Analysis of Target Genes Regulated by Chronic Electroconvulsive Therapy Reveals Role for *Fzd6* in Depression

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Background: Chronic electroconvulsive seizure (chr-ECS), one of the most efficacious treatments for depressed patients, increases the levels of transcription factor cyclic adenosine monophosphate response element binding protein (CREB) in rodent models and mediates the effects of chronic antidepressant treatment. The objective of this study was to determine the changes in CREB occupancy at gene promoters and subsequent gene expression changes induced by chr-ECS.

Methods: We use chromatin immunoprecipitation followed by microarray analysis to identify CREB binding promoters that are influenced by chr-ECS ($n = 6$/group). Selected genes are confirmed by secondary validation techniques, and the functional significance of one target was tested in behavioral models ($n = 8$/group) by viral mediated inhibition of gene expression.

Results: The results demonstrate that chr-ECS enhances CREB binding and activity at a select population of genes in the hippocampus, effects that could contribute to the efficacy of chr-ECS. Viral vector-mediated inhibition of one of the CREB-target genes regulated by chr-ECS, *Fzd6*, produced anxiety and depressive-like effects in behavioral models of depression.

Conclusions: The results identify multiple gene targets differentially regulated by CREB binding in the hippocampus after chr-ECS and demonstrate the role of *Fzd6*, a Wnt receptor in behavioral models of depression.

Key Words: ChIP-chip, chr-ECS, CREB, depression, frizzled receptor, Wnt signaling

Depression is a devastating illness affecting up to 17% of the population and one of the leading causes of disability worldwide. It likely results from a combination of genetic, biochemical, and environmental factors (1). Current chemical antidepressants have limited efficacy in approximately two-thirds of depressed patients, and electroconvulsive therapy (ECT) is often successfully used to treat patients who are resistant to antidepressants. However, the molecular mechanisms underlying the therapeutic efficacy of ECT are not known. In rodents, both acute and repeated administration of electroconvulsive seizure (chr-ECS) have been shown to regulate the expression of a variety of genes, including angiogenic and neurotrophic factors (2–4), and to increase the proliferation of neural stem-like cells in the hippocampus (5).

In the central nervous system, the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) is a key modulator of various functions, including neuroplasticity, learning, and memory, and has been implicated in multiple psychiatric disorders (6,7). In depression, the role for CREB is both regional and temporal specific, and CREB has been shown to produce both pro-depressive and anti-depressive behaviors, depending on the brain regions examined (8,9). In the hippocampus, CREB has been shown to mediate the effects of both chemical and non-chemical antidepressant treatments. Reduced CREB levels in postmortem brains of depressed patients, increased levels with antidepressant treatments (10–13), and increased antidepressant behavioral responses by induction of CREB in hippocampus of rodents (14) suggest an important role of CREB in the pathophysiology and treatment of depression.

CREB binds to the cAMP response element and, upon ser-133 phosphorylation, can lead to activation of multiple transcriptional pathways (15–17). Electroconvulsive seizure increases ser-133 phosphorylation of CREB in rodent brain (18). With chromatin immunoprecipitation and microarray technology (ChIP-chip), we previously identified >860 CREB binding sites in rodent brain and altered CREB occupancy and/or ser-133 phosphorylation of CREB (pCREB) at a subset of these targets within 15 min of an initial (acute) ECS treatment (19). However, the underlying adaptive changes that contribute to the enhanced therapeutic efficacy produced by repeated ECS, similar to the time course for therapeutic intervention in depressed patients, are not fully understood. The current study addresses this question by determining the changes in CREB binding/activity resulting from chr-ECS. The results demonstrate enhanced binding of CREB and pCREB at select gene promoters with chr-ECS relative to acute ECS. The functional consequences of altered CREB binding/activity were validated for a select number of genes, including Frizzled 6 (*Fzd6*), one of the receptors involved in Wnt signaling.

Wnts are secreted glycoproteins that signal through the Fz receptors (20). *Wnt2* was previously shown to be upregulated by different classes of antidepressants, and viral-vector mediated overexpression of *Wnt2* has been shown to produce an antidepressant-like effect in rodents (21). Activation of the canonical Wnt signaling pathway results in inhibitory phosphorylation of glycogen-synthase kinase-3 (GSK-3)β, resulting in increased accumulation of β-catenin in the nucleus leading to activation of Wnt target genes. Prior research has shown that the Wnt-GSK-3 system is involved in the adaptive responses underlying antidepressant treatment (22–24), and selective inhibitors of GSK-3 have antidepressant efficacy in behavioral models of depression (25,26). However, little is known about the Fz receptor subtypes that mediate the actions of Wnt signaling. After confirming that chr-ECS increased *Fzd6* messenger RNA (mRNA) and showing that chronic unpredictable stress (CUS) had the opposite effect, we determined the behavioral

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Received Feb 14, 2011; revised Jul 15, 2011; accepted Aug 11, 2011.

0006-3223/$36.00
doi:10.1016/j.biopsych.2011.08.004

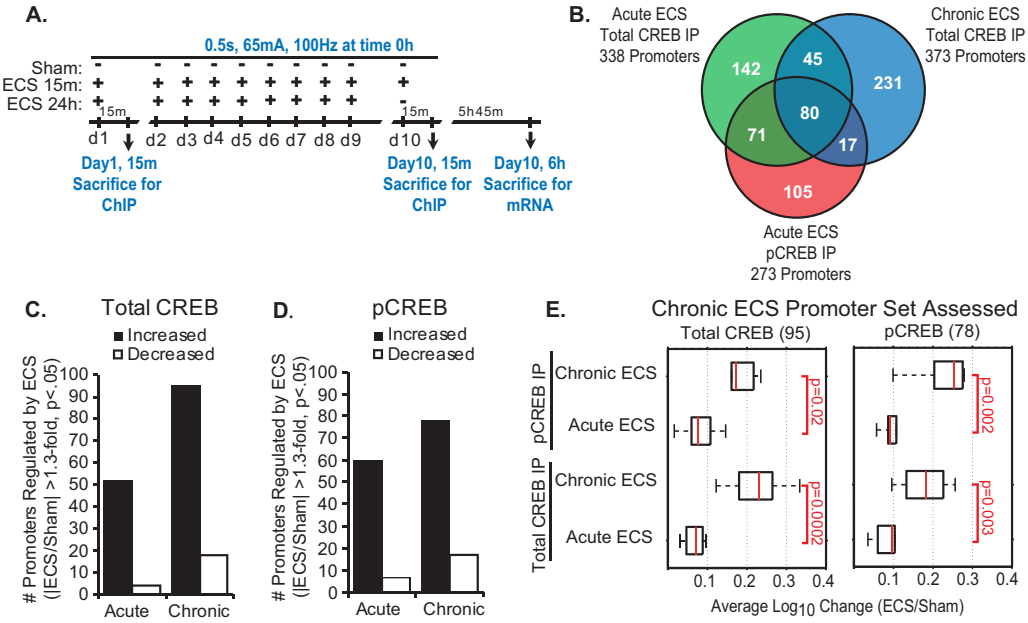

Figure 1. Identification of chronic electroconvulsive seizure (chr-ECS)-induced cyclic adenosine monophosphate-response element binding (CREB) occupied promoters after chr-ECS. **(A)** Schematic diagram showing details of the experimental timeline for analysis of hippocampus. **(B)** Venn diagram of the overlap between promoters enriched 1.5-fold, $p < .05$ relative to pre-immune immunoglobulin G by total CREB antibody 15 min after a single ECS (acute), ser-133 phosphorylation of CREB (pCREB) antibody 15 min after acute ECS, and total CREB antibody 15 min after the 10th ECS (chronic). **(C)** Bar graph indicates number of promoters in the hippocampus with ECS/sham differences in total CREB **(C)** or pCREB **(D)** occupancy of >1.3-fold, $p < .05$ (black), or <−1.3-fold, $p < .05$ (white), after acute or chr-ECS. **(E)** Box plots indicate average log10 ratio (ECS/sham) for total CREB antibody after acute ECS (bottom), total CREB antibody after chr-ECS (lower middle), pCREB antibody after acute ECS (upper middle), and pCREB antibody after chr-ECS (top) for the 95 promoters exhibiting >1.3-fold, $p < .05$, enhancement of total CREB occupancy or the 78 promoters exhibiting >1.3-fold, $p < .05$, enhancement of pCREB occupancy relative to sham treatment after chr-ECS. ChIP, chromatin immunoprecipitation; IP, immunoprecipitation; mRNA, messenger RNA.

significance of viral-mediated knockdown of *Fzd6* expression in the hippocampus.

Methods and Materials

Animals

Male Sprague-Dawley rats (180–220 g for chr-ECS treatment; 250–300 g for adeno-associated virus [AAV] studies) (Charles River Labs, Wilmington, Massachusetts) were housed, two or three/cage, under standard illumination parameters (12-hour light/dark cycle) and were given free access to food and water. All procedures were in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Yale University Animal Care and Use Committee.

chr-ECS

Bilateral ECS was administered as described previously (2). For ChIP-chip experiments, animals were killed by decapitation 15 min after the tenth sham/ECS treatment ($n = 6$) or 24 hours and 15 min after the ninth ECS treatment with sham treatment 15 min before sacrifice ($n = 6$) (Figure 1A). For reverse transcriptase polymerase chain reaction (RT-PCR), in situ hybridization and immunohistochemistry experiments, a different cohort of animals were killed by decapitation 6 hours after the last ECS treatment.

ChIP-chip Processing, Hybridization, and Analysis

The ChIP-chip experiments, quality control, and analysis were performed exactly as described (19). Briefly, chromatin isolated from 10 mg tissue was sonicated into 200–500 base pair (bp) fragments. Resulting samples were immunoprecipitated with 4 μg total CREB antibody (Upstate #06863; Upstate, Ithaca, New York), 2 μg pCREB antibody (Upstate #06519), or 5 μg normal rabbit immunoglobulin G (IgG) (Sigma I8140; Sigma, St. Louis, Missouri). The ChIP DNA was amplified to linear phase by ligation-mediated PCR, and amplified DNA was labeled with Genesphere's DNA900 labeling system (Genesphere, Hatfield, Pennsylvania) and hybridized to DNA microarrays from the Beta Cell Biology Consortium containing 1-kilobase pair (Kb) sequences immediately upstream of the transcription start site (TSS) of 12,000 genes and 2-Kb sequences from 3 Kb to 1 Kb upstream of the TSS for half of the represented genes (http://www.betacell.org). Mean Cy3/Cy5 ratios from 6 independent animal/ChIP/array replicates were calculated on a per-spot, per-array basis with GenePix Pro 6.0 (Axon Instruments, Foster City, California) and Genespring 7.2 (Silicon Genetics, Redwood City, California) after intensity-dependent Lowess normalization. Analysis of ratio data was performed in Matlab R2007a (Mathworks, Natick, Massachusetts).

Quantitative Real-Time RT-PCR

Total RNA from hippocampus stored at −80°C was purified with RNAqueous according to the directions of the manufacturer (Ambion, Austin, Texas). One microgram of total RNA was reverse transcribed as before (2). Gene-specific primers were designed with PRIMER3 software.

In Situ Hybridization Analysis

In situ hybridization was conducted on coronal brain sections (14 μm) by hybridization with the 35S-labeled riboprobes for *Fzd6*, *Slc6a9*, and *Per1* as previously described (2,27). Gene-specific primers were designed with PRIMER3 software. Relative gene expression changes were determined with the software IMAGEJ (http://rsb.info.nih.gov/ij/). Primers to make riboprobes for in situ hybridization: