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Altered CREB binding to activity-dependent genes in serine racemase deficient mice, a mouse model of schizophrenia

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Abstract

cAMP-response-element-binding protein (CREB) is a transcription factor ubiquitously expressed in the brain that regulates neuroplasticity by modulating gene expression. The influx of calcium through *N*-methyl-D-aspartate receptors (NMDARs) is a well-defined mechanism that leads to the increased expression of CREB-dependent genes, including brain derived neurotrophic factor (BDNF), microRNA-132, and activity-regulated cytoskeleton-associated protein (Arc). These molecules are implicated in the pathophysiology of schizophrenia. We previously demonstrated that serine racemase knockout (SR^{-/-}) mice, which exhibit NMDAR hypofunction due to a lack of the forebrain NMDAR co-agonist D-serine, also have reduced expression of CREB-dependent genes in the hippocampus. Using chromatin immunoprecipitation, we show here that in SR^{-/-} mice, there is less CREB bound to the promoter regions of BDNF, microRNA-132, and Arc. These data suggest that NMDAR hypofunction in SR^{-/-} mice leads to reduced CREB binding on known activity-dependent genes, in turn contributing to their reduced expression.

Keywords

Serine racemase; D-serine; NMDA receptor; CREB; Arc; Brain derived neurotrophic factor

N-methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors that are essential mediators of synaptic plasticity, learning and memory¹. What makes the activation of these receptors unique, is that in addition to the binding of its agonist glutamate, they also require the concomitant binding of a co-agonist, glycine or D-serine, to the GluN1 subunit of the NMDAR²⁻³. D-serine is converted from L-serine by the neuronal enzyme serine racemase (SR)⁴. Both the enzyme and D-serine are enriched in cortico-limbic regions of the

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Author contributions

D.T.B. performed the experiments, analyzed the data, and wrote the manuscript. D.T.B. and J.T.C. wrote the manuscript.

Conflicts of Interest

JTC has served as a consultant to Forum Pharmaceuticals in the last 2 years. A patent owned by Massachusetts General Hospital for the use of D-serine as a treatment for serious mental illness could yield royalties for Dr. Coyle. DTB declares no competing financial interests.

brain and are localized to the same areas as NMDARs⁵. Importantly, the D-serine binding site on GluN1 is not saturated *in vivo*⁶ even though the endogenous co-agonists glycine and D-serine are present in the extracellular space³. D-serine is important for normal NMDAR functioning, NMDAR-dependent long-term potentiation (LTP), and it is believed to be the primary co-agonist for synaptic, but not extra-synaptic NMDARs⁷⁻¹¹. From studies with mice with a constitutive deletion in SR (SR^{-/-}) mice, D-serine was shown to also be important for maintaining glutamatergic dendritic integrity in the adult cortex and hippocampus^{8, 12-13}.

Over the past two decades, there has been a convergence of pharmacologic, genetic, and biochemical evidence pointing to alterations in excitatory signaling, particularly involving hypofunction of NMDAR, as a key contributor to the schizophrenia disease process¹⁴. Genetic animal models have also provided a wealth of data suggesting that reduced NMDAR activity can lead to changes in the brain and behavior that are similar to what is observed in schizophrenia¹⁵⁻¹⁷. We have demonstrated that mice lacking SR (SR^{-/-}) have reduced D-serine and display NMDAR hypofunction⁹. Similar to what is observed in schizophrenia, SR^{-/-} mice have excitatory neurons with reduced dendritic spines, enlarged lateral ventricles, reduced cortico-hippocampal volume, oxidative stress, and an increased concentration of GABA in the prefrontal cortex, which are associated with learning and cognitive deficits^{8-9, 18-21}.

SR^{-/-} mice also have reduced expression of genes that are dependent on NMDAR activity, such as brain derived neurotrophic factor (BDNF), activity-regulated cytoskeleton-associated protein (Arc), and microRNA-132 (miR-132)^{8, 18}. cAMP-response-element-binding protein (CREB) is a transcription factor ubiquitously expressed in the brain that regulates neuroplasticity by modulating gene expression. The influx of calcium through NMDARs is a well-defined mechanism that leads to the increased expression of CREB-dependent genes²², including BDNF, Arc, and miR-132. Therefore, we were interested in determining whether the amount of CREB bound to the promoter regions of BDNF, Arc, and miR-132 was altered in SR^{-/-} mice.

In order to assess CREB occupancy at various genes from the brains of WT and SR^{-/-} mice, we utilized a chromatin immunoprecipitation (ChIP) assay that can probe protein-DNA interactions within the natural chromatin context of the cell. Before we assessed CREB occupancy between our WT and mutant mice, we first confirmed that the ChIP assay was functioning optimally. Using hippocampal tissue from WT mice, we demonstrated that our digestion produced chromatin fragments of adequate sizes, being digested to 1 to 5 nucleosomes in length (150 to 900 bp; Fig. 1A). We then incubated this DNA with either positive (Histone H3) or negative (rabbit IgG) control antibodies and performed qPCR on the immunoprecipitated and purified DNA using primers (provided by the manufacturer) against the mouse RLP30 gene locus. As shown in Fig. 1B, there was an enrichment of immunoprecipitated DNA compared to input DNA in our positive control antibody, but not negative control antibody sample (Fig. 1B), thereby confirming the specificity of our assay. Thus, we next aimed to determine whether the amount of CREB bound to DNA at various NMDAR activity-dependent genes was altered in SR^{-/-} mice. We have previously

demonstrated that the total levels of CREB protein are unaltered in the hippocampus of SRKO mice⁸.

Aberrant microRNA processing might contribute to the pathophysiology of schizophrenia²³, as the expression of the neuron-enriched miR-132 is reduced in the disorder²⁴. microRNAs are non-coding RNAs that modulate neural plasticity by regulating the translation of target mRNA transcripts. miR-132 regulates basal and activity-induced neurite outgrowth²⁵. The expression of this microRNA is enhanced *in vivo* in response to external stimuli and after NMDAR activation^{23, 26-27}. We first examined the miR132 gene in WT and SR^{-/-} mice. As expected, we did not detect any signal from chromatin incubated with control IgG antibody (Fig. 1C). To validate that our primers were directed against the miR-132 CRE region, we used DNA obtained from CREB antibody ChIP in PCR reactions containing primers directed against a sequence within the miR-132 CRE²⁵ or 800 base pairs upstream of the CRE. We found significantly more immunoprecipitated DNA relative to input with the miR132 CRE primers compared to the upstream CRE primers (Fig. 1C), demonstrating that our primers are targeting the DNA sequence where CREB is binding to miR132 promoter. Using these validated CRE primers, we found that SR^{-/-} mice have approximately 50% less CREB bound at the miR-132 promoter region than WT mice in chromatin isolated from the hippocampus (Fig. 1D; $t(9) = 3.46$, $p < 0.01$).

Recent genetic studies have demonstrated a convergence of genetic variants on a set of synaptic proteins that interact with the protein Arc in subjects with schizophrenia²⁸⁻²⁹. Arc (Arg3.1) is an immediate early gene that is robustly induced by learning and its expression is almost exclusively in excitatory neurons of the hippocampus and neocortex³⁰. The upregulation of Arc mRNA is induced, in part, by calcium influx through NMDARs, where it is trafficked to dendrites and synthesized at synaptic sites³¹, as well as to the nucleus where it suppresses GluA1 transcription³². Arc is also involved in regulating dendritic spines via actin remodeling³³. A major synaptic activity response element (SARE) was identified as a ~100-bp element located at >5 kb upstream of the Arc transcription initiation site in the mouse genome, where CREB binding leads to enhanced transcription³⁴. We found that SR^{-/-} mice had 80% less CREB binding to the SARE region of the Arc gene (Fig. 1E; $t(8) = 3.57$, $p < 0.01$).

Evidence suggests that impaired neurotrophic signaling could underlie the morphological abnormalities in schizophrenia. BDNF regulates numerous aspects of synaptic plasticity and neuronal dendritic fidelity by signaling through tropomyosin receptor kinase B (TrkB), its high-affinity receptor³⁵. BDNF mRNA and protein³⁶⁻³⁸ levels are reduced in the brains of subjects with schizophrenia. The transcription of BDNF is quite complex, as the rodent gene is composed of at least eight untranslated 5' exons and one 3' exon encoding the protein. The different transcripts are expressed by splicing each of the non-coding exons to the coding exon³⁹. This complex structure allows BDNF transcripts to be differentially modulated at subcellular locations and in response to distinct stimuli⁴⁰. Using previously validated exon-specific BDNF primers⁴¹, we found that SR^{-/-} mice had significantly less CREB binding at the BDNF I promoter region (Fig. 1F; $t(9) = 2.88$, $p < 0.05$), but not at the BDNF IV promoter region (Fig. 1F; $t(9) = 1.50$, $p > 0.05$). As the expression of both of these splice variants BDNF (exon I and IV) are induced by neuronal activity³⁹⁻⁴⁰, it is possible

there could be a difference in CREB binding to BDNF pIV in SR^{-/-} mice following stimulation.

Finally, we were interested in determining whether SR^{-/-} mice had a more global deficit in the binding of CREB to the promoter regions of genes whose transcription is enhanced by NMDAR activity. Therefore, we looked at CREB binding to cFos and activating transcription factor 3 (ATF3)⁴². Interestingly, the levels of CREB occupancy on these genes did not significantly differ between WT and SR^{-/-} mice under baseline conditions (Fig. 2A-B; cFos: $t(8) = 1.84$, $p > 0.05$; ATF3: $(8) = 0.93$, $p > 0.05$).

In sum, these data demonstrate that NMDAR hypofunction caused by the selective removal of the NMDAR co-agonist, D-serine, leads to altered CREB binding on known activity-dependent genes. Our ChIP results suggest that reduced CREB binding contributes to the lower levels of BDNF, miR-132, and Arc that we previously observed in SR^{-/-} mice. There are several factors to take into consideration when interpreting these results. First, it is possible that the reduced levels of these targets in SR^{-/-} mice are due to other epigenetic changes in addition to reduced CREB binding, such as histone modifications, methylation, and differential binding of other transcriptional activators or repressors. Second, our ChIP was performed using an antibody against total CREB. The phosphorylation of CREB and other coactivators (i.e. CREB binding protein) affects transcriptional activity²². For example, phosphorylation of CREB at serine 133 is a well-established site that enhances its activity. Third, as we performed ChIP using whole hippocampal homogenate, it is possible that CREB binding could be vary between subfields of the hippocampus, as well as between different brain regions. As our data were collected from naïve animals, it will be of interest to determine whether SR^{-/-} mice show a blunted recruitment of CREB to the promoters of these genes following a stimulus that increases NMDAR activity, such as learning. Finally, future studies will determine whether pharmacological enhancement of NMDAR (i.e D-serine administration) function in SR^{-/-} mice, which reverses these abnormalities at the RNA and protein levels, also normalizes the epigenetic perturbations.

METHODS

Animals

Adult male mice (3-5 months old) on a C57BL/6 background were used for all the experiments. Animals were group housed in polycarbonate cages and maintained on a 12:12 h light/dark cycle in a temperature (22°C) and humidity controlled vivarium. Animals were given access to food and water *ad libitum*. All animal procedures were approved by the McLean Hospital Institutional Animal Care and Use Committee.

Chromatin immunoprecipitation

Wild-type (WT; $n = 5-6$) and SR^{-/-} ($n = 5$) mice were killed and their hippocampi were flash frozen on dry ice. ChIP was performed on hippocampal tissue using the SimpleChIP Plus Enzymatic Chromatin IP kit with magnetic beads (Cell Signaling Technology; Danvers, MA; #9005). Briefly, proteins were crosslinked to DNA using 1.5% formaldehyde (37% stock by weight with methyl alcohol; Fisher Scientific) with rocking for 20min at room

temperature. Adding glycine stopped the crosslinking. The tissue was disaggregated into a single-cell suspension using a dounce homogenizer. The cells were lysed and 0.13 μ l of micrococcal nuclease (37°C with frequent mixing for 16min) was used to digest the chromatin. Nuclear membranes were broken by incubating all the samples simultaneously in a bath sonicator (amplitude = 80) for 6x 20s (30s in between sonications on ice). Chromatin was incubated with rabbit anti-CREB (CST #9197) antibody at 1:50 dilution overnight at 4°C on a rotisserie. A 2% input aliquot was removed from each sample. The remaining chromatin was incubated with Protein G magnetic beads, followed by low and high-salt washes. Chromatin was eluted from the beads using Elution Buffer (heat at 65°C for 5min then on rotisserie for 25min). The cross-links were reversed using Proteinase K and incubating for 2h at 65°C. The DNA was then purified using the kit-supplied columns.

qPCR

To determine the levels of CREB bound at each gene of interest, PCR primers were directed near the CRE or SARE sequence of each promoter. A list of the primer sequences is included in the Supplemental Table S1. A serial dilution of chromatin DNA was used to generate a standard curve (power regression fitting) and determine the efficiency of amplification. A 2 μ l aliquot of each input and IP sample was used for qPCR (Platinum Sybr Green qPCR SuperMix-UDG; Invitrogen), with a total reaction volume of 20 μ l. Each standard and sample was run in triplicate and melting temperature analysis was performed after every PCR to ensure accuracy. The C_t values for each IP and input sample were normalized using the standard curve. The averaged normalized values were used to calculate the fold-enrichment for each animal (average normalized IP / average normalized Input $\times$ 2). The fold-enrichment values were then averaged for each genotype and used for statistical analyses. Unpaired student's *t* test was used to compare values between experimental groups. Values of $P < 0.05$ were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Arc	activity-regulated cytoskeleton-associated protein
BDNF	brain derived neurotrophic factor
ChIP	chromatin immunoprecipitation
CRE	cAMP-response-element
CREB	cAMP-response-element-binding protein

miR-132	microRNA-132
NMDAR	<i>N</i> -methyl-D-aspartate receptors
SR	serine racemase
WT	wild-type

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Figure 1.

Reduced CREB occupancy to the *cis* regulatory regions of the miR-132, BDNF, Arc genes in the hippocampus of SR^{-/-} mice. (A) Representative agarose gel demonstrating proper chromatin digestion from wild-type hippocampal tissue. (B) Positive control (histone H3 antibody) showing RLP30 intron 2 enrichment compared to a negative control rabbit IgG antibody. (C) Validation of the CRE site in the miR-132 gene showing more enrichment using primers directed to the CRE sequence in the promoter region compared to ones directed 800bp upstream from it (white bars; CREB antibody). No enrichment was detected when the negative control antibody was used for IP (black bars; IgG). (D) Relative amount of CREB bound to the miR-132 promoter in the hippocampus of WT (n = 6; black bar) and SR^{-/-} (n=5; white bar) mice. (E) Relative amount of CREB bound to the synaptic activity response element (SARE) of the Arc gene in the hippocampus of WT (n = 6; black bar) and SR^{-/-} (n=5; white bar) mice. (F) Relative amount of CREB bound to the promoter regions of BDNF exons I (pI) and IV (pIV) in the hippocampus of WT (n = 6; black bar) and SR^{-/-} (n=5; white bar) mice. (B-F) Values are expressed as signal relative to the total amount of input chromatin. Asterisk (*) indicates significant differences from WT (p < 0.05). All values represent the mean ± SEM.

Figure 2.

CREB binding at the promoter regions of other activity-dependent genes is unaltered in SR^{-/-} mice. (A) CREB promoter occupancy on the cFOS and (B) ATF3 genes was quantified in the hippocampus of WT (n = 6; black bars) and SR^{-/-} (n=5; white bars) mice. Values are expressed as signal relative to the total amount of input chromatin. All values represent the mean $\pm$ SEM.