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Imipramine Treatment and Resiliency Exhibit Similar Chromatin Regulation in the Mouse Nucleus Accumbens in Depression Models

Matthew B. Wilkinson,[1]* Guanghua Xiao,[2]* Arvind Kumar,[3]* Quincey LaPlant,[1] William Renthal,[2] Devanjan Sikder,[4] Thomas J. Kodadek,[4] and Eric J. Nestler[1]

[1]Fishberg Department of Neuroscience, Mount Sinai School of Medicine, New York, New York 10029-6574, Departments of [2]Clinical Sciences, [3]Psychiatry, and [4]Internal Medicine and Division of Translational Research, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390

Although it is a widely studied psychiatric syndrome, major depressive disorder remains a poorly understood illness, especially with regard to the disconnect between treatment initiation and the delayed onset of clinical improvement. We have recently validated chronic social defeat stress in mice as a model in which a depression-like phenotype is reversed by chronic, but not acute, antidepressant administration. Here, we use chromatin immunoprecipitation (ChIP)-chip assays—ChIP followed by genome wide promoter array analyses—to study the effects of chronic defeat stress on chromatin regulation in the mouse nucleus accumbens (NAc), a key brain reward region implicated in depression. Our results demonstrate that chronic defeat stress causes widespread and long-lasting changes in gene regulation, including alterations in repressive histone methylation and in phospho-CREB (cAMP response element-binding protein) binding, in the NAc. We then show similarities and differences in this regulation to that observed in another mouse model of depression, prolonged adult social isolation. In the social defeat model, we observed further that many of the stress-induced changes in gene expression are reversed by chronic imipramine treatment, and that resilient mice—those resistant to the deleterious effects of defeat stress—show patterns of chromatin regulation in the NAc that overlap dramatically with those seen with imipramine treatment. These findings provide new insight into the molecular basis of depression-like symptoms and the mechanisms by which antidepressants exert their delayed clinical efficacy. They also raise the novel idea that certain individuals resistant to stress may naturally mount antidepressant-like adaptations in response to chronic stress.

Introduction

Depression, a common debilitating illness, is a heterogeneous syndrome comprised of multiple subtypes that remain poorly defined (Krishnan and Nestler, 2008). Although many brain regions are implicated in depression, our laboratory has focused on the nucleus accumbens (NAc) as one substrate for depressive-like behavior in rodent models. Cellular and molecular manipulations within this brain region exert dramatic effects in animal models of depression (Willner, 1983; Zacharko and Anisman, 1991; Willner et al., 1992; Nestler and Carlezon, 2006), and there is increasing evidence for abnormalities within the NAc in human depression (Tremblay et al., 2005; Krishnan et al., 2007, 2008).

We have validated a chronic social defeat stress paradigm in mice, which causes depressive-like symptoms that are reversed by chronic, but not acute, administration of antidepressant medications (Berton et al., 2006; Tsankova et al., 2006; Krishnan et al., 2007). Interestingly, inbred mice exhibit two distinct responses to chronic defeat stress: a susceptible response characterized by depressive symptoms as well as an unsusceptible response where the animals appear "resilient," avoiding most deleterious effects of defeat stress. This model can be used to study naturally occurring variations in vulnerability to chronic stress. Contrasting with defeat stress, a type of "active stress," we have demonstrated that prolonged social isolation of adult mice, a form of "passive stress," induces depression-like behavior, although with partly distinct symptoms seen under these two conditions (Wallace et al., 2009). Chronic defeat stress and prolonged social isolation could model subtypes of human depression or, alternatively, show phenotypic differences resulting from the differential severity of the stressors involved (Antonijevic, 2008).

We set out to characterize long-lasting changes in gene transcription within the NAc in these two depression models. We used chromatin immunoprecipitation (ChIP)-chip assays that provide a genome-wide view of chromatin regulation at gene promoters (Renthal et al., 2009). We analyzed dimethylation of Lys9 and Lys27 in histone H3 (dimethylK9/K27-H3), well characterized repressive modifications associated with reduced gene expression (Felsenfeld and Groudine, 2003; Kouzarides, 2007). We also analyzed binding of the transcriptionally active form of

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*M.B.W., G.X., and A.K. contributed equally to this manuscript.

Correspondence should be addressed to Eric J. Nestler, One Gustave L. Levy Place, Box 1065, New York, NY 10029-6574. E-mail: eric.nestler@mssm.edu.

A. Kumar's present address: Centre for Cellular and Molecular Biology, Habsiguda, Uppal Road, Hyderabad 500 007, A.P., India.

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cAMP response element binding protein (CREB), phospho-CREB, since increased CREB activity within the NAc induces depression-like behavior in several models (Pliakas et al., 2001; Barrot et al., 2002; Newton et al., 2002; Carlezon et al., 2005; Blendy, 2006). Using the social defeat model, we also used the dimethylK9/K27-H3 and phospho-CREB markers of transcriptional regulation to analyze changes in chromatin structure associated with antidepressant treatment as well as with the susceptible versus resilient phenotypes.

These approaches make it possible for the first time to analyze the stable epigenetic changes that occur in brain in depressive-like states and in response to treatment. Our findings indicate that imipramine treatment and resiliency share a common set of regulated genes, supporting the notion that certain individuals are naturally resistant to stressors in the environment (Charney, 2004; Charney and Manji, 2004; Yehuda, 2004; Hoge et al., 2007), in part by mounting active responses that overlap with those induced by antidepressant treatment.

Materials and Methods

Social defeat and imipramine treatment. Male C57BL/6ByJ (hereafter, C57) mice, 7–9 weeks old, were obtained from Jackson Laboratory and housed on a 12 h light/dark cycle with access to food and water *ad libitum*. Male CD1 retired breeder mice (9–13 months old) were obtained from Charles River Laboratories. Animals were permitted to acclimate to the facility for 1 week before experimentation. Mice were subjected to chronic social defeat exactly as described previously (Berton et al., 2006; Krishnan et al., 2007). Briefly, CD1 mice were screened for aggressive behavior after intrusion into their home cages. Those mice exhibiting aggression were used to defeat the C57 mice. The C57 mice were defeated for 10 min per day and then housed for the remainder of the day with the aggressor but separated by a Plexiglas screen. This process was repeated daily for 10 consecutive days with a different CD1 aggressor each day to minimize interaggressor variability. Control animals were housed in divided cages with another C57 mouse. On day 11, defeated animals were subjected to the social interaction test and sorted into either susceptible or unsusceptible (resilient) phenotypes based on interaction scores as described (Krishnan et al., 2007).

Mice were then divided into treatment groups. For each group (control, susceptible, unsusceptible/resilient), one-half of the animals received daily intraperitoneal injections of imipramine (20 mg/kg) for 28 d. The other half in each group received vehicle. Previous work has shown that this imipramine treatment paradigm completely reverses the social interaction deficit seen in susceptible animals, which otherwise persists unabated in vehicle-treated susceptible animals (Berton et al., 2006; Tsankova et al., 2006). All mice were killed 24 h after the last imipramine or vehicle injection, and NAc dissections were obtained by punch dissection as described previously (Kumar et al., 2005). We did not analyze tissue from the nondefeated control mice and resilient mice treated with imipramine since no behavioral consequences of imipramine have been seen under these conditions (Berton et al., 2006; Tsankova et al., 2006).

Social isolation. Male C57 mice, 7–9 weeks old, were housed on a 12 h light/dark cycle with access to food and water *ad libitum*. Animals were permitted to acclimate to the facility for 1 week before experimentation. Test animals were housed individually for 8 weeks, whereas control animals were group housed for the same period of time. This paradigm has been shown to induce a range of depression- and anxiety-like symptoms (Wallace et al., 2009). After the 8 week treatment period, NAc samples were obtained as above.

Chromatin immunoprecipitation and microarray analysis. ChIP was performed for dimethylK9/K27-H3 and for phospho-CREB as described previously (Kumar et al., 2005). Briefly, for each ChIP, bilateral 14 gauge NAc punches were pooled from approximately eight mice, the tissue was lightly fixed to cross-link DNA with associated proteins, and the material was sheared and immunoprecipitated with an antibody that recognizes dimethylK9/K27-H3 or phospho-CREB, and then repaired and amplified (Sikder et al., 2006). Resulting immunoprecipitated DNA and total (input) genomic DNA were labeled with Cy5 and Cy3 fluorescent dyes, respectively. Independent, duplicate samples were then hybridized to NimbleGen mouse promoter arrays that span ~20,000 genes. The resulting array data were subjected to rigorous statistical analysis (see supplemental Materials and Methods, available at www.jneurosci.org).

Briefly, data were initially preprocessed to $\log_2$ ratios and scaled using Tukey's Biweight Method to prevent inclusion of outliers in the final data set. Using median normalization and the floor method, a final data set was created for analysis. Significant alterations between treatment and control conditions were determined by first averaging the mean signal intensity for the mark of interest at each probe across the replicated samples. Since the gene array is tiled, a moving average that took into account three adjacent probes on the chip was used to stabilize the signal, and the p value for each probe was calculated using the ChIPOTle algorithm. False discovery rates for H3 methylation were determined by fitting the p values with a β-uniform mixture model. This method provides an acceptable false discovery rate that is in line with our previous work, as well as the work of others (Renthal et al., 2009). By only taking those probes with p values <0.001 (i.e., >3.1 SDs from the mean), false discovery rates are calculated at 0.18 and 0.16 for repressive histone methylation in social defeat and social isolation, respectively. We have previously shown that this method of data analysis allows us to estimate true false positive rates. (Renthal et al., 2009). Moreover, the overlap rate for the independent data sets in these experiments is in line with other published work on false discovery rates (Pyne et al., 2006), further alleviating concerns with regard to false discovery of genes. Overlap between the replicates was also calculated as a quality control measure. On average, the duplicates in each case showed a 70–80% similarity with Pearson's correlations of 0.6–0.9.

Promoter plots and genome-wide spatial binding patterns. We used mpeak software (Zheng et al., 2007) to derive the signal peaks and overall spatial pattern of the locations of dimethylK9/K27-H3 and phospho-CREB binding relative to transcriptional starting sites. The empirical densities of the peak location were plotted and compared between social defeat (or isolation) condition and control condition. The empirical densities were estimated using R statistical software with all default settings (adjust = 1). Then, the bandwidth was determined by R density function automatically according to the number of enrichment probes, identified from the mpeak package. The bandwidths for H3 methylation and phospho-CREB binding are 271 and 623, respectively, in social defeat. The bandwidths for H3 methylation and phospho-CREB binding are 315 and 649 respectively, in social isolation.

Heat maps. The heat maps included in Figures 1–4 depict the signal strength averaged across probes for each gene. Genes listed in the Tables are those with >1.1-fold change in social defeat or isolation that were also present as significantly changed in DNA expression arrays form our previous work (Berton et al., 2006; Krishnan et al., 2008; Wallace et al., 2009). The genes were rearranged using the Spearman hierarchical clustering method (Genespring).

Results

Repressive histone methylation is regulated in two mouse models of depression: evidence of similarly regulated genes

As a first step toward characterizing epigenetic regulation associated with depression, we mapped the entire genome of mice that were subjected to one of two recently validated models of chronic stress, chronic social defeat (Berton et al., 2006; Tsankova et al., 2006; Krishnan et al., 2007) or prolonged adult social isolation (Wallace et al., 2009), for levels of dimethylK9/K27-H3 in the NAc. This dimethylation mark has been shown to be a highly reliable indicator of gene repression in many systems (Felsenfeld and Groudine, 2003; Kouzarides, 2007). For this first set of social defeat analyses, only animals that exhibited a susceptible/defeated phenotype—as measured by a social avoidance scale (Berton et al., 2006)—on day 11, the day after 10 d of chronic defeat, were used. Mice were then analyzed 28 d later, a time when the avoidance phenotype remains stable. Control animals received

nonaggressive social interactions as described previously (Berton et al., 2006). Socially isolated animals were subjected to isolated housing for 8 weeks compared with group-housed controls.

In both social defeat and social isolation animals, compared with their respective controls, we observed a significant difference ($p < 0.001$) in the relative levels of dimethylK9/K27-H3 in regions of gene promoters immediately upstream to their start sites (−2000–0 bp), as evidenced by the curves in Figure 1*A*. Note that these data are averaged across the entire genome, which lends credence to the idea that there are significant and far-reaching changes that occur in the NAc at a genomic level in response to either form of chronic stress.

Heat maps were generated from these data using Genespring software (Agilent Technologies) to visually represent the enrichment or attenuation of dimethylK9/K27-H3 at individual gene promoters (Fig. 1*C*). For socially defeated mice, a total of 1285 genes showed increased H3 methylation (supplemental Table S1, available at www.jneurosci.org as supplemental material), whereas 799 exhibited decreased methylation (supplemental Table S2, available at www.jneurosci.org as supplemental material). In the social isolation model, 1448 genes showed increased methylation (supplemental Table S3, available at www.jneurosci.org as supplemental material), and 615 displayed decreased methylation (supplemental Table S4, available at www.jneurosci.org as supplemental material). We identified 436 genes—~20% of the total (supplemental Table S5, available at www.jneurosci.org as supplemental material)—that displayed alterations in H3 methylation (increased or decreased) ($p < 0.001$) in response to chronic social defeat and social isolation.

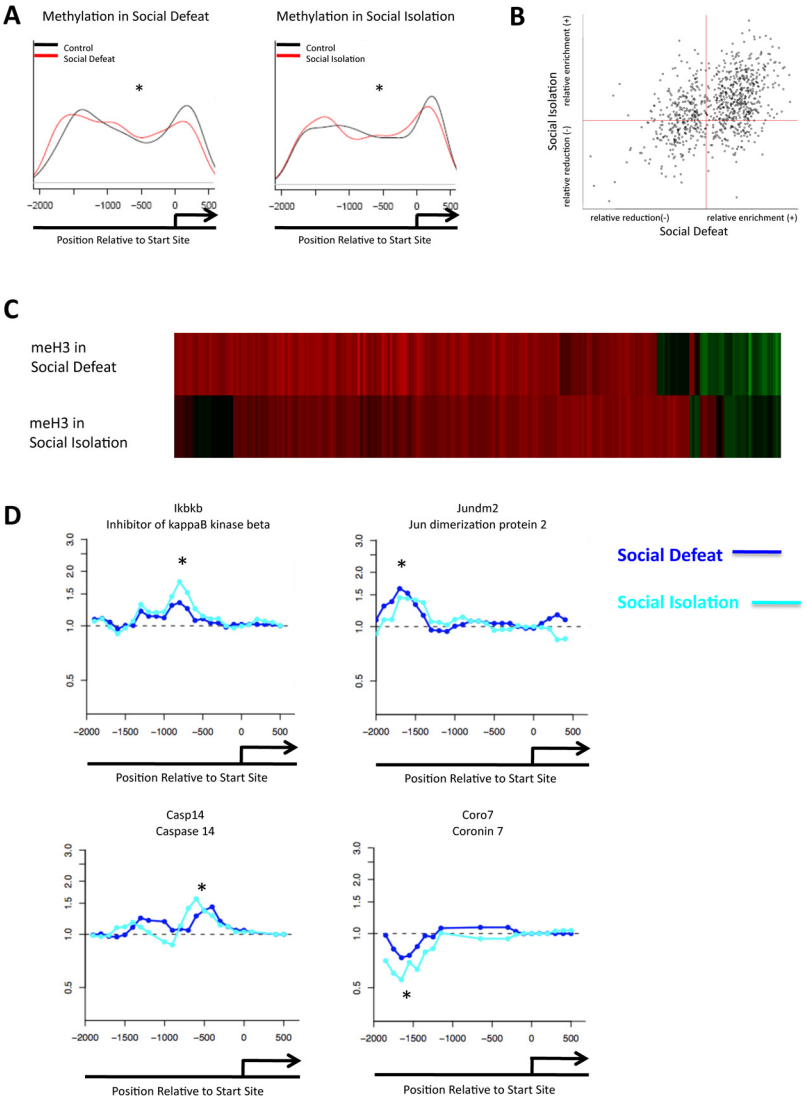

Figure 1. Similar regulation of H3 methylation in two mouse models of depression. ***A***, Promoter plots show significant alterations in repressive dimethylK9/K27-H3 methylation in the NAc between control animals and those subjected to chronic social defeat or chronic social isolation. ***B***, Positive correlation is seen between the two models for H3 methylation ($p < 0.0001$). ***C***, The top heat map illustrates the fold change of H3 methylation in social defeat, compared with the fold change of H3 methylation of the same genes in the social isolation model. ***D***, Patterns of H3 methylation at representative gene promoters. Light blue is social isolation; dark blue is social defeat. *$p < 0.05$.

Two clear results were apparent from these analyses. First, the vast majority of these regulated genes exhibited an increase in dimethylK9/K27-H3, which would be expected to correspond to a decrease in gene transcription. Moreover, the majority of genes (~75%) were regulated in a similar manner in social defeat and in social isolation (i.e., those genes that showed increased H3 methylation in social defeat also showed increased H3 methylation in social isolation). Of these, 265 genes showed increased H3 methylation in both models (supplemental Table S6, available at www.jneurosci.org as supplemental material), whereas 60 genes showed a decrease (supplemental Table S7, available at www.jneurosci.org as supplemental material) ($p < 0.001$). Figure 1*B* depicts this information graphically. The positive correlation ($p < 0.0001$) between enrichment and attenuation of dimethylK9/K27-H3 binding between the two depression models suggests that social defeat and social isolation involve many similar gene regulatory events in terms of this repressive mark despite the very different type of stress involved. This is consistent with the fact that the two models induce some similar behavioral abnormalities such as depressive symptoms (e.g., anhedonia) and anxiety-like behavior (Krishnan et al., 2007; Wallace et al., 2009).

Genes that showed interesting patterns of methylated H3 regulation in these depression models were studied by Ingenuity molecular pathway analysis. Full lists of regulated genes with fold changes in methylation status are provided in supplemental Tables S1–S7, available at www.jneurosci.org as supplemental material. Interestingly, many of the genes that display increased H3 methylation in social defeat and social isolation are those involved in either inflammatory or cell death pathways (Table 1*A*). For example, inhibitor of κ-B kinase β (encoded by *Ikbkb*) usually allows for increased nuclear factor κB (NF-κB) signaling by causing degradation of inhibitor of κ-B (IKB), which normally constitutes a molecular brake on the system. Increased H3 methylation at the *Ikbkb* promoter (Fig. 1*D*) may contribute to a decreased ability to activate this pathway, as reduced *Ikbkb* expression would be associated with higher levels of IKB (Schmid and

Table 1. Genes demonstrating similar dimethylK9/K27-H3 states in both stress models

Genes	Gene activity	Reference
A		
Aktip/Ft1	General regulator of apoptosis via PKB/Akt pathway	Remy and Michnick, 2004
Casp14	Expressed during ischemic neuronal cell death	Krajewska et al., 2004
CD84	Enhances Ifn-γ secretion during inflammatory responses	Yan et al., 2007
Fcrlb	Involved in cell-mediated cytotoxicity, inflammation, and hypersensitivity	Daëron, 1997
Gstm4	Reduces oxidative stress damage by inactivating reactive intermediates	Hayes et al., 2005
Ikbkb	Activity allows for activation NfκB-dependent pathways	Schmid and Birbach, 2008
IL-16	Marker of microglial activation in inflammatory states	Guo et al., 2004
Il1rl1	Involved in induction and signaling in TNFα, IL-2, IL-6, IL-12, and IL-18 pathways	Subramaniam et al., 2004
IL8rα	Increased expression following inflammatory stimuli (LPS)	Lee et al., 2002
NEDD9	Implicated in NFκB signaling through Tax	Singh et al., 2007
Sep-15	Possesses anti-oxidant properties; dysregulation is implicated in neurodegenerative disorders	Chen and Berry, 2003; Ashrafi et al., 2007
SLAM7	Involved in NK cytotoxity and B-cell activation	Calpe et al., 2008
SLAM9	Unknown function; homology suggests similar function to SLAM7	Calpe et al., 2008
B		
Fgd5	RhoGEF predicted to be involved in actin remodeling via Rac-Pac pathways	Rossman et al., 2005; Buchsbaum, 2007
Sorbs3/vinexin	Involved in cell– cell adhesion and actin dynamics	Kioka et al., 1999
C		
Jdp2/Jundm2	Binding to c-jun causes transcriptional repression via binding to AP-1 sites; binding to ATF-2 causes transcriptional repression via effects on HDAC3	Eitoku et al., 2008
Mcm8	Required for eukaryotic genome replication	Maiorano et al., 2006
Spic	Transcription factor similar to Pu0.1 involved in B-cell development; other transcriptional functions unclear	Bemark et al., 1999
TFIIF	Part of the required core of transcriptional initiation machinery	Thomas and Chiang, 2006

A, Genes implicated in cellular inflammation, death, or redox reactions; *B*, genes implicated in cellular plasticity; *C*, genes implicated in gene regulation. LPS, Lipopolysaccharide.

Birbach, 2008). Another gene of interest is *Sep-15*, which encodes a selenoprotein. Its primary function is to serve as an antioxidant in cells, thereby removing toxic intermediates (Chen and Berry, 2003; Ashrafi et al., 2007). This gene is dysregulated in several neurodegenerative disorders such as Alzheimer's disease. Increased H3 methylation at this gene promoter may contribute to a decreased ability of NAc neurons to deal with oxidative stress after exposure to chronic social defeat or social isolation stress.

Also prominent among gene promoters in which H3 methylation is regulated similarly after social defeat and social isolation are those involved in the regulation of gene transcription (Table 1*C*). One example is Jun dimerization protein 2 (*Jundm2/Jdp2*) (Fig. 1*D*). The binding of this protein to its partner c-Jun allows the complex to bind to AP-1 responsive genes and mediate transcriptional repression. Moreover, Jdp2 causes additional repressive effects on transcription via binding to ATF-2 (activating transcription factor 2) (Eitoku et al., 2008). Consequently, a decrease in the availability of the *Jdp2* gene for transcription would result in "de-repression" of AP-1 and ATF-2 regulated genes.

Opposite regulation of phospho-CREB binding in two mouse models of depression

In addition to studying H3 methylation, we analyzed levels of phospho-CREB binding genome-wide in the same extracts of NAc of mice subjected to chronic social defeat or social isolation stress. This analysis was of interest given the fact that these two depression models are associated with opposite changes in phospho-CREB levels in the NAc, despite the fact that some common depressive symptoms are seen. Thus, active forms of stress like social defeat are associated with increased levels of phospho-CREB in the NAc, which has been shown to induce depression-like behaviors (Pliakas et al., 2001; Barrot et al., 2002; Newton et al., 2002; Carlezon et al., 2005; Blendy, 2006), whereas prolonged social isolation decreases phospho-CREB levels in this brain region (Wallace et al., 2009). ChIP-chip has previously been used to characterize CREB binding to gene promoters in brain in response to electroconvulsive seizures (Tanis et al., 2008), but not yet applied to depression models.

In contrast to the similar genome-wide regulation of H3 methylation observed in social defeat and social isolation, phospho-CREB analysis revealed strikingly different results. Furthermore, whereas changes in H3 methylation were confined primarily to the transcription start site of the genes, phospho-CREB binding was seen throughout the promoter region as far as 2000 bp upstream. As with the repressive methylation mark, phospho-CREB binding was significantly altered when averaged across all genes in the region immediately upstream to their start sites. Whereas social defeat increased phospho-CREB binding close to the start site and decreased it further upstream, social isolation decreased phospho-CREB binding around the region of the start site and increased binding further upstream. These data (shown in Fig. 2*A*) are averaged across the entire genome and provide a global view of the widespread epigenetic changes that occur in the NAc in these two depression models. Figure 2*B* illustrates the negative correlation between the social defeat and social isolation paradigms in terms of phospho-CREB binding as opposed to the positive correlation found for dimethylK9/K27-H3 binding (see Fig. 1*B*).

Genes that showed altered levels of phospho-CREB binding after chronic social defeat are listed in supplemental Tables S8 and S9, available at www.jneurosci.org as supplemental material, whereas those that showed altered levels of phospho-CREB binding after chronic social isolation are listed in supplemental Tables S10 and S11, available at www.jneurosci.org as supplemental material ($p < 0.0001$). Heat maps were generated to visually represent the enrichment or attenuation of phospho-CREB binding at these individual gene promoters (Fig. 2*C*). In total, 473 genes (supplemental Table S12, available at www.jneurosci.org as sup-

plemental material) exhibited altered phospho-CREB binding in both the social defeat and social isolation model ($p < 0.001$). Although a larger number of genes exhibited changes in one model or the other, we focused on those that were altered in both. Confirming the negative trend seen globally (Fig. 2*B*), we found that 346 genes (~73%) of these genes exhibited opposite changes in phospho-CREB binding in social defeat versus social isolation. Figure 2*D* illustrates representative examples of these genes.

Molecular pathway analysis indicated that similar gene families showed altered phospho-CREB binding after chronic stress as found for altered dimethylK9/K27-H3 binding, including those involved in inflammation, cell death, redox state, or gene regulation (Table 2). For instance, *Ndufa3* and *Ndufa5* are subunits of NADH [nicotinamide adenine dinucleotide (reduced form)]:ubiquinone oxireductase (Loeffen et al., 1998) and *Gstm4* and *Gstm5* are capable of reducing oxidative stress by consuming reactive intermediates (Hayes et al., 2005). All four show altered phospho-CREB binding. This corresponds with the regulation of H3 methylation at the *Gstm4* and *Sep-15* gene promoters. Additionally, genes such as *Edar*, the product of which is capable of activating the NF-κB pathway, exhibit altered phospho-CREB binding (Kumar et al., 2001). As these changes are in the opposite direction in social defeat versus social isolation, CREB regulation of these inflammatory pathways presumably proceeds differently in these two models.

Several other genes that are differentially regulated via phospho-CREB after chronic social defeat and chronic social isolation regulate the neuronal cytoskeleton (Table 2*B*). Several encode proteins that are involved in the reorganization of actin (*Ank3*, *Arghef9*, *Kptn*, and *Lasp1*). The products of some of these genes function in cell–cell signaling as well. The product of *Ank3* regulates sodium channel inactivation (Shirahata et al., 2006), whereas that of *Lasp3* accumulates in the cell membrane and contains an SH3 domain that operates in signal transduction pathways (Schreiber et al., 1998).

Last, numerous genes that show altered phospho-CREB binding after social defeat or social isolation encode proteins that themselves control gene activation and expression (Table 2*C*). The product of *Cxxc1* is capable of binding to unmethylated regions of DNA and serving as a gene activator (Voo et al., 2000). Several genes (*EII*, *Gtf2f2*, *Gtf2h3*, and *Med31*) that display altered phospho-CREB binding encode proteins that are part of the general transcription machinery (Armache et al., 2005; Guglielmi et al., 2007). This suggests that CREB in the NAc acts as a large scale genomic "switch," which orchestrates a variety of transcriptional changes in response to environmental perturbations.

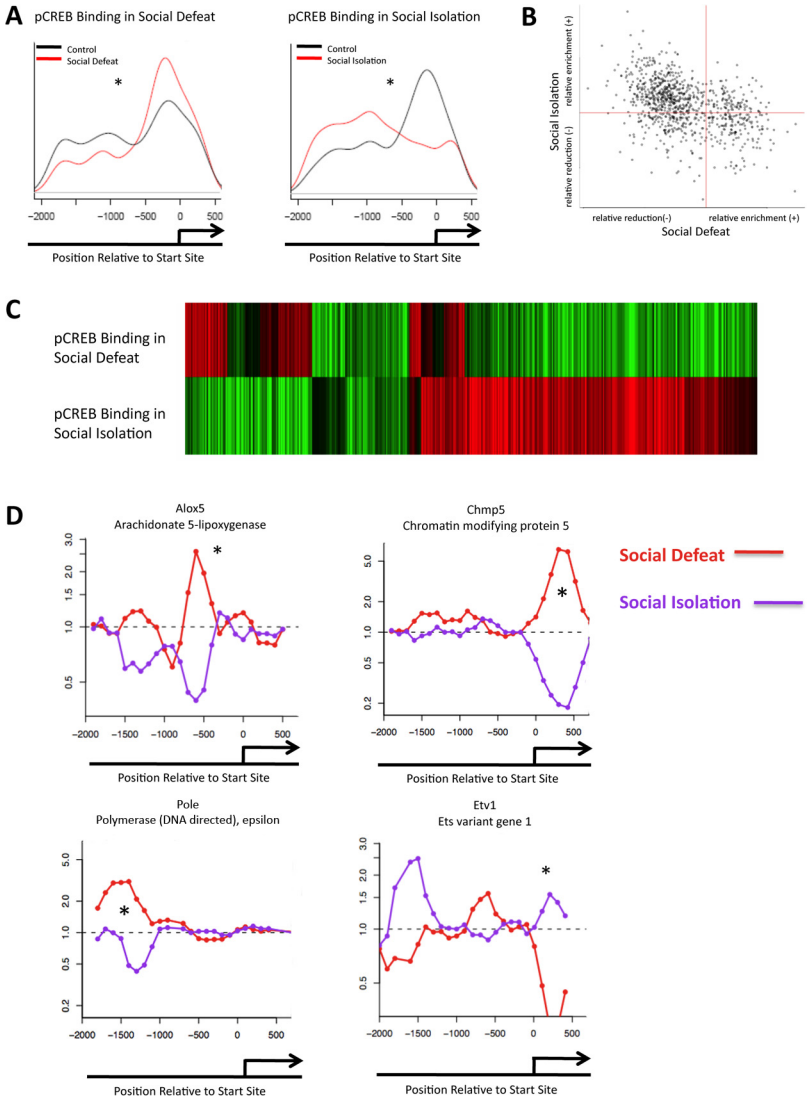

Figure 2. Opposite regulation of phospho-CREB binding in two mouse models of depression. ***A***, Promoter plots show significant alterations in phospho-CREB binding in the NAc between control animals and those subjected to chronic social defeat or chronic social isolation. ***B***, Negative correlation is seen between the two models for phospho-CREB binding ($p < 0.0001$). ***C***, The top heat map illustrates the fold change of phospho-CREB binding in social defeat, compared with the fold change of phospho-CREB binding of the same genes in the social isolation model. ***D***, Patterns of phospho-CREB binding at representative gene promoters. Red is social isolation; purple is social defeat. *$p < 0.05$.

Imipramine treatment reverses the histone methylation and phospho-CREB changes induced by social defeat

Having established changes induced by chronic social defeat in dimethylK9/K27-H3 and phospho-CREB binding in the NAc, we were next interested in determining the effect of chronic imipramine treatment, which is known to reverse the behavioral deficits associated with this model (Berton et al., 2006; Tsankova et al., 2006). In this experiment, we built on the data shown in Figures 1 and 2, which show chromatin changes in chronically defeated mice 28 d after the last defeat; these animals received vehicle injections. Animals were now analyzed that displayed equivalent levels of social defeat on day 11 but were treated with imipramine for this 28 d period to reverse the behavioral deficits.

Figure 3*A* shows that, consistent with the behavioral reversal, a large portion of H3 methylation changes induced by social defeat are reversed by chronic imipramine treatment. The top heatmap in Figure 3*A* shows the stable H3 methylation changes apparent in the NAc 4 weeks after chronic social defeat in the

Table 2. Genes demonstrating opposite pCREB binding in both stress models

Genes	Gene activity	Reference
A		
⇓ In defeat ⇑ in isolation		
Alox5/5-LO	Catalyzes the first step in leukotriene biosynthesis	Murphy and Gijón, 2007
Etv/ERM	Involved in PKC signaling to CD44 and ICAMs	Larsson, 2006
Ndufa3	Subunit of NADH:ubiquinone oxireductase	Loeffen et al., 1998
Ndufb5	Subunit of NADH:ubiquinone oxireductase	Loeffen et al., 1998
Sep-15	Possesses anti-oxidant properties; dysregulation is implicated in neurodegenerative disorders	Chen and Berry, 2003; Ashrafi et al., 2007
Thap1	Zinc finger protein; pro-apoptotic factor; also implicated in interactions with chromatin remodeling enzymes	Bessière et al., 2008
⇓ In isolation ⇑ in defeat		
CD5l	Unknown; inflammatory by similarity	
Edar	Receptor capable of activating NfκB, JNK, and caspase independent death pathways	Kumar et al., 2001
Gstm4	Reduces oxidative stress damage by inactivating reactive intermediates	Hayes et al., 2005
Gstm5	Reduces oxidative stress damage by inactivating reactive intermediates	Hayes et al., 2005
IL-2	Pro-inflammatory cytokine implicated in depression via activation of degradation pathways for W and 5-HT	Müller and Schwarz, 2007
Klrb1b	Inhibitor of NK cell-mediated cytotoxicity	Calpe et al., 2008; Lanier et al., 1994
B		
⇓ In defeat ⇑ in isolation		
Ank3	Involved in linking spectrin/actin network to the cytoplasmic part of integral membrane proteins; involved in neuronal sodium channel inactivation	Shirahata et al., 2006
Ankra2	Identified as a binding partner for class II HDACs	Wang et al., 2005
Arhgef9	Critically involved in neuronal gephyrin clustering and actin dynamics	Harvey et al., 2004
Dctn3	Smallest subunit of dynactin; involved in dynein transport, spindle formation, chromosomal organization, protein transport	Karki et al., 1998
Dync1li2	Subunit of dynein; may play roles similar to that for dynactin	Karki et al., 1998
Etv5/ERM	Involved in PKC signaling to allow microfilament rearrangement	Larsson, 2006
Kptn	Actin binding protein involved with cytoskeletal rearrangements in platelets	Bearer and Abraham, 1999
Lasp1	Actin binding protein involved in cystoskeletal remodeling and extension of cellular outgrowths; contains SH3 domain	Schreiber et al., 1998
C		
⇓ In defeat ⇑ in isolation		
Ankra2	Identified as a binding partner for class II HDACs	Wang et al., 2005
Cxxc1	Binds to unmethylated CpG regions of DNA; acts as a gene activator	Voo et al., 2000
Ddx28	RNA helicase; involved in transcription and translation	Abdelhaleem, 2005
Ddx42	RNA helicase; involved in transcription and translation	Abdelhaleem, 2005
E4f1	Transcription factor regulated by E1A	Le Cam et al., 2004
Ell	Elongation factor of RNA polymerase II	Armache et al., 2005
FoxN3	Member of fork head/ winged transcription factors	Katoh and Katoh, 2004
Gabpb2	Transcription factor; capable of binding to purine rich regions of genome	Muller and Schwarz, 2007; Le Cam et al., 2004
Gtf2f2	General transcription factor; binds to RNA pol II	Armache et al., 2005
Gtf2h3	Integral member of the TFIIH transcription factor involved in NER	Armache et al., 2005
Lmo4	Transcription factor with GC rich regions; required for hematopoeisis	Ferreira et al., 2005
Med31	Part of the mediator complex; involved in the transcription of almost all RNA pol II dependent genes	Guglielmi et al., 2007
Nolc1	Involved in nucleolar organization and genes transcribed by RNA pol I	Tsai et al., 2008
Pola1	DNA polymerase	Loeb and Monnat, 2008
Pole	DNA polymerase	Loeb and Monnat, 2008
Rnaseh2a	Ribonuclease; implicated in neuroinflammatory disorders	Rigby et al., 2008
Smad4	Involved in TGF-β signaling via binding to DNA along with SMAD1 or 2	Ross and Hill, 2008
Sox12	Group C Sry-HMG related transcription factor	Dy et al., 2008
Sox17	Sry-HMG related transcription factor important in early development	Jang and Sharkis, 2007
Sox4	Group C Sry-HMG related transcription factor	Dy et al., 2008
Sp1	Transcription factor capable of binding to GC rich domains	Li et al., 2004
Tsen 34	One of two subunits important for tRNA splicing and pre-mRNA 3′-end formation	Paushkin et al., 2004
⇓ In isolation ⇑ in defeat		
Ints7	Subunit of the integrator complex which associates with RNA pol II	Nakagawa et al., 2008
Mcm3	Required for eukaryotic genome replication	Maiorano et al., 2006
Msx2	Transcriptional repressor of cells derived from the neural crest	Ramos and Robert, 2005
Tmem34	Possible tumor suppressor gene	Akaishi et al., 2007

A, Genes implicated in cellular inflammation, death, or redox reactions; *B*, genes implicated in cellular plasticity; *C*, genes implicated in gene regulation. Up arrows indicate increased pCREB binding. Down arrows indicate decreased pCREB binding. HDAC, Histone deacetylase; HMG, high mobility group; NK, natural killer; NER, nucleotide excision repair; JNK, c-Jun N-terminal kinase.

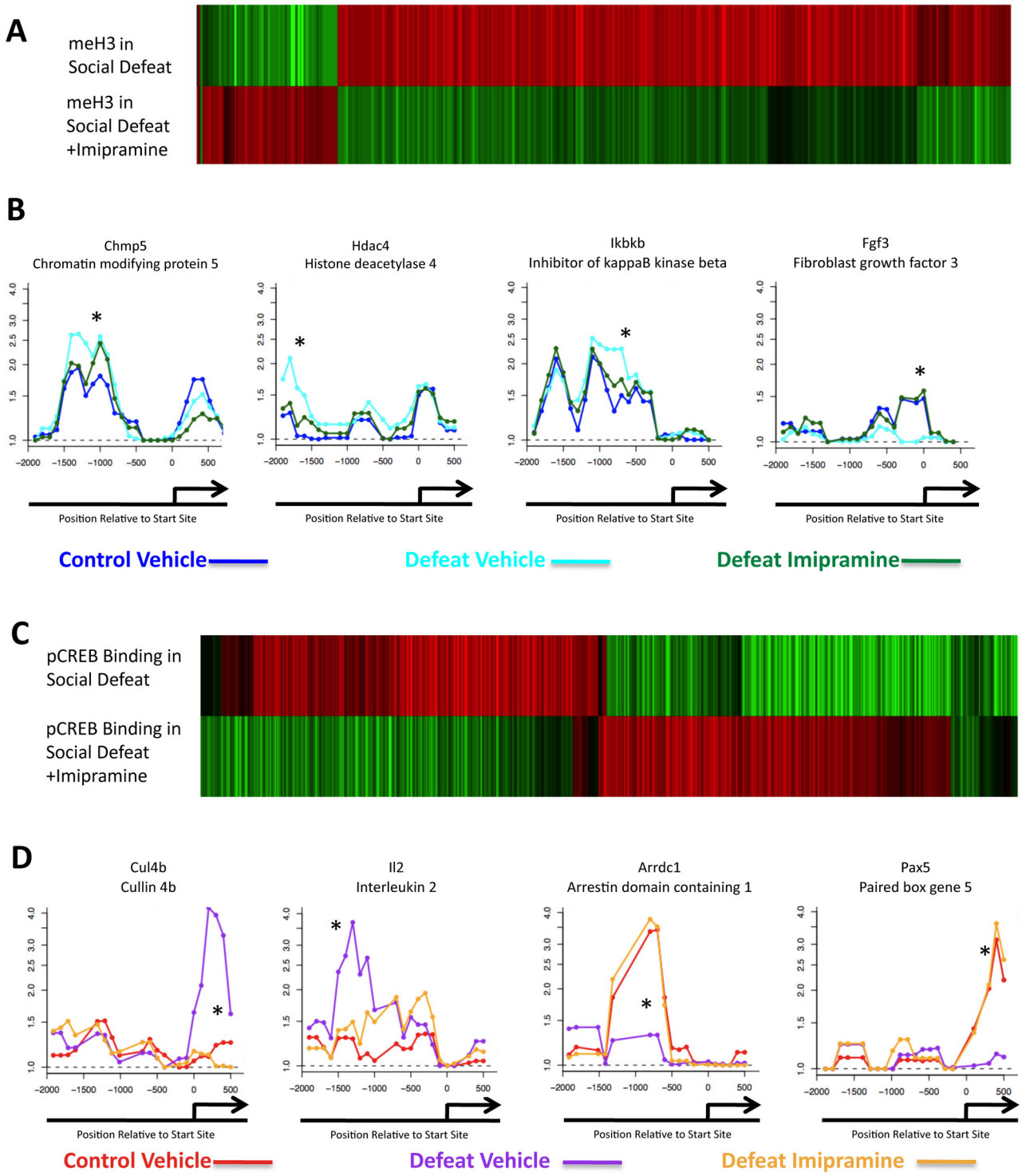

Figure 3. Reversal of H3 methylation and phospho-CREB binding patterns by imipramine treatment. ***A***, Heat maps of genes whose repressive dimethylK9/K27-H3 methylation status seen in the NAc after chronic social defeat stress is reversed by chronic imipramine treatment. The top panel shows the state of the genes 4 weeks after social defeat (with vehicle injections), whereas the bottom shows the same genes after 4 weeks of imipramine injections. The bottom panel is represented as change from the −imipramine condition. ***C*** illustrates a similar reversal of phospho-CREB binding. Data are presented in the same manner as in ***A***. ***B***, ***D***, Promoter plots for representative genes. ***B*** shows genes for H3 methylation, whereas ***D*** shows genes for phospho-CREB binding. For H3 methylation, dark blue is for control animals given vehicle, light blue for defeated animals given vehicle, and dark green for defeated animals given imipramine. For phospho-CREB binding, red is for control animals given vehicle, purple for defeated animals given vehicle, and orange for defeated animals given imipramine. *$p < 0.05$.

absence of imipramine treatment; the bottom heat map shows the relative H3 methylation states for these same genes after 4 weeks of imipramine treatment. Note that data in the +imipramine map are presented as change compared with the −imipramine state; that is, an equal intensity change in the opposite direction from the −imipramine condition corresponds to a return to baseline methylation status seen in nondefeated controls. Seven hundred and eighty-five total genes were reversed in their methylation pattern after imipramine treatment. Lists of genes in which imipramine reversed decreases or increases in H3 methyl-

ation induced by chronic social defeat are shown in supplemental Tables S13 and S14, available at www.jneurosci.org as supplemental material. Together, these data demonstrate that imipramine significantly reversed ~38% of all of the H3 methylation changes seen after chronic defeat stress. Among the genes normalized by imipramine are several involved in inflammation, redox state, and gene regulation (e.g., *Sep-15*, *CD84*, *FcrlB*, *Ikbkb*, *Il16*, and *Spi-C*). Although we did not study the chromatin effects of imipramine in the social isolation paradigm, it should be noted that each of the genes mentioned was commonly regulated after social defeat and social isolation.

We next analyzed phospho-CREB binding to gene promoters in the NAc 4 weeks after chronic social defeat stress and the effects of chronic imipramine on these adaptations. Heat maps are shown in Figure 3*C*. The top heat map depicts those genes that exhibited stable alterations in phospho-CREB binding 4 weeks after social defeat; the bottom heat map reveals how those genes were influenced by chronic imipramine treatment. As for the H3 methylation data, the +imipramine heat map shows data compared with the −imipramine heat map, such that a bar of equal intensity but opposite color corresponds to a return to baseline (nondefeated control) levels of phospho-CREB binding.

Analysis of these data revealed that chronic imipramine treatment reversed ~29% of the genes that showed stable alterations in phospho-CREB binding after chronic social defeat. In this experiment, 350 (~49%) of these genes showed increased phospho-CREB binding after social defeat that was reversed by imipramine, whereas 280 (~19%) displayed decreased phospho-CREB binding that was reversed by imipramine. These imipramine-corrected genes were again involved in the same 3 molecular pathways described above (e.g., *Acbd4*, *EII*, *Prkcsh*, *Thoc1*, *Gstm5*, *Il2*, and *Mcm3*). Full gene lists are provided in supplemental Tables S15 and S16, available at www.jneurosci.org as supplemental material.

Susceptible and resilient mice exhibit differential regulation of histone methylation and phospho-CREB binding

An important feature of the social defeat model is that not all animals subjected to the stress exhibit the characteristic defeat phenotype. Such unsusceptible or resilient animals can be distinguished from susceptible animals based on a measure of social avoidance (Krishnan et al., 2007). These groups, so defined, also show numerous other differences in behavioral and physiological parameters, with the resilient animals exhibiting values closer to those of control animals that are not defeated. This difference led us to examine levels of dimethylK9/K27-H3 and phospho-CREB binding in the NAc in these two distinct populations of mice. Animals were examined 4 weeks after the chronic (10 d) defeat procedure when the susceptible and resilient phenotypes remain stable.

In general, resilient mice displayed patterns of H3 methylation in the NAc that more closely approximated those exhibited by control mice, with susceptible mice being significantly different from both control and resilient animals. Five hundred and forty-six total genes showed such differential levels of H3 methylation in resilient mice compared with susceptible animals. 124 genes showed increased H3 methylation in resiliency that was not seen in susceptible animals, whereas 422 showed increased methylation in susceptible animals, but a significant similarity to baseline (control) status in resilient animals (Fig. 4*A*; supplemental Tables S17 and S18, available at www.jneurosci.org as supplemental material). The data are shown in Figure 4*A* such that the top heat map represents susceptible animals compared with controls, with the bottom heat map reflecting resilient animals compared with susceptible animals. Thus, oppositely colored bars of equal intensity suggest that resiliency is associated with normalization of these genes to baseline status, possibly by preventing the modifications in the first place. Among these differentially regulated genes are several involved in inflammation, redox state, and gene regulation (e.g., *Sep-15*, *Cenpt*, *Cox6c*, *Itgad*, and *Spi-C*) (Fig. 4*B*). Importantly, the resilient state was also associated with significant changes in levels of H3 methylation at other gene promoters compared with nondefeated control animals. Some of these genes that showed modified dimethylK9/K27-H3 levels in resilient animals were the same that showed opposite changes in susceptible animals, whereas others were regulated uniquely in the resilient state. A total of 2122 genes showed altered H3 methylation in the resilient state as different from the control state. These findings support the view that resiliency is an active process, associated with chromatin modifications of its own, and not just the absence of modifications seen in defeated animals.

Analogous results were obtained for phospho-CREB binding. In this experiment, 247 genes (Fig. 4*C*; supplemental Table S19, available at www.jneurosci.org as supplemental material) showed decreased phospho-CREB binding in susceptible mice in which such binding was normalized to control levels in resilient animals, whereas 267 genes (supplemental Table S20, available at www.jneurosci.org as supplemental material) showed increased phospho-CREB binding in susceptible mice, with normal (i.e., control) levels of phospho-CREB binding seen in resilient animals. Again, the top heat map in Figure 4*C* reflects susceptible animals relative to control, and the bottom heat map reflects resilient animals compared with susceptible animals, such that equal intensity but oppositely colored bars show control levels of phospho-CREB binding in resilient animals. Thus, ~24% of the genes that showed significant changes in phospho-CREB binding in susceptible and resilient animals exhibited differential regulation under these two conditions. These regulated genes were again involved in the same three molecular pathways stated above (e.g., *Etfb*, *Il2*, *Mcm3*, *Ndufb9*, *Thoc1*) (Fig. 4*D*).

Resiliency and imipramine treatment show similar patterns of chromatin regulation

The observation that imipramine and resiliency each offset a large proportion of the chromatin changes that occur in the social defeat paradigm raised the novel possibility that they do so by modulating similar pathways. Indeed, we found appreciable overlap in H3 methylation patterns under these two conditions. For example, 124 genes showed decreased dimethylK9/K27-H3 in the NAc of susceptible mice that was not observed in resilient mice. One hundred ninety-one genes showed decreased dimethylK9/K27-H3 in the NAc of susceptible mice that was reversed with chronic imipramine treatment. There are 51 overlapping genes in these two datasets. Regulation in the opposite direction (i.e., increased H3 methylation in susceptible mice, but normal H3 methylation in imipramine treatment or in resiliency) shows an overlap of 275 genes (~31% of the imipramine genes; ~40% of the resiliency genes) (Fig. 5*A*). Many of these genes have been previously mentioned, including *Cenpt*, *Cox6c*, *Dctn3*, and *Spi-C*. Another overlapping gene not mentioned previously is *Jmjd1a*, a histone demethylase involved in gene regulation.

Levels of phospho-CREB binding likewise showed similarities between resiliency and imipramine treatment. Of genes that showed decreased phospho-CREB binding in the NAc of susceptible animals, with no changes in either resiliency (247) or after imipramine treatment (280), there are 140 overlapping

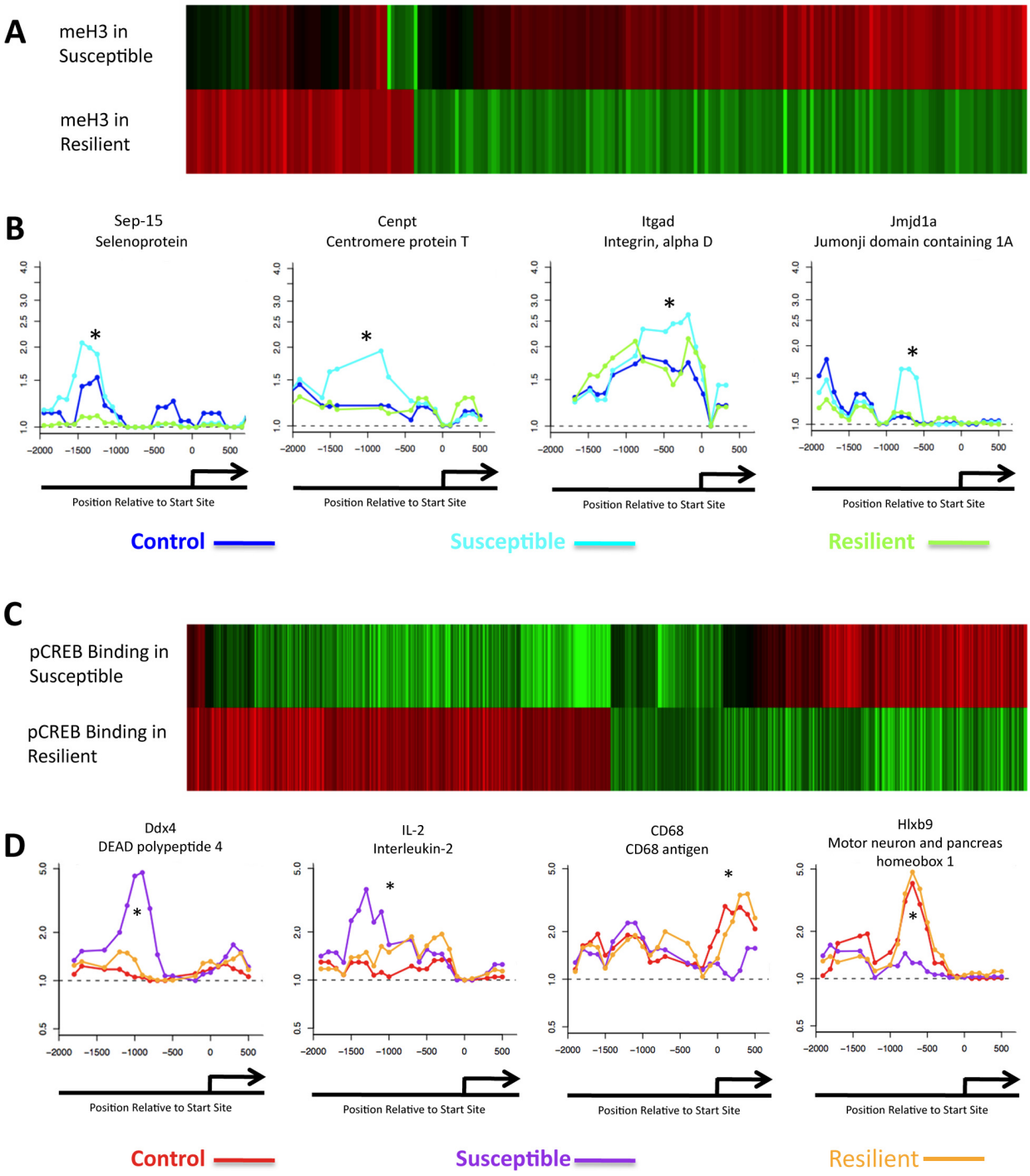

Figure 4. Differential patterns of H3 methylation and phospho-CREB binding in resiliency. ***A***, Heat maps of genes whose repressive dimethylK9/K27-H3 methylation status in the NAc is mostly opposite in susceptible versus resilient animals 4 weeks after chronic social defeat. The top heat map depicts the state of the gene in susceptible animals compared with nondefeated controls, whereas the bottom shows the same genes in resilient animals represented as change from the susceptible condition. ***C*** illustrates a similar differential effect for phospho-CREB binding in susceptible versus resilient animals. Data are presented in the same manner as in ***A***. ***B***, ***D***, Promoter plots for representative genes. ***B*** shows genes for H3 methylation, whereas ***D*** shows genes for phospho-CREB binding. For methylation, dark blue is for control animals given vehicle, light blue for vulnerable animals given vehicle, and dark green for resilient animals given vehicle. For phospho-CREB binding, red is for control animals given vehicle, purple for vulnerable animals given vehicle, and orange for resilient animals given vehicle. $^*p < 0.05$.

genes. Of those that showed increased phospho-CREB binding in the NAc of susceptible mice, and no change in resiliency (561) or after imipramine treatment (478), 211 are overlapping (Fig. 5*B*). In correspondence with earlier findings, many of these genes are involved in inflammation, redox state, or gene regulation (e.g., *Cul4b*, *Ddx4*, *Hist1h2bc*, *IL2*, *Ndufb9*, and *Zfp535*). Full gene lists are provided in supplemental Tables S21–S24, available at www.jneurosci.org as supplemental material.

Discussion

Recent work has demonstrated the utility of using ChIP-chip analyses of the NAc to study psychiatric phenomena such as drug addiction (Renthal et al., 2009). Our current study extends these

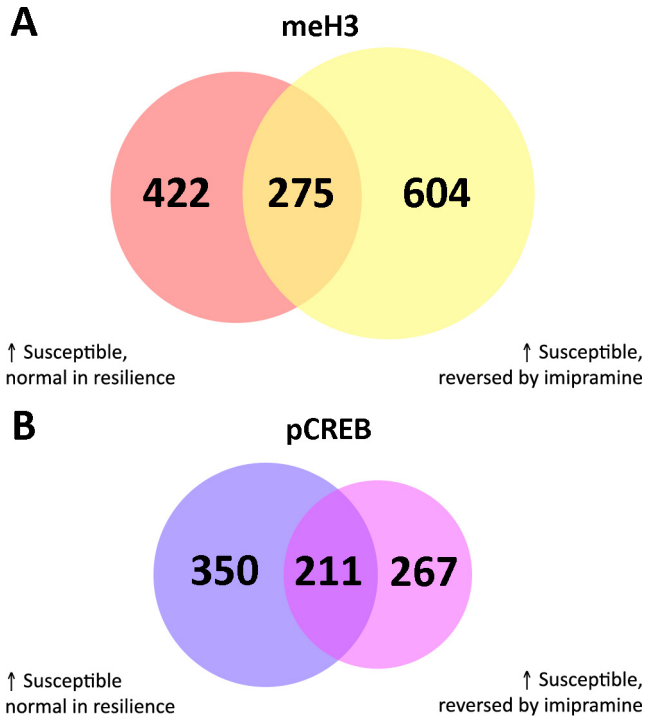

Figure 5. Overlap of chromatin changes in resiliency and after imipramine treatment. ***A***, Venn diagram of genes whose H3 methylation status is regulated in the NAc of susceptible animals after chronic social defeat and reversed by imipramine treatment and not seen in resiliency. ***B***, Venn diagram of genes whose phospho-CREB binding is regulated in the NAc of vulnerable animals after chronic social defeat and reversed by imipramine treatment and not seen in resiliency.

techniques to another common psychiatric illness, major depressive disorder, by using two animal models of depression: chronic social defeat stress (Berton et al., 2006; Tsankova et al., 2006; Krishnan et al., 2007) and prolonged social isolation of adult mice (Wallace et al., 2009).

We show that levels of dimethylK9/K27-H3, a marker of gene repression, are highly increased in both the social defeat and social isolation models. Despite the fact that one model involves an active form of stress and the other a type of passive stress, we have shown some common depressive-like symptoms (e.g., anhedonia-like symptoms) and anxiety-related behavior in the two paradigms (Krishnan et al., 2007; Wallace et al., 2009). The increased H3 methylation observed would reduce the ability of the affected genes to be transcribed either at baseline or in response to other stimuli, and the consequences of such increased methylation may be far-reaching. Indeed, a key advantage of this ChIP-chip analysis is that it provides a novel look at genes that are largely repressed or silenced.

Among the regulated genes are several involved in inflammatory pathways and regulation of redox state. One possibility is that the repression of such genes may be protective (e.g., by limiting the consequences of the initial activation of these biochemical pathways), but that this comes at the cost of decreased ability to activate the pathways later in the face of further challenges. *Sep-15*, which shows differential H3 methylation between stressed and control animals, has been shown to have antioxidant properties, and its dysregulation is implicated in neurodegenerative disorders, such as Alzheimer's disease (Chen and Berry, 2003; Ashrafi et al., 2007). Activity of the Na^+/K^+ ATPase, as well as activity of enzymes in the mitochondrial respiratory chain, are also disrupted in animal models of stress (Rezin et al., 2009). This corresponds with our findings in that we see regulation of *Cox6c* and *Cox8c*, subunits of cytochrome *c* oxidase. Likewise, the repression of genes involved in NF-κB signaling is interesting in that we have recently demonstrated a prodepressive effect of inhibiting the NF-κB pathway in the NAc (LaPlant et al., 2009). This is in contrast to the effect of NF-κB in hippocampus, where activation of the pathway decreases neurogenesis and depressive-like phenotypes (Pace et al., 2006; Koo and Duman, 2008). This work adds to an evolving literature that has implicated a range of inflammatory, cell stress, and cytokine pathways in depression-related phenomena. Chronic inflammation, through cytokine release, induces depression-like behavior in animals, and treatment of hepatitis C with interferon α induces depression in a large subset of patients (Dantzer et al., 2008). Conversely, behavioral stress, as shown here, activates cytokine signaling in the brain, which may contribute to a range of stress-related behavioral abnormalities (Shintani et al., 1995; Merali et al., 1997; Maier et al., 1999; Anisman et al., 2008).

Another class of genes highly regulated in common in the social defeat and social isolation models are those involved in transcriptional regulation. *Jdp2* shows greatly induced H3 methylation in stressed animals. The product of this gene binds to c-Jun, with the resulting complex inducing transcriptional repression by binding to AP-1 sites and preventing the binding of active AP-1 transcription factor complexes. Therefore, repression of *Jdp2* would lead to decreased repression of c-Jun and hence to an increase in AP-1 mediated transcription. Moreover, Jdp2 protein binds to ATF-2 and causes transcriptional repression via HDAC3 (Eitoku et al., 2008), and we have recently demonstrated antidepressant-like effects of ATF-2 when overexpressed in the NAc (Green et al., 2008), making this protein an attractive target for future investigations.

For phospho-CREB, our results demonstrate that, unlike H3 methylation state, binding of phospho-CREB to gene promoters is differentially regulated in the social defeat and social isolation models of depression. It is interesting, however, that the same general categories of genes showed altered levels of phospho-CREB binding as found for H3 methylation. Additionally, prominent among the genes that displayed opposite regulation of phospho-CREB binding in the two stress models are those related to actin remodeling. It is well documented that hippocampal volume is decreased in depressed patients (Egger et al., 2008), and that there is hippocampal dendritic atrophy in chronic stress paradigms (Magariños et al., 1996). Identification of stress-induced alterations in several genes involved in actin remodeling in the NAc raises the novel possibility that related morphological changes may occur in NAc neurons. Our data suggest that CREB activity may function as an overall switch that coordinates such regulation in the context of chronic stress.

The clinical efficacy of antidepressants, all of which alter monoaminergic neurotransmission, is well documented; however, it is unclear how chronic antidepressant administration changes the behavioral state of the organism. Our analyses provide new insight into the long-term effects of antidepressant treatments on the brain. Specifically, our data demonstrate that chronic imipramine treatment dramatically reverses many of the effects of chronic social defeat stress on H3 methylation and on phospho-CREB binding in the NAc. Although such actions would not be expected to account for the complete behavioral effects elicited by antidepressant treatment, they provide a beginning framework and suggest several novel molecular pathways to pursue. Among the genes that show reversal of stress regulation by imipramine is *Chrh2*, which encodes the corticotropin releas-

ing hormone receptor, which has been highly implicated in stress responses (Nemeroff and Vale, 2005).

One of the most intriguing aspects of the social defeat paradigm is its consistent ability to generate animals that have undergone equivalent levels of defeat stress but do not exhibit a depression-like phenotype (Krishnan et al., 2007). Consistent with previous studies, in which we demonstrated that such resilient animals display some unique changes in mRNA expression in the NAc compared with susceptible animals (Krishnan et al., 2007), we now show that resiliency is likewise associated with a set of unique chromatin modifications in the NAc with respect to H3 methylation and phospho-CREB binding, further supporting the notion that resilience is an active process and not simply the absence of changes that occur in vulnerable animals. The identification of such "resilience genes," therefore, defines a novel drug discovery pathway whereby new antidepressant treatments can be developed to promote resiliency. Given that susceptible and resilient responses are seen among inbred mice raised in identical environmental settings, the factors responsible for these differential adaptations to chronic defeat stress remain unknown. One possibility is that preexisting epigenetic modifications contribute to these distinct responses (Krishnan and Nestler, 2008). Regardless, the deleterious adaptations in susceptible animals may involve "allostatic overload." Stress in the short-term normally elicits adaptive responses that are beneficial to the animal; in a more chronic context, the system may be overtaxed in some individuals, resulting in dysregulation of multiple neural processes (McEwen, 2008).

One of the most striking findings of the present study is the significant overlap in the chromatin changes in the NAc associated both with chronic imipramine treatment and with resiliency. This observation supports the highly novel view that resilient individuals are less vulnerable to the deleterious effects of chronic stress in part by mounting naturally some of the same changes in gene expression that occur in the NAc. Such overlapping genes provide a rich array of possible mediators of resiliency and antidepressant responses. However, since less than half of all patients treated with available antidepressants show a complete response, the genes that show chromatin regulation in resilient animals and not after antidepressant treatment may provide an even better set of novel targets for the development of new agents with a broader range of clinical efficacy.

The experiments undertaken in this study examined adult male mice only. Previous work has shown that there are important effects of both gender and age that should be considered going forward. Females are more susceptible to depression and more likely exhibit atypical symptoms of depression (Gorman, 2006). Furthermore, a better understanding of childhood and geriatric depression, and improved treatments, are sorely needed (Lenze et al., 2008; Tsapakis et al., 2008). It would also be interesting in future studies to examine individual differences in chromatin mechanisms in susceptible and resilient mice, something not yet feasible because of the current need to pool tissue from multiple animals for ChIP analysis.

The results of the present study illustrate the power of epigenetic investigations in psychiatric illness. ChIP-chip techniques provide novel insight into the detailed transcriptional mechanisms underlying the regulation of previously known gene targets in depression models. They also reveal many previously unknown gene targets and thereby provide a rational framework for the development of new treatment agents. Ultimately, a detailed step by step manipulation of individual genes, or sets of genes, identified by these methods will be needed to validate them as bona fide mediators of depression or antidepressant action. In the meantime, this work enables a more complete appreciation of the molecular changes induced in the brain that underlie vulnerability or resistance to depression and its effective treatment.

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