nature neuroscience

Resilience to social stress coincides with functional DNA methylation of the *Crf* gene in adult mice

Evan Elliott, Gili Ezra-Nevo, Limor Regev, Adi Neufeld-Cohen & Alon Chen

DNA methylation regulates gene transcription and has been suggested to encode psychopathologies derived from early life stress. We found that methylation regulated the expression of the *Crf* (also known as *Crh*) gene and that chronic social stress in adult mice induced long-term demethylation of this genomic region. Demethylation was observed only in the subset of defeated mice that displayed social avoidance and site-specific knockdown of *Crf* attenuated the stress-induced social avoidance.

Maintenance of homeostasis in the presence of diverse challenges requires adaptive responses involving changes in the central nervous and neuroendocrine systems. The pathways by which the brain translates stressful stimuli into the final integrated biological response are incompletely understood. Nevertheless, it is clear that dysregulation of these physiological responses to stress can have severe psychological consequences and is linked to the etiology and pathophysiology of mood disorders[1,2].

The biological system that has been most closely linked to the stress response in mammals is the neuroendocrine limbic-hypothalamic-pituitary-adrenal (LHPA) axis. Perception of physical or psychological stress is followed by a series of events that result in changes in activation of the LHPA axis and the secretion of glucocorticoids from the adrenal cortex. The neuropeptide corticotrophin-releasing factor (CRF), expressed and secreted from the parvocellular neurons of the paraventricular nucleus (PVN) in the hypothalamus, represents the final common path for the integration of the neuroendocrine stress response in the brain[3,4] and has a well-established role in the regulation of the LHPA axis. Chronic hyperactivation of the CRF system has been linked to stress-related emotional disorders such as anxiety, anorexia nervosa and depression. However, the molecular mechanisms underlying the increase in CRF levels are undetermined[4,5].

Recent work has shown that early life stress in rodents and humans can change methylation patterns at specific loci of the genomic DNA, which in turn permanently alter gene expression in the brain and induce increases in anxiety behavior in the adult[6–9]. Methylation of DNA at CpG dinucleotides may inhibit local gene transcription by interfering with transcription factor binding or by recruiting methylated-DNA binding proteins that alter transcription efficiency. Considering the relative stability of DNA methylation, this may be a robust mechanism for inducing long-term changes in gene transcription and animal behavior. Nonetheless, open questions in the field of DNA methylation include its involvement in adult stress-induced neuropsychopathologies, as well as its specific role in the intact behavioral response to stressful events.

Toward this end, we examined the functional role of methylation in the *Crf* gene locus and studied its involvement in adult stress–induced behavioral changes in a mouse model of depression. We found a pattern of relatively high methylation levels in the *Crf* promoter region and low methylation levels in the intronic region (**Fig. 1a** and **Supplementary Methods**). Notably, methylation levels were high at CpG site –226, which resides in the middle of the cAMP-responsive element (CRE)

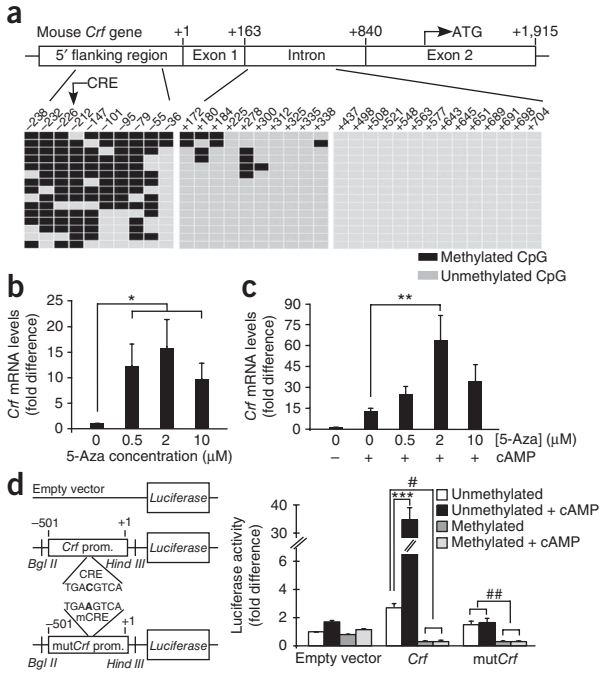

Figure 1 Methylation of the *Crf* promoter regulates gene expression. (**a**) Analysis of DNA methylation at the mouse *Crf* locus by sequencing of PCR clones derived from sodium bisulfite–treated mouse genomic DNA extracted from the PVN. Each row represents an independent clone. (**b**) Incubation of N42 immortalized mouse hypothalamic cells with 5-Aza increased *Crf* mRNA expression ($n = 4$). Bars represent mean ± s.e.m. $^{*}P = 0.029$. (**c**) Co-incubation of 5-Aza and N6,02′-dibutyryl-cAMP increased *Crf* mRNA expression ($n = 4$). $^{**}P = 0.0021$. (**d**) Luciferase assays with constructs containing the *Crf* promoter or *Crf* promoter with a point mutation in the CRE sequence (mut*Crf*). Basal and cAMP-activated *Crf* promoter activity was attenuated by methylation ($n = 4$). $^{***}P = 0.0088$; effect of methylation, $^{\#}P = 0.0017$; effect of methylation, $^{\#\#}P < 0.0001$. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel. Correspondence should be addressed to A.C. (alon.chen@weizmann.ac.il).

Received 14 June; accepted 24 August; published online 3 October 2010; doi:10.1038/nn.2642

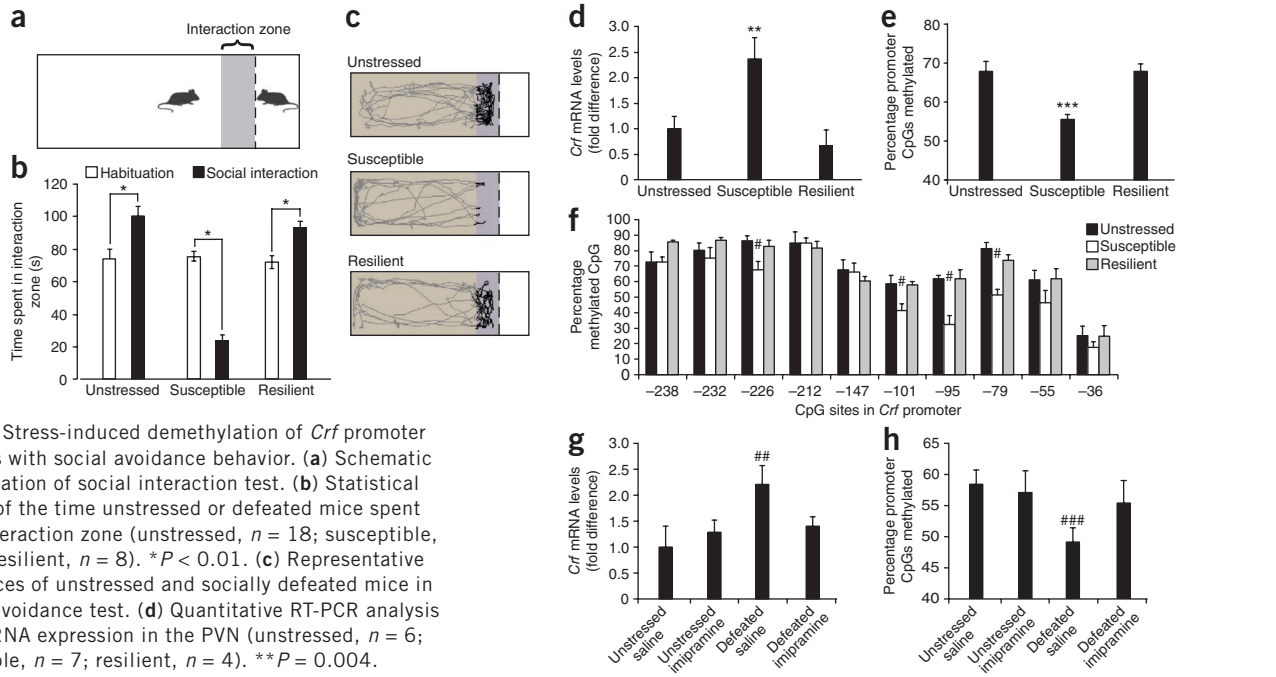

Figure 2 Stress-induced demethylation of *Crf* promoter coincides with social avoidance behavior. (**a**) Schematic representation of social interaction test. (**b**) Statistical analysis of the time unstressed or defeated mice spent in the interaction zone (unstressed, $n = 18$; susceptible, $n = 24$; resilient, $n = 8$). $^{*}P < 0.01$. (**c**) Representative video traces of unstressed and socially defeated mice in a social avoidance test. (**d**) Quantitative RT-PCR analysis of *Crf* mRNA expression in the PVN (unstressed, $n = 6$; susceptible, $n = 7$; resilient, $n = 4$). $^{**}P = 0.004$. (**e**) Methylation of the combined CpGs examined in the promoter region ($n = 5$ animals, 16 clones per animal (unstressed and susceptible) or 4 animals, 19 clones per animal (resilient)). $^{***}P = 0.001$. (**f**) Percentage of methylated CpG at each CpG. $^{\#}P < 0.05$. (**g**) Imipramine attenuated the defeat-induced increase in *Crf* mRNA expression ($n = 8$). $^{\#\#}P = 0.028$. (**h**) Imipramine attenuated the defeat-induced decrease in *Crf* promoter methylation ($n = 5$). $^{\#\#\#}P = 0.046$.

nucleotide sequence. Methylation of CRE sites has been shown to inhibit CREB-induced gene transcription in other genomic contexts[10].

To assess the functionality of methylation at the *Crf* locus in the hypothalamus, we treated the immortalized hypothalamic N42 cells with the DNA-methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-Aza). Quantitative reverse transcription (RT)-PCR determined an increase of *Crf* mRNA levels after 5-Aza treatment (**Fig.1b** and **Supplementary Fig. 1**), suggesting that DNA methylation is involved in the inhibition of basal *Crf* mRNA expression. As the CRE domain is important in CRF transcription[11], we treated cells with a cell-permeable analog of cAMP in the presence or absence of 5-Aza. cAMP stimulation alone increased *Crf* levels, whereas cAMP stimulation in the presence of 5-Aza induced a significantly larger increase in *Crf* transcript levels ($P = 0.0021$; **Fig. 1c**), suggesting that methylation regulates cAMP-induced transcription of *Crf* mRNA.

We used luciferase assays to further explore the effect of methylation on *CRF* transcription. *In vitro* methylation of the *Crf* promoter–luciferase construct completely abolished both basal and cAMP-induced *Crf* promoter activity (**Fig. 1d**). A mutation in the CRE site substantially reduced cAMP-induced *Crf* promoter activity. Notably, methylation of the mutated *Crf* promoter further downregulated its activity, suggesting that attenuation of the *Crf* promoter activity cannot be explained by effects at the CRE site alone (**Fig. 1d**).

To determine whether stress can induce changes in methylation of the *Crf* locus in adult mice, we subjected mice to 10 consecutive days of social defeat, which is an established protocol for inducing anhedonia and social avoidance[12]. We assessed the social behavior of defeated mice with a social interaction test. Defeated mice were allowed to habituate to an open field and then an unfamiliar mouse was placed in a neighboring chamber; the mice were able to interact through open slits (**Fig. 2a**). The majority of defeated mice substantially avoided the vicinity of the neighboring chamber in the presence of an unfamiliar mouse. However, a subset of defeated mice preferentially entered the interaction zone to interact with the unfamiliar mouse, displaying behavioral resiliency to chronic social defeat (**Fig. 2b**,**c** and **Supplementary Fig. 2**).

To determine the long-term effects of social defeat on *Crf* mRNA expression and DNA methylation, we housed defeated mice for an additional 2 weeks. Quantitative RT-PCR revealed a significant increase in *Crf* mRNA levels in the PVN of mice that were susceptible to social defeat stress ($P = 0.0086$) but not in resilient mice ($P = 0.3057$; **Fig. 2d**). Sodium bisulfite sequencing determined that methylation of the *Crf* promoter was significantly decreased in susceptible mice ($P = 0.0009$) but not in resilient mice ($P = 0.9457$; **Fig. 2e**). DNA methylation levels were decreased at four specific CpGs in the *Crf* promoter in defeated mice that displayed social avoidance (**Fig. 2f** and **Supplementary Fig. 3**), including CpG –226, which is located in the CRE regulatory element.

Next, we determined the effect of an antidepressant, imipramine, on *Crf* mRNA expression and promoter methylation. After social defeat, a 3-week injection regimen of imipramine attenuated the social avoidance behavior (**Supplementary Fig. 4**), and changes in *Crf* mRNA expression and promoter methylation levels (**Fig. 2g**,**h**). Thus, antidepressant treatment can modulate DNA methylation levels and this modulation correlates with both mRNA transcription and social behavior.

We determined the social defeat–induced changes in mRNA levels of chromatin-modulating enzymes in the PVN. We detected significant decreases in *Dnmt3b* and *Hdac2* expression 1 h after the last social defeat, whereas the demethylation-promoting factor *Gadd45* was substantially increased after defeat (**Supplementary Fig. 5** and **Supplementary Table 1**). Considering the role of *Gadd45b* in DNA demethylation[13], this protein may be important for the demethylation of the *Crf* promoter. In addition, multiple infusions of the DNA-methyltransferase inhibitor RG108 into the PVN had no effects on

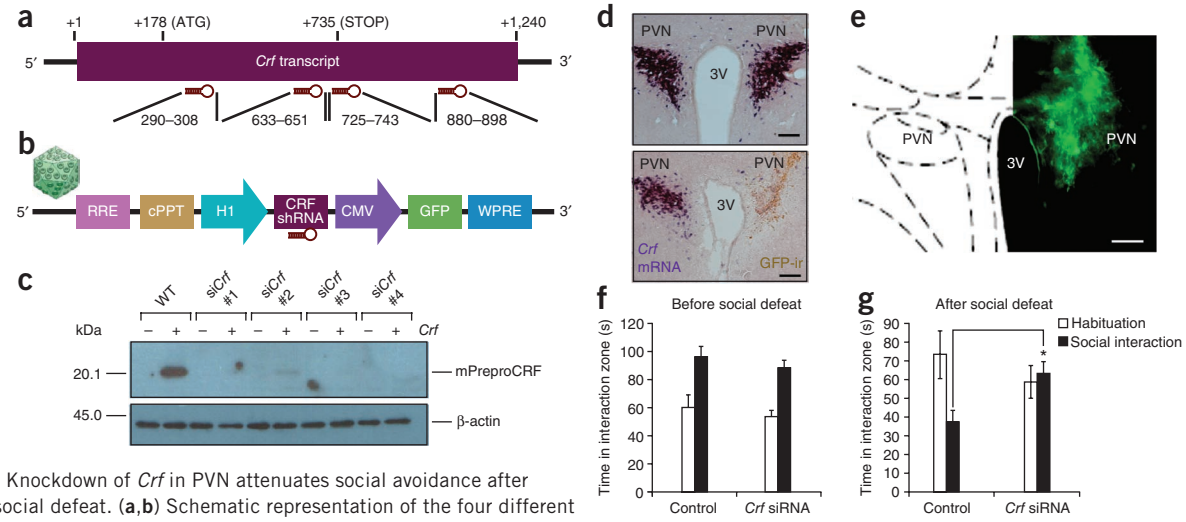

Figure 3 Knockdown of *Crf* in PVN attenuates social avoidance after chronic social defeat. (**a**,**b**) Schematic representation of the four different siRNA target sequences designed from the open reading frame of the mouse *Crf* gene and the lentiviral construct designed to knockdown *Crf*. (**c**) Western blot analysis revealed that all of the si*Crf* lentiviruses were able to reduce *Crf* expression in HEK293T cells transfected with a *Crf*-expressing plasmid. PreproCRF, CRF precursor peptide. (**d**) Brain slices of a mouse injected with si*Crf* #3 viruses or control were *in situ* hybridized for *Crf* and immunostained for GFP. Purple-stained cells represent *Crf* mRNA and DAB-stained cells represent GFP immunoreactivity. Scale bar represents 100 µm. 3V, third ventricle. (**e**) GFP was expressed at the site of injection in mice injected with si*Crf*-expressing lentiviruses in the PVN. Section map adapted from ref. 15. Scale bar represents 100 µm. (**f**,**g**) Mice injected with scramble or *Crf* siRNA–expressing lentiviruses were tested for social interaction behavior both before and after social defeat ($n = 8$–11). $^*P = 0.048$

social behavior, *Crf* DNA methylation levels or *Crf* mRNA expression, suggesting that a demethylation factor may be necessary to induce *Crf* demethylation (**Supplementary Fig. 6** and **Supplementary Methods**). Only the decrease in *Hdac2* expression remained 2 weeks after social defeat and there were no substantial differences in enzyme transcript expression between the susceptible and resilient mice. Thus, acute changes in chromatin-regulating enzymes during the stress protocol may regulate changes in DNA methylation, and differential methylation between resilient and susceptible mice may be a result of promoter-specific protein interactions.

We used lentiviral-mediated knockdown of *Crf* in the PVN to further examine the involvement of PVN CRF-expressing neurons in mediating the behavioral changes associated with social defeat. Short interfering RNA (siRNA) sequences targeting mouse *Crf* were designed, cloned into lentiviral constructs (**Fig. 3a**,**b**) and tested for knockdown efficiency both *in vitro* and *in vivo* (**Fig. 3c**,**d**). Validated siCRF lentiviruses were then used to knockdown *Crf* specifically in the PVN (**Fig. 3e**). Knockdown of *Crf* had no effect on social interaction behavior before social defeat (**Fig. 3f**). However, after social defeat, mice injected with *Crf* knockdown viral particles spent significantly more time ($P = 0.048$) engaged in social interaction than those injected with control viruses (**Fig. 3g**). Thus, depletion of *CRF* from the PVN attenuated stress-induced social avoidance, suggesting that *Crf* expression is involved in the social avoidance behavior induced by chronic social defeat.

Taken together, our data suggest that direct epigenetic regulation of *Crf* may be a mechanism for regulating long-term behavioral responses to stress. In addition, the finding that epigenetic regulation of *Crf* correlates with the behavioral response to stress further implicates epigenetics as a primary regulator of behavior. Stress-induced changes in DNA methylation have been characterized in early life stress[7], whereas adulthood stress has been correlated with changes in histone modifications[14]. Our results indicate that stress regulates DNA methylation in the adult mouse, suggesting that regulation of DNA methylation may be a primary molecular mechanism underlying stress-induced behavioral changes in the adult mouse.

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

This work was supported by Roberto and Renata Ruhman, Mark Besen and the Pratt Foundation, the Israel Science Foundation, the Legacy Heritage Biomedical Science program of the Israel Science Foundation, the Nella and Leon Benoziyo Center for Neurosciences, the Nella and Leon Benoziyo Center for Neurological Diseases, the Carl and Micaela Einhorn-Dominic Brain Research Institute, the Irwin Green Alzheimer's Research Fund, and Gerhard and Hannah Bacharach. A.C. is incumbent of the Philip Harris and Gerald Ronson Career Development Chair.

AUTHOR CONTRIBUTIONS

E.E. performed the cell culture experiments, *in vivo* DNA methylation and mRNA analysis, and behavioral experimentation. G.E.-N. performed stereotaxic surgery and viral injections. L.R. and A.N.-C. verified the *Crf* knockdown lentivirus. E.E. and A.C. designed the experiments, interpreted the results and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/natureneuroscience/.
Reprints and permissions information is available online at http://www.nature.com/reprintsandpermissions/.

1. de Kloet, E.R., Joels, M. & Holsboer, F. *Nat. Rev. Neurosci.* **6**, 463–475 (2005).
2. McEwen, B.S. *Physiol. Rev.* **87**, 873–904 (2007).
3. Vale, W., Spiess, J., Rivier, C. & Rivier, J. *Science* **213**, 1394–1397 (1981).
4. Bale, T.L. & Vale, W.W. *Annu. Rev. Pharmacol. Toxicol.* **44**, 525–557 (2004).
5. Joels, M. & Baram, T.Z. *Nat. Rev. Neurosci.* **10**, 459–466 (2009).
6. McGowan, P.O. *et al. Nat. Neurosci.* **12**, 342–348 (2009).
7. Murgatroyd, C. *et al. Nat. Neurosci.* **12**, 1559–1566 (2009).
8. Roth, T.L., Lubin, F.D., Funk, A.J. & Sweatt, J.D. *Biol. Psychiatry* **65**, 760–769 (2009).
9. Mueller, B.R. & Bale, T.L. *J. Neurosci.* **28**, 9055–9065 (2008).
10. Zhang, X. *et al. Proc. Natl. Acad. Sci. USA* **102**, 4459–4464 (2005).
11. Aguilera, G., Kiss, A., Liu, Y. & Kamitakahara, A. *Stress* **10**, 153–161 (2007).
12. Krishnan, V. *et al. Cell* **131**, 391–404 (2007).
13. Ma, D.K. *et al. Science* **323**, 1074–1077 (2009).
14. Tsankova, N.M. *et al. Nat. Neurosci.* **9**, 519–525 (2006).
15. Paxinos, F. & Franklin, K.B.J. *The Mouse Brain in Stereotaxic Coordinates* (Academic Press, San Diego, California, 2001).