Suri et al., 1993; Widnell et al., 1994). CATH.a cells were 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. C6 cells were grown in nutrient mixture F12 Ham (Kaighn's modification) (Sigma, St. Louis, MO) supplemented with 2.5g/L sodium bicarbonate, 15% horse serum, and 2.5% fetal bovine serum (Gibco) under the same incubation conditions. CATH.a and C6 cells were propagated and subcultured at 1:2-4 or 1:4-10, respectively.

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 (Coven et al., 1998; Delfino and Walker, 1999; Meyer et al., 1993; Shell et al., 2002; Walker et al., 1995; Widnell et al., 1994, 1996). 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. The -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 (Meyer et al., 1993). This 5' regulatory region encompasses 1210 bps that include restriction sites for *Sau* 3AI at both termini.

The wt *CREB1* promoter and variant promoter containing the A to G transition located at position -115 were cloned using genomic DNA prepared from research subjects with REMDD who were heterozygous for these alleles. The methods for cloning the wt *CREB1* promoter and promoter containing the A₋₆₅₆ variant have been previously described (Zubenko and Hughes, 2008, 2009). To achieve this, a 1580 bp region containing the 1210 bp *Sau* 3AI fragment was amplified using the primers 5'-CCAGAATCGAACCCTCTCGCTTCC-3' and 5'-CCTCCTCCTGCTCCTC 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, 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 created by the A to G transition in the variant promoter (see Figure 1). Large scale preparation of the plasmids from cultures was performed using the Wizard® *Plus* Maxipreps DNA Purification System (Promega, Madison, WI). The base sequences of the cloned *CREB1* 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 base at position –115 and were devoid of PCR or cloning artifacts.

The research subjects whose genomic DNA was used to clone the wt and variant *CREB1* promoters 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 Cell Lines

Approximately 18 hrs prior to transfection, 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 (McEwen and Alves, 1999; Cornil et al., 2006). 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 (McEwen and Alves, 1999; Cornil et al., 2006).

Cells were transfected with the *CREB1* promoter-CAT reporter constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or FuGENE6 (Roche Applied Science, Indianapolis, IN) that were optimized for CATH.a or C6 cells, respectively (Zubenko and Hughes, 2008, 2009). The transfection cocktail for CATH.a cells 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 mixing. The transfection cocktail for C6 cells was formed by diluting 8 μ l of FuGENE 6 reagent in 92 μ l of serum free culture medium, and then adding 3 μ g of plasmid DNA followed by 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. Following incubation at room temperature, the transfection cocktails were added dropwise to the respective cell culture dish, which was rocked gently to distribute the complex. Cells were grown overnight for approximately 20 hrs before further manipulation.

Chloramphenicol Acetyltransferase (CAT) Assay

CAT assays were performed using the CAT Enzyme Assay System With Reporter Lysis Buffer (Promega, Madison, WI). Aliquots of clarified cell lysate were assayed in 125 μ l reactions containing 40 μ M chloramphenicol with 0.20 μ Ci of 3 H-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),

mixed, 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 promoter genotype, gonadal steroid hormones, and their interactions on *CREB1* promoter activity 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.

Results

Effects of gonadal steroid hormones on the activity of the wt and variant *CREB1* promoters in CATH.a neuronal cells

Cultures of CATH.a cells were grown in medium that lacked or contained physiologically-relevant (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) a CAT reporter construct containing either the wild-type A₋₁₁₅ *CREB1* promoter or the G₋₁₁₅ variant *CREB1* promoter, and (b) the 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.

Approximately 20 hours 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 no significant 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 2, the A to G transition at position -115 significantly reduced *CREB1* promoter activity compared to the wt promoter (two-way ANOVA genotype effect; $F = 633.78$, $df = 1,40$, $p < 0.000001$). The hormonal environment also had a significant effect on the activity of the *CREB1* promoter (two-way ANOVA hormone effect; $F = 102.28$, $df = 3,40$, $p < 0.000001$). All pairwise comparisons of hormone effects reflected significant differences ($p < 0.0002$, post hoc Tukey HSD), except for 17 β -estradiol and progesterone whose effects on promoter activity were similar. A significant hormone-genotype interaction was observed ($F = 25.67$, $df = 3,40$, $p < 0.000001$), reflecting the differential effects of hormonal environment on the activities of the wt and variant promoter. The activity of the variant promoter was significantly lower than that of the wt promoter both in the absence of gonadal steroids (N: $t = 4.41$, $df = 6.92$, $p = 0.003$), and in the presence of each gonadal steroid (E: $t = 18.14$; P: $t = 24.67$; T: $t = 13.02$; all $df = 10$; all $p < 0.000001$).

Effects of gonadal steroid hormones on the activity of the wt and variant *CREB1* promoters in C6 Glial cells

Cultures of C6 glial cells were grown in medium that lacked or contained 100 nM concentrations of gonadal steroids, transfected, harvested, and assayed as described for the CATH.a neuronal cells in the previous section. As observed for experiments that employed CATH.a cells, growth of transfected C6 cells in the presence of these hormones had negligible effects on β -galactosidase specific activity, and negligible CAT specific activity was found in cells that lacked the *CREB1* promoter-CAT reporter construct.

Consistent with the results for CATH.a cells, the A to G transition at position -115 significantly reduced *CREB1* promoter activity compared to the wild type promoter in C6 cells (Figure 3; two-way ANOVA genotype effect; $F = 187.65$, $df = 1,40$, $p < 0.000001$). The hormonal environment also had a significant effect on the activity of the *CREB1* promoters (two-way ANOVA hormone effect; $F = 116.11$, $df = 3,40$, $p < 0.000001$). All pairwise comparisons of hormone conditions reflected significant differences in promoter activity ($p < 0.03$, post hoc Tukey HSD). A significant hormone-genotype interaction also was observed ($F = 36.29$, $df = 3,40$, $p < 0.000001$), reflecting the observation that the reduction in *CREB1* promoter activity produced by the A₋₁₁₅ variant was hormone-dependent. Significant differences between the activity of the wt and variant *CREB1* promoters occurred only in the absence of hormones (N: $t = 17.65$, $df = 10$, $p < 0.000001$) and in the presence of 17 β -estradiol ($t = 28.51$, $df = 10$, $p < 0.000001$). The greater relative decrease occurred for the variant *CREB1* promoter in the absence of hormones, while the greater absolute decrease in the activity of the variant promoter occurred in the presence of 17 β -estradiol.

Discussion

These results reveal that the A to G transition at position -115 of the *CREB1* promoter significantly reduced promoter activity in noradrenergic CATH.a neuronal cells, regardless of the hormonal environment. Consistent with the results for CATH.a cells, the G₋₁₁₅ variant also reduced *CREB1* promoter activity in C6 cells, but only in the absence of gonadal steroids or in the presence of a physiologically relevant concentration of 17 β -estradiol. The findings from the transfection experiments employing CATH.a neuronal cells are more consistent with the observation that the increased risk for unipolar Mood Disorders conferred by the G₋₁₁₅ promoter allele that was segregating in three RE-MDD families was not limited by sex.

These findings differ qualitatively from the clinical and molecular phenotypes of the A₋₆₅₆ *CREB1* promoter variant, another rare, highly-penetrant risk allele for unipolar Mood Disorders in RE-MDD families (Zubenko et al., 2003a; Zubenko and Hughes, 2008, 2009). In contrast to the G₋₁₁₅ allele, the results of linkage and association studies indicated that the increased risk for depressive disorders conferred by the A₋₆₅₆ promoter allele was limited to women who carried this allele. Moreover, published transfection experiments analogous to those employed in the current study support the hypothesis that the A₋₆₅₆ allele contributes to the development of depressive disorders in women by selectively increasing the activity of the *CREB1* promoter in both CATH.a and C6 cell lines during exposure to physiologically-relevant concentrations of 17 β -estradiol (Zubenko and Hughes, 2008, 2009).

A probable mechanism by which these sequence variants influence the activity of the *CREB1* promoter is by affecting the biological activity of a transcription factor binding site at their respective locations. Several lines of evidence from an *in silico* analysis suggest that the effects of the G to A transition at position -656 may be mediated by CP2 binding (Zubenko and Hughes, 2008). The pathogenic A₋₆₅₆ allele creates a perfect match to the core of the CP2 binding motif (CCCAG), 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 (Zubenko et al., 2003a). In contrast, the G₋₁₁₅ variant disrupts the core sequence of a CP2 binding motif that exists in the wt promoter (see Figure 1). Among its target genes, CP2 appears to regulate the expression of glycogen synthase kinase 3 β (Lau et al., 1999), which has been implicated in the pathophysiology of both mood disorders and AD (Bhat et al., 2004; Jope et al., 2007; Manji et al., 2001). In addition, a non-coding polymorphism in the 3' untranslated region of the CP2 gene has been reported to affect the risk of MDD (Schahab et al., 2006) and Alzheimer's disease (Lambert et al., 2000), both of which aggregate in RE-MDD families (Zubenko et al., 2001).

How can sequence variants that have opposite effects on *CREB1* promoter activity confer increased risk of developing the same phenotype? The clinical syndrome of MDD is a common manifestation of brain dysfunction produced by a large number and a wide variety of causes. In addition to unfavorable genetic and environmental influences, syndromic MDD can result from trauma, infection, anoxia, hemorrhage, neoplasm, and degenerative or vascular diseases of the central nervous system; endocrine, metabolic, or neoplastic systemic disorders; exposure to drugs or toxins; and interactions among predisposing factors. *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 (Mayr and Montminy, 2001). Because

of this breadth of influence, it seems plausible that the dysregulation of *CREB1* expression (either increased or decreased) may adversely affect brain function and present clinically as MDD or a related condition. It should also be noted that the overexpression or reduced activity of a gene can produce the same aberrant phenotype at the cellular level (Rose and Fink, 1987).

Animal studies suggest that depressive-like symptoms in rodents may result from decreases in CREB levels in some brain regions (e.g. in hippocampus, depressed mood and impaired cognition), or increases in CREB levels in other regions (e.g. in nucleus accumbens, anhedonia), implying that the clinical characteristics of major depressive episodes (MDEs) may result from the anatomic distribution of particular alterations in CREB expression (for review see Carlezon et al., 2005). No differences were apparent in the clinical presentation of the MDEs of affected RE-MDD family members who carried the G₋₁₁₅ *CREB1* promoter allele compared to those who carried the A₋₆₅₆ allele. However, these comparisons had several important limitations including the small numbers of carriers, the sex-specific effects of the A₋₆₅₆ allele, the reduced variance in depressive symptoms inherent in comparing symptom profiles of episodes that met diagnostic criteria for an MDE, and reliance on the results from structured diagnostic assessments and available medical records aimed at establishing reliable and valid lifetime psychiatric diagnoses (Zubenko et al., 2001; Zubenko et al., 2003b). Further characterization of the clinical and biological correlates of these risk alleles seems warranted.

CREB and other transcription factors appear to participate at the top level of a molecular and cellular cascade that controls aspects of neuronal plasticity that regulate mood, cognition, and related phenotypes. The effects of *CREB1* promoter variants on promoter activity, their influence on the development of Mood Disorders and related clinical features, and the interaction of their phenotypic expression with sex, seem likely to be complex and allele-specific rather than a general property of the *CREB1* locus. In addition to the characteristics of the *CREB1* promoter variants at positions -115 and -656, recent reports have 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 (Perlis et al., 2007a,b). This phenomenon complicates the design and interpretation of genomic studies aimed at identifying sequence variants that influence the development of behavioral traits and psychiatric disorders.

Dissecting the clinical biology that underlies these observations is a challenging goal that will most likely proceed through incremental steps. *In silico* analysis did not identify an estrogen receptor binding motif that overlapped with the variants identified at *CREB1* positions -656 or -115, 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 genotype at position -656, 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 (McEwen and Alves, 1999; Mayr and Montminy, 2001). 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.

Preclinical studies that explore the functional significance of these *CREB1* alleles, and clinical studies that employ individuals who carry these susceptibility alleles, seem likely to advance our understanding of the clinical biology and experimental therapeutics of Mood

Disorders. Humanized recombinant mice that bear *CREB1* susceptibility alleles may also provide relevant animal models. By analogy, considerable insight into the pathophysiology of typical forms of Alzheimer's disease (AD) has been gleaned from studies that examined the effects of rare autosomal dominant mutations that confer risk for early-onset, familial AD, yet account for only a small fraction (< 1%) of all AD cases (Selkoe and Podlisny 2002).

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Figure 1.

Electropherograms Illustrating the A(-115)G *CREBI* Promoter Variant. (Left) Electropherogram from automated sequencing of genomic DNA from an A/G heterozygote. The sequence is shown (5' to 3', plus strand) from position -121 to -104, inclusive. (Right) Autoradiogram showing RFLP genotyping of an A/A homozygote (labeled 90 bp *Msp* I digestion fragment) and an A/G heterozygote (labeled 90 bp and 59 bp *Msp* I digestion fragments). RFLP-based genotyping of the alleles at position -115 of the *CREBI* promoter was performed by PCR amplification of the variable region, followed by digestion of the amplified product with *Msp*I, whose recognition site (5'-CCGG-3') was created by the presence of the G₋₁₁₅ variant. Amplification was performed using the ³²P end-labeled forward primer (5' to 3') CCG CGG AAC CCC TTC TCG TC and the unlabeled reverse primer AGT CCG CCG CCA TTA TTC TTT G (Zubenko et al., 2002b).

Figure 2.

Effects of the A(-115)G *CREB1* Promoter Variant and Gonadal Steroid Hormones on *CREB1* Promoter Activity in Noradrenergic CATH.a Neuronal Cell Line. WT (A₋₁₁₅) promoter, solid bars. Variant (G₋₁₁₅) promoter, hatched bars. Corresponding means ($\pm$ SD) for wt and variant promoter activity were: No Hormone (N), 3.00 (0.15) and 2.35 (0.33); 17 β -Estradiol (E), 4.01 (0.16) and 2.25 (0.18); Progesterone (P), 3.99 (0.12) and 2.20 (0.13); and Testosterone (T), 5.22 (0.26) and 3.11 (0.30). Results of two-way ANOVA: Genotype effect, $F = 633.78$; $df = 1,40$; $p < 0.000001$; Hormone effect, $F = 102.28$; $df = 3,40$; $p < 0.000001$; Genotype-hormone interaction, $F = 25.67$, $df = 3,40$; $p < 0.000001$. All pairwise *post hoc* comparisons of hormone effects, $p < 0.0002$, Tukey HSD, except for E vs. P ($p = 0.98$, Tukey HSD). Significant pairwise comparisons of the two genotypes within hormone conditions are indicated on the Figure by an asterisk: N, $t = 4.41$, $df = 6.92$, $p = 0.003$; E, $t = 18.14$, $df = 10$, $p < 0.000001$; P, $t = 24.67$, $df = 10$, $p < 0.000001$; T, $t = 13.02$, $df = 10$, $p < 0.000001$.

Figure 3.

Effects of the A(-115)G *CREB1* Promoter Variant and Gonadal Steroid Hormones on *CREB1* Promoter Activity in C6 Glial Cell Line. WT (A₋₁₁₅) promoter, solid bars. Variant (G₋₁₁₅) promoter, hatched bars. Corresponding means ($\pm$ SD) for wild-type and variant promoter activity were: No Hormone (N), 1.07 (0.07) and 0.33 (0.08); 17 β -Estradiol (E), 1.85 (0.04) and 0.91 (0.07); Progesterone (P), 1.00 (0.08) and 0.93 (0.07); and Testosterone (T), 1.61 (0.19) and 1.44 (0.23). Results of two-way ANOVA: Genotype effect, $F = 187.65$; $df = 1,40$; $p < 0.000001$; Hormone effect, $F = 116.11$; $df = 3,40$; $p < 0.000001$; Genotype-hormone interaction, $F = 36.29$, $df = 3,40$; $p = 0.000001$. All pairwise *post hoc* comparisons of hormone effects, $p < 0.03$, Tukey HSD. Significant pairwise comparisons of the two genotypes within hormone conditions are indicated on the Figure by an asterisk and occurred only for N, $t = 17.65$, $df=10$, $p < 0.00001$; and for E, $t = 28.51$, $df = 10$, $p < 0.000001$.