Supporting Information

Barr et al. 10.1073/pnas.0902863106

SI Text

Comparative Genome Information. Comparative genomic analyses were performed using the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway). We looked at the degree of interspecific and intraspecific sequence conservation (placental mammalian conservation (1) and indel-purified sequences) (2) for the sequence 1 kb 5′ and 1.6 kb 3′ to the CRH transcription start site (*H. sapiens* Mar 2006 assembly, chr8:67,251,637–67,254,257). For the proximal regulatory region (−350 → +1, *H. sapiens* Mar 2006 assembly, chr8:67,253,252–67,253,601), we assessed the putative functional significance of −248 C → T by looking for conserved transcription factor-binding sites (http://www.gene-regulation.com/pub/databases.html), cis-regulatory potential (3, 4), and vertebrate conservation. The UCSC Genome Browser was also used to determine whether there were SNPs within the corresponding region of humans (5).

Electrophoretic Mobility Shift Assay. Double-stranded oligonucleotides containing the −248 C (5′-CA AGt cat aag aag ccc ttc cat ttt agg gct-3′) and T (5′-CA AGt cat aag aag c*T*c ttc cat ttt agg gct-3′) alleles were generated for performance of gel shift assays using nuclear extract generated from an immortalized hypothalamic cell line (IVB cells) (6). Assays were performed using the Gel Shift Assay System (Promega) per the manufacturer's instructions. After annealing complementary oligonucleotides (95 °C 5 min, 25 °C 30 min), double-stranded probes were [^{32}P]-ATP labeled using T4 kinase (Promega) and purified using a Bio-Spin 30 chromatography column (Bio-Rad). Incorporation of radiolabel was → 1×10^5 cpm/ng DNA. Binding assays were performed using the Gel Shift Assay System (Promega) per the manufacturer's instructions. Nuclear extract (5 μg) was incubated for 20 min with 100,000 cpm of each oligonucleotide probe. Competitor oligonucleotides were added at 10× the concentration of the labeled probes. Samples were immediately separated by electrophoresis (250 V for 20 min) on a Novex 6% DNA retardation gel (Invitrogen), after which gels were dried and bands visualized by autoradiography. Gel shift assays were repeated four times.

Reporter Assay. Genomic DNA was extracted from macaques homozygous for the most common CRH alleles for PCR-based cloning of rhCRH. The forward primer was 5′-GCG *GAA TTC* GGC TCA TAA CTC CTT TAT GTG CTT GC-3′ (containing an *Eco*RI site) and the reverse primer was 5′-AAA *GGA TCC* GAG GGA CGT CTC CGG GGC-3′ (containing a *Bam*HI site). These primers produced a 783-bp amplification product (from rhCRH −660 to + 123). Reaction mixtures (50 μL) contained 100 ng DNA, 0.1 mM dNTPs, 0.5 μM of each primer, 2.5 U PfuUltra High Fidelity DNA Polymerase and PfuUltra Buffer (Stratagene). Amplifications were performed using a Perkin-Elmer thermocycler (9700) with one cycle at 95 °C, 30 cycles of 95 °C/30 s, 61.5 °C/30 s, 72 °C/5 min, and a final 10-min extension at 72 °C. Following cleavage using *Eco*R1 and *Bam*HI, PCR product was separated by electrophoresis and isolated using a QIAquick gel extraction kit. Cleaved products were then ligated into *Eco*RI/*Bam*HI-digested pDsRed-2.1 (BD Biosciences) using standard molecular cloning techniques. The −248 T allele was introduced using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Fidelity of resultant constructs was verified by sequencing.

The −248 C → T SNP is located in a region important to CRH transcriptional activity. We used an in vitro system to test whether CRH transcriptional activity was altered as a function of this variant following treatment with agents known to influence CRH promoter activity (TPA, forskolin and dexamethasone). Many studies examining effects of various agents on CRH expression have been performed using immortalized hypothalamic cells (IVB). However, this cell line exhibits levels of transfection efficiency that were prohibitively low for the purposes of testing functional effects of the −248 C → T SNP in a reporter system. Therefore, we used a mouse HT22 hippocampal cell line, the kind gift of David Schubert (The Salk Institute, La Jolla, CA) (7) Using this cell line, we were able to demonstrate treatment effects on CRH promoter activity that are consistent with observations made in studies examining CRH expression levels in hypothalamic (IVB) and placental cell lines (Fig. S1).

HT22 cells were propagated in DMEM (Gibco) supplemented with 10% FBS and maintained at 37 °C in a humidified incubator (5% CO_2). Cells were seeded at a density of 5×10^4/well in a 12-well tissue culture plate. When 60–70% confluent, either the −248 C or T rhCRH pDsRed reporter constructs (1 μg) were co-transfected with a GFP reporter (0.5 μg pGlow-TOPO, Invitrogen,) using Lipofectamine 2000 (Invitrogen,), according to the manufacturer's instructions. To determine promoter responses to PKC, PKA and high doses of corticosteroids, cells were treated with the phorbol ester, TPA (12-O-tetradecanoylphorbol 13-acetate, 20 nM), 30 μM forskolin (Sigma) or 30 μM forskolin + 100 nM dexamethasone (Sigma) (6, 8) 12 h following transfection. Expression of DsRed was monitored 48h following treatment using an Olympus IX70 microscope (Olympus America) interfaced with a Hamamatsu ORCA-ER digital camera (Hamamatsu). Region of interest analysis was performed using OpenLab software. A CRH promoter expression value was obtained by dividing the DsRed intensity by that for GFP. Experiments for each treatment were performed at least two times, with treatments performed in duplicate within experiment. Measurements were made by two observers (MG and CSB) and averaged. Data were analyzed by ANOVA, with treatment (No treatment, TPA, forskolin, forskolin + dex) and genotype (−248 C and −248 T) included as independent variables.

Genotyping for rhCRH −248 C → T. DNA was isolated from whole blood using standard extraction methods. Genomic DNA (5 ng) was dispensed into 96-well plates, dried and stored at room temperature for up to a month before use. Genotyping was performed by the 5′ exonuclease method using fluorescent, allele-specific MGB probes. Oligonucleotide primers (Forward: 5′-GGC CTT TCA TAG TAA GAG GTC AAT ATG T-3′; Reverse: 5′-CGC CTC TTG GTG ACG TCA A-3′) and probe sets (−248C, 6FAM-TCA TAA GAA GCC CTT CCA TT and −248T, VIC-GTC ATA AGA AGC TCT TCC ATT) were designed based on the rhesus macaque sequence. Each reaction well contained 12.5 μL PCR Master Mix (Applied Biosystems), 800 nmol forward and reverse primer, and 160 nmol each reporter and quencher probe. PCR parameters were: 50 °C for 2 min; 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, and 60 °C for 1 min; reactions were then stored at 4 °C. PCR was carried out with a GeneAmp PCR system 7700 (Applied Biosystems). Allele-specific signals were detected as endpoint 6-FAM or VIC fluorescence intensities at 508 nm and 560 nm, respectively, and genotypes were generated using

Sequence Detection System Software Version 1.7 (Applied Biosystems). Genotyping error rate was directly determined by re-examining 10% of the samples, randomly chosen, and by comparing to sequencing data from the genotyped animals (n = 40 chromosomes). The overall error rate was 0.5%, and genotype completion rate was 93%.

Rhesus macaque (*Macaca mulatta*) infants were randomly selected to be reared

1. Felsenstein J, Churchill GA (1996) A Hidden Markov Model approach to variation among sites in rate of evolution. *Mol Biol Evol* 13:93–104.
2. Lunter G, Ponting CP, Hein J (2006) Genome-wide identification of human functional DNA using a neutral indel model. *PLoS Comput Biol* 2:e5.
3. King BR, Nicholson RC (2007) Advances in understanding corticotrophin-releasing hormone gene expression. *Front Biosci* 12:581–590.
4. Kolbe D, et al. (2004) Regulatory potential scores from genome-wide three-way alignments of human, mouse, and rat. *Genome Res* 14:700–707.
5. Sherry ST, et al. (2001) dbSNP: The NCBI database of genetic variation. *Nucleic Acids Res* 29:308–311.
6. Kasckow J, et al. (2003) Corticotropin-releasing hormone (CRH) expression and protein kinase A mediated CRH receptor signaling in an immortalized hypothalamic cell line. *J Neuroendocrinol* 15:521–529.
7. Maher P, Schubert D (2000) Signaling by reactive oxygen species in the nervous system. *Cell Mol Life Sci* 57:1287–1305.
8. Wagner U, et al. (2006) Promoter polymorphisms regulating corticotrophin-releasing hormone transcription in vitro. *Horm Metab Res* 38:69–75.

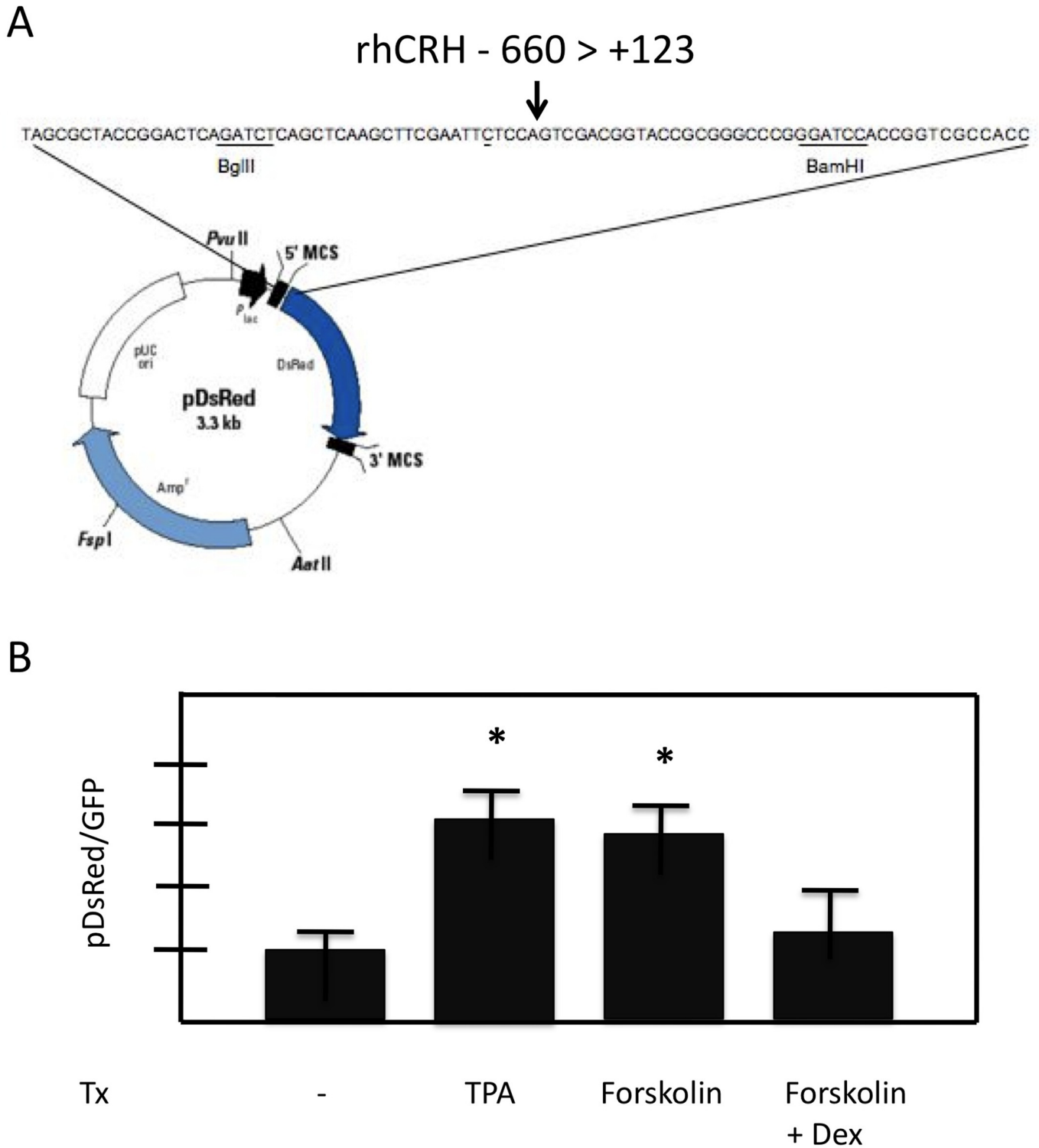

Fig. S1. Construct design and effects of treatment in transfected HT22 cells. HT22 cells were treated with TPA, forskolin, or forskolin + dexamethasone, and rh*CRH* promoter-driven pDsRed levels were assessed. Similar treatment effects to those observed in hypothalamic and placental cell lines were observed in the hippocampual HT22 cell line.