DOCUMENT SUMMARY

This 2009 research paper from Nature Neuroscience explores the molecular basis of how adverse early-life experiences create long-lasting changes in the brain. The study demonstrates that early-life stress (ELS) in mice leads to persistent hyperactivity of the stress response system (HPA axis) and behavioral changes, driven by the sustained overexpression of the arginine vasopressin (AVP) gene. This is caused by a lasting epigenetic change—specifically, the hypomethylation (a reduction in DNA methylation) of the Avp gene, which prevents the repressive protein MeCP2 from binding and silencing it.

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FORMATTED CONTENT

Dynamic DNA methylation programs persistent adverse effects of early-life stress

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Adverse early life events can induce long-lasting changes in physiology and behavior. We found that **early-life stress (ELS)** in mice caused enduring hypersecretion of **corticosterone** and alterations in passive stress coping and memory. This phenotype was accompanied by a persistent increase in **arginine vasopressin (AVP)** expression in neurons of the hypothalamic paraventricular nucleus and was reversed by an **AVP** receptor antagonist. Altered **Avp** expression was associated with sustained **DNA hypomethylation** of an important regulatory region that resisted age-related drifts in methylation and centered on those CpG residues that serve as DNA-binding sites for the methyl CpG-binding protein 2 (**MeCP2**). We found that neuronal activity controlled

the ability of **MeCP2** to regulate activity-dependent transcription of the **Avp** gene and induced epigenetic marking. Thus, **ELS** can dynamically control **DNA methylation** in postmitotic neurons to generate stable changes in **Avp** expression that trigger neuroendocrine and behavioral alterations that are frequent features in depression.

Epigenetic regulation of gene expression allows the integration of intrinsic and environmental signals in the genome[1]. Greater emphasis is being placed on the role of epigenetic mechanisms in facilitating the adaptation of organisms to changing environments through alterations in gene expression. Evidence that dietary or pharmacological interventions have the potential to reverse environment-induced modification of epigenetic states[2–5] has provided an additional impetus for understanding the epigenetic basis of disease, including disorders of the brain. It has been suggested that epigenetic mechanisms underlie brain plasticity, a process requiring stable modulation of gene expression [6,7]. **DNA methylation** is one of the most intensely studied epigenetic mechanisms and recent work has suggested that this form of gene regulation may determine risk for psychiatric disorders[3,9,10].

Exposure to stress during neurodevelopment has an effect on the quality of physical and mental health [11,12]. Periodic infant-mother separation during early postnatal life is one of the most commonly used procedures for inducing **ELS** in rodents. It is characterized by lifelong elevated glucocorticoid secretion, heightened endocrine responsiveness to subsequent stressors and disruption of the homeostatic mechanisms that regulate the activity of the **hypothalamo-pituitary adrenal (HPA) axis**, all of which are considered to be pathogenetic factors in disorders of mood and cognition[13–15].

Here we examined the coupling of experience-driven neuronal activity with **DNA methylation** and gene expression. We focused on the expression of the two hypothalamic secretagogues that regulate **HPA axis** activity by increasing the synthesis and release of pituitary adrenocorticotropin, namely, **AVP** and corticotropin-releasing hormone (CRH). Abundant evidence links **AVP** and CRH to mood and cognitive behaviors [16,17], making their receptors the targets of psychopharmacological agents [18,19]. In addition to being important in the postnatal development and functional maturation of the pituitary-adrenal axis, **AVP** potentiates the actions of CRH under circumstances that demand sustained activation of the pituitary and adrenal glands [20]. We found that **ELS** induces persistent hypomethylation of the **Avp** enhancer, accompanied by sustained upregulation of **Avp** expression, increased **HPA axis** activity and behavioral alterations.

In the course of exploring the molecular mechanisms underlying these changes, we found that **MeCP2** is important in the epigenetic programming of neuroendocrine and behavioral functions.

RESULTS

ELS-induced phenotypes

Consistent with previous studies [15,21], **ELS** during the first 10 d of life led to sustained hyperactivity of the **HPA axis**, characterized by **corticosterone** hypersecretion under basal conditions, hyper-responsiveness to acute stressors applied later in life and escape from the inhibitory constraints of dexamethasone (Fig. 1a). Although it had no effect on body mass, **ELS** induced involution of the thymus, hypertrophy of the adrenals (Supplementary Fig. 1) and increased expression of pituitary pro-opiomelanocortin (Pomc) mRNA, which encodes the adrenocorticotropin pro-hormone (Fig. 1b). Pomc expression is induced by the hypothalamic

neuropeptides **AVP** and CRH, and all of them are under negative feedback control by the glucocorticoid receptor. Conspicuously, levels of Nr3c1 (the gene that encodes the glucocorticoid receptor) mRNA in the hippocampus, hypothalamic paraventricular nucleus (PVN) and pituitary were either unchanged or upregulated in **ELS**-treated mice (Supplementary Fig. 1), arguing against impaired **corticosterone** feedback as the primary cause of the observed increases in Pomc expression and glucocorticoid secretion. Although **ELS** did not influence hypothalamic Crh mRNA expression (Supplementary Fig. 1), the procedure resulted in a significant upregulation of **Avp** mRNA ($P<0.05$; Fig. 1c). The changes in **Avp** expression persisted for at least 1 year and were restricted to the parvocellular subpopulation of neurons in the PVN, that is, in those neurons that drive the pituitary-adrenal axis (Supplementary Fig. 2).

AVP exerted its regulatory role on Pome expression levels via activation of pituitary **AVP** V1b receptors. Application of SSR149415, a selective V1b receptor antagonist, normalized the elevated Pomc mRNA levels (Fig. 1b) and **corticosterone** secretion (data not shown), verifying the critical role of **AVP** in driving the disturbed endocrine phenotype in **ELS** mice.

ELS also produced long-lasting behavioral changes. Adult **ELS**-exposed mice showed memory deficits in an inhibitory avoidance task (Fig. 1d). In addition, they had increased immobility in the forced swim test (Supplementary Fig. 3). In contrast, anxiety-like behavior was unaffected by **ELS** in the elevated plus-maze, novelty-induced hypophagia and light-dark avoidance tests (data not shown). The **ELS**-induced behavioral phenotypes were reproduced in two further independent replications. Treatment with the SSR149415 partially reversed the impaired memory in **ELS** mice (Fig. 1d) and abolished the changes in behavioral stress coping (Supplementary Fig. 3) without influencing the behavioral performance of control mice.

Differential methylation of the Avp gene

Methylation of cytosine residues in CpG dinucleotides can result in epigenetic gene silencing; such CpGs are conspicuously under-represented in mammalian genomes and typically cluster in glucocorticoid-rich regions called CpG islands (CGIs)[22]. Computational analysis and a recent genome-wide classification of promoter CGIs[23] predicted 4 CGIs in **Avp**: CG11 (intermediate CpG frequency in the promoter region), CGI2 (high CpG frequency covering the second and third exons), CGI3 and CG14 (intermediate CpG frequency in the -3.6-kb downstream region) (Fig. 2a and Supplementary Fig. 4). The latter region, also referred to as the intergenic region (IGR) separates the neighboring, tail-to-tail-orientated **Avp** and oxytocin genes and includes a composite enhancer region in the first 2.1 kb proximal to **Avp** that is important for expression[24].

Sequence analysis of bisulfite-converted DNA isolated from the PVN of naive C57BL/6N mice showed sparse methylation in the promoter CGII and exonic CGI2. In contrast, we found high levels of CpG methylation clustered at the more distal enhancer encompassing CpG7 to CpG32 and spanning CG13 (Fig. 2b). The latter region is highly conserved between species and is important for **Avp** regulation[24]. Less-dense methylation was observed in CGI4 and the adjacent oxytocin tissue-specific enhancer region had only a few, irregularly spaced and highly methylated CpG residues (Fig. 2b). A similar methylation of CpG residues at the **Avp** locus was found in unrelated CD1 mice, supporting the idea that this pattern is unlikely to be strain specific (data not shown). Together, these results support the idea that CGIs in intergenic regions are more likely to be methylated than those at gene promoters and that CGIs with intermediate CpG densities are methylated more frequently [25].

Persistent hypomethylation of CG13 after ELS

We compared PVN tissue from **ELS** and control mice aged 6 weeks, 3 months and 1 year and found hypomethylation of multiple CpG residues throughout the downstream **Avp** enhancer region in **ELS** mice (Fig. 3a-c). Analysis of overall methylation of the enhancer revealed substantial reductions in methylation in **ELS** mice of all ages (Fig. 3d). Significantly marked ($P<0.05$) CpG residues largely mapped to CGI3 of the enhancer. For many of these, the degree of **ELS**-induced hypomethylation was consistently greater than that observed for overall CG13 hypomethylation; for example, CpG10 showed uniformly strong reductions in methylation (by 37% at 6 weeks, ($P<0.005$), 21% at 3 months ($P<0.05$) and 66% at 1 year ($P<0.005$)). This finding reveals that **ELS** triggers a heterogeneous response in CpG hypomethylation and indicates a functional role for marked changes.

To obtain a functional measure of those CpG residues that are likely to control **Avp** expression, we sought correlations between **Avp** mRNA levels and the degree of methylation of individual CpG residues that were significantly hypomethylated ($P<0.05$) in **ELS** mice. Therefore, in situ hybridization (ISH) and **DNA methylation** analyses were performed on tissues from the same individual mice. Of the 11 CpGs that were significantly hypomethylated ($P<0.05$) in 6-week-old **ELS** mice (Fig. 3a), only seven (CpGs 7, 10, 12, 13, 14, 15 and 17) had methylation patterns that were strongly correlated with **Avp** mRNA levels (Fig. 3e). For example, although residues CpG10 and CpG22 had similar levels of methylation (methylation at CpG10 in controls and **ELS** was 60.3±5.9% and 37.6%, respectively; methylation at CpG22 in controls and **ELS** was 69.5±8.6% and 39±9.8% respectively; Fig. 3a), the methylation status of CpG10, but not of CpG22, correlated strongly with differences in **Avp** expression (CpG10, $r2=0.44$, $P<0.05$; CpG22, $r2=0.07$, $P>0.1$). Thus, **ELS**-induced alterations in CpG methylation appear to be important for **Avp** mRNA levels, although individual CpG residues located in CGI3 seem to contribute, in different degrees, to altered expression. Those CpGs that failed to show a priori significant differences ($P>0.05$) in their methylation status in response to **ELS** correlated poorly with **Avp** mRNA levels (data not shown). Notably, differential methylation of CGI3 was not evident when DNA from the hypothalamic supraoptic nucleus of 6-week-old control and **ELS** mice were compared (Supplementary Fig. 5); the latter is consistent with the observation that **Avp** transcript levels in this nucleus were not influenced by **ELS** (Supplementary Fig. 2).

Methylation landmarks correlate with Avp expression

These data led us to hypothesize that **ELS**-induced changes in the methylation status of relevant CpG residues are persistent and sustain elevated **Avp** expression, whereas changes in functionally less-significant CpGs ($P>0.05$) wane over time. To identify CpGs predictive of persistently increased **Avp** expression, we examined each CpG residue in detail. Residues considered to be of predictive value were those that were significantly methylation ($P<0.05$) marked by **ELS** at one age at least and nominally altered by more than 25% at two other ages. By these criteria, CpG residues 10, 12, 13, 14 and 15 were revealed as methylation landmarks in the **Avp** enhancer (Fig. 3f). The number of CpG residues that were significantly marked by **ELS** decreased with age (11 in 6-week-old, 7 in 3-month-old and 3 in 1-year-old mice; Fig. 3f). With the exception of CpG10, which localized to the upstream boundary (Fig. 2a), all of the emerging methylation landmarks mapped to the center of CGI3 (CpGs 12, 13, 14 and 15). We corroborated the functional role of these residues by correlating their individual methylation status with **Avp** mRNA levels in control and **ELS** 6-week-old, 3-month-old and 1-year-old mice (Fig. 3g). This revealed that the composite methylation status of these residues faithfully reflected longitudinal **Avp** expression. Poor, if any, correlations were found between **Avp** mRNA expression (at any age) and those CpG residues that showed either initial (CpGs 7, 17, 21, 22, 23 and 32) or otherwise transient (CpGs 11, 20 and 31) differences in methylation status (data

not shown). This set of findings highlights the functional importance of CpG residues 10, 12, 13, 14 and 15 in the regulation of **Avp** expression.

Although significant hypomethylation ($P<0.0001$) of the **Avp** enhancer occurred with age in control mice (Fig. 3d), we did not observe age-dependent changes in **Avp** mRNA levels (Fig. 1c). In contrast, **ELS** mice did not show age-related hypomethylation of the **Avp** enhancer (Fig. 3d), but nevertheless maintained higher levels of **Avp** mRNA, as compared to controls (Fig. 1c). This raised the question of whether single CpG residues might be differentially sensitive to age- versus **ELS**-induced hypomethylation. Analysis of the effects of aging on hypomethylation of all 32 CpGs of the **Avp** enhancer in 6-week-old and 1-year-old control and **ELS** mice showed that age-associated hypomethylation only occurred in 16% of the CpGs in the **Avp** enhancer region of **ELS** mice, as compared with 38% in control mice (Fig. 3h). Notably, those CpG residues with an assigned regulatory role (methylation landmarks 10, 12, 14 and 15) did not show significant hypomethylation ($P>0.05$) with aging (Fig. 3h).

Enhancer methylation directs Avp expression

An AVP-expressing N6 mouse hypothalamic cell line [26] was used to examine whether **Avp** enhancer methylation modulates **Avp** expression (Fig. 4a). We analyzed of the methylation profile in the CGI3 region that spans CpG10-14 and found a pattern that was similar to the one that we observed in the mouse PVN (Supplementary Fig. 6). Treatment of N6 cells with 5-azacytidine, a potent inhibitor of **DNA methylation**, reduced the level of methylation of the **Avp** enhancer and, concomitantly, increased **Avp** expression (Fig. 4b and Supplementary Fig. 6). In transfection assays (Fig. 4c), deletion of the CGI3 region reduced reporter activity by 37% and deletion of the entire enhancer resulted in almost complete abolition of reporter activity (Fig. 4d). We examined **Avp** gene reporter activity after in vitro methylation of the entire **Avp** vector, including the promoter and transcribed regions. Methylation led to a tenfold decrease in reporter activity (Fig. 4e). Moreover, reporter activity was reduced by 50% when methylation was targeted specifically to CG13 (Fig. 4e). Together with the results obtained in hypothalamic tissue, this finding suggests that CGI3-specific methylation is critical for the control of **Avp** expression.

MeCP2 selectively binds CG13 and represses Avp expression

DNA methylation is interpreted by a family of methyl CpG-binding domain (MBD) proteins comprising **MeCP2**, MBD1, MBD2, MBD3 and MBD4, the first three of which couple **DNA methylation** to transcriptional repression. Although hypothalamic N6 cells expressed **MeCP2**, MBD1 and MBD2 (Supplementary Fig. 7), we found **MeCP2** to be the most potent repressor of the CG13 methylated vector in co-transfection experiments (Fig. 4f).

We directly assessed the binding of **MeCP2** at the **Avp** locus by immunoprecipitation of cross-linked chromatin from N6 cells with antibodies to **MeCP2** or control IgG, followed by PCR analysis of the recovered DNA using seven primer pairs bracketing the **Avp** locus (Fig. 4g). As expected, **MeCP2** did not occupy the poorly methylated **Avp** promoter and exonic CpG islands (CGI1 and 2); moreover, **MeCP2** was also absent at CGI4, which is methylated to a relatively high extent. In contrast, **MeCP2** was strongly enriched at the CG13 region (Fig. 4g). Notably, CGI3, but not CGI4, was poorly recovered when the same chromatin samples were immunoprecipitated with antibodies to MBD1 and MBD2 (Supplementary Fig. 7). Thus, **MeCP2** preferentially and selectively occupies CGI3 of the **Avp** enhancer.

Pre-treatment of N6 cells with 5-azacytidine robustly decreased **MeCP2** occupancy at CGI3 and increased promoter binding of activated (pSer5) RNA polymerase II and **Avp** transcription in

parallel (Fig. 4b,h). Therefore, **MeCP2** occupancy at the **Avp** locus is **DNA methylation**-dependent and, once bound, **MeCP2** acts to repress transcription. High-affinity binding of **MeCP2** to methylated DNA requires a local sequence context, namely a symmetrical methyl-CpG dinucleotide that localizes close to a run of four or more A/T bases that facilitate DNA binding[27]. We identified four CpG dinucleotides (CpGs 13, 14 and 21, as well as the highly relevant CpG10) that matched the latter criterion in the CGI3 sequence (Fig. 4i). Their function in the context of **MeCP2** binding was tested by in vitro DNA-binding electrophoretic mobility shift assays (EMSAs), using recombinant **MeCP2** and oligonucleotides that spanned CpG10, CpG12 or CpG14. As anticipated, methylation of these motifs proved to be essential for **MeCP2** binding and effective self-competition (Fig. 4j and Supplementary Fig. 8). **MeCP2** specifically bound to the key motif CpG10 with a KD of 2.6 nM (comparable to that previously reported[27]) and DNA binding was strongly impaired (KD>5C nM) after mutation of the A/T run adjacent to the CpG10 dinucleotide (CpG10A/Tmut) (Fig. 4k). Compared with CpG10, the neighboring motifs CpG12 and CpG14 bound **MeCP2** with lower affinity (KD≈9.6 and 10.5 nM, respectively) and competed poorly with CpG10 for forming a complex with **MeCP2** (Supplementary Fig. 8). Together, these results indicate that the **Avp** enhancer contains context-specific, high-affinity **MeCP2** DNA-binding sites that are important for the regulation of **Avp**.

Phosphorylation of MeCP2 prevents Avp enhancer occupancy

Neuronal depolarization has been shown to trigger Ca2+-dependent phosphorylation of **MeCP2**, causing dissociation of **MeCP2** from the Bdnf promoter and increased Bdnf transcription [28],[29]. Recently, de novo Ca2+/calmodulin-dependent protein kinase II (**CaMKII**) was shown to mediate phosphorylation of rat **MeCP2** at serine 421 (S438 in mouse) [30]. To explore whether this mechanism might be responsible for regulating **MeCP2** occupancy at the **Avp** enhancer, we transfected N6 cells with either **MeCP2** and/or a constitutively active form of **CaMKII** (**CaMKII***) together with the CG13-methlyated **Avp** vector. Transfection of **CaMKII*** increased **Avp** reporter activity slightly, and completely reversed **MeCP2**-mediated repression (Fig. 5a). Furthermore, we observed that **CaMKII** markedly reduced the occupancy of Flag-tagged **MeCP2** at the **Avp** enhancer, but failed to release DNA-bound nonphosphorylatable **MeCP2** (S438A; Fig. 5b). Subsequently, MeCP2-S438A and (to a lesser degree) **MeCP2** prevented **CaMKII*** activity-dependent increases in **Avp** expression (Fig. 5c); this result is consistent with a repressive role of **MeCP2**.

Additional experiments showed that K+-induced depolarization of N6 cells faithfully reproduced the effects of **CaMKII*** transfection, that is, relieved **MeCP2** occupancy at the **Avp** enhancer. A role of **CaMKII** in mediating MeCP2-S438 phosphorylation was confirmed by the complete reversal of this regulation after pretreatment with the **CaMKII** inhibitor KN-93. Moreover, K+ -induced depolarization increased the presence of activated RNA polymerase II at the **Avp** promoter (Fig. 5d), verifying the ability of **MeCP2** to repress activity-dependent gene expression. Lastly, an antibody to the regulatory MeCP2-S438 phosphorylation site (MeCP2-pS438; Supplementary Fig. 9) reacted strongly with extracts from membrane-depolarized N6 cells, but only weakly with extracts from nonstimulated N6 cells (Fig. 5e). Thus, membrane depolarization directly leads to phosphorylation of **MeCP2** at S438.

ELS reduces MeCP2 occupancy at Avp enhancer

We next asked whether the sustained increased expression of **Avp** after **ELS** is triggered by MeCP2-S438 phosphorylation and subsequent relief of **MeCP2** occupancy at the **Avp** enhancer. This hypothesis was supported by the observation that MeCP2-S438 phosphorylation was prominently increased in parvocellular AVP-expressing neurons in the PVN of 10-d-old **ELS** mice (Fig. 6). In addition, the PVN of 10-d-old **ELS** mice had increased phospho-CaMKII

immunoreactivity in AVP-positive neurons, a finding that is compatible with a role for this kinase in the mediation of activity-dependent MeCP2-S438 phosphorylation (Supplementary Fig. 10). The extents to which **CaMKII** and MeCP2-S438 were phosphorylated in the parvocellular division of the PVN did not differ between 6-week-old control and **ELS** mice (Fig. 6 and Supplementary Fig. 10). In addition, neither Mecp2 mRNA nor total **MeCP2** and **CaMKII** protein expression differed between the two groups (Supplementary Fig. 10). Lastly, MeCP2-pS438 immunoreactivity in the supraoptic nucleus did not differ between control and **ELS** mice at all ages, demonstrating the site-specificity of the effects (data not shown). These results indicate that there is an age- and cell type-specific role for **ELS**-induced MeCP2-S438 phosphorylation, prompting us to examine its relevance for enhancer occupancy.

Although RNA polymerase II occupancy at the **Avp** promoter and **Avp** expression were markedly increased in 10-d-old **ELS** mice (Fig. 7a), CG13 methylation did not differ between control and **ELS** mice of this age (Supplementary Fig. 11). Notably, in vivo chromatin immunoprecipitation (ChIP) experiments on PVN tissue revealed that, of the various MBDs, **MeCP2** was selectively enriched at CGI3 in 10-d-old and 6-week-old control mice (Fig. 7b and data not shown) and that binding of **MeCP2** to the **Avp** enhancer was reduced in **ELS** mice of both ages (Fig. 7b). Given that 10-d-old control and **ELS** mice have identical methylation patterns, the measured differences in **MeCP2** occupancy suggest that **ELS**-induced MeCP2-S438 phosphorylation results in relief of **MeCP2** occupancy at the **Avp** enhancer. The repressive function of **MeCP2** at the **Avp** enhancer was substantiated by sequential in vivo ChIP experiments, which revealed strong coupling of **MeCP2** to transcriptionally inactive chromatin marks (data not shown), comparable with those reported for Crh occupancy[31].

In sum, de-repression of **Avp** transcription in 10-d-old **ELS** mice appears to involve increased MeCP2-S438 phosphorylation, whereas reduced enhancer occupancy in 6-week-old mice most likely reflects **ELS**-induced CG13 hypomethylation (Figs. 3a and 7b).

DISCUSSION

Adverse experiences during early life contribute to the etiology of psychiatric conditions in later life [14,32]. Our results suggest that **ELS** in mice leads to epigenetic marking (hypomethylation) of a key regulatory region of the **Avp** gene in the PVN. These epigenetic events are accompanied by persistent upregulation of **Avp** expression in the parvocellular subdivision of the PVN and, consequently, sustained hyperactivity of the **HPA axis**. Notably, the **ELS**-induced endocrine phenotype lasted for at least 1 year following the initial adverse event and could be normalized through administration of an **AVP** V1b receptor antagonist.

Studies in humans and in animal models suggest that stress or elevated glucocorticoid secretion are important for the function of interdependently regulated behavioral domains[33,34]. Here, **ELS**-treated mice showed increased immobility in the forced-swim test, which assesses stress-coping ability[35], and had deficits in step-down avoidance learning. Although acute rises in glucocorticoid secretion can facilitate inhibitory avoidance learning[13], our data support the notion that sustained elevated glucocorticoid levels impair memory performance in **ELS** mice. Notably, the behavioral phenotypes induced by **ELS** were shown to be partly reversible after antagonism of **AVP** V1b receptors, thus highlighting **AVP** as an important mediator of these processes, but not necessarily the only [14].

Our results identify CpG residues in the CG13 region of the **Avp** enhancer whose persistent hypomethylation after **ELS** is critical for the regulation of **Avp** expression.

Recent work defined these residues as being high-affinity, context-specific **MeCP2** DNA-binding sites[27]. On the basis of previous reports [28],[29], we hypothesized that signaling mechanisms controlling **MeCP2** occupancy are critical for gene repression and the dynamic methylation of CGI3 in response to **ELS**. Supporting this, we found that depolarization of hypothalamic cells can regulate **MeCP2** function by inducing its site-specific phosphorylation via **CaMKII** activity. Taken together, our results indicate that phosphorylation of **MeCP2** at S438 is critical for **MeCP2** to function as a reader and interpreter of the **DNA methylation** signal at the **Avp** enhancer.

That experience-dependent stimuli dynamically control the methylation of CG13 is supported by the observation that **ELS** induced contemporaneous increases in **CaMKII** activation, MeCP2-S438 phosphorylation and **Avp** expression in 10-d-old mice. On the other hand, MeCP2-S438 and **CaMKII** were phosphorylated to similar extents in adult control and **ELS** mice, indicating that **ELS**-induced **MeCP2** phosphorylation is important for the establishment of epigenetic marks. Once established, the observed differences in **Avp** enhancer methylation centered on **MeCP2** binding sites, which appeared to be actively maintained in **ELS** and control mice. This interpretation is compatible with the view that **MeCP2** serves as an epigenetic integration platform on which synergistic cross-talk between histone deacetylation, H3K9 methylation and **DNA methylation** act to confer gene silencing[22].

From a physiological perspective, it is conceivable that increased methylation of the **Avp** enhancer during early postnatal life serves to restrain the **HPA axis** in critical periods when homeostatic thresholds are set; this would facilitate adaptation of the endocrine system to future environmental stimuli. Our data suggest that **ELS** tilts the balance toward persistent hypomethylation and **Avp** overexpression by inducing reductions in **MeCP2** binding. Thus, phosphorylation of **MeCP2** appears to be a conduit of experience-driven changes in gene expression, serving as an important mediator of the persistent effects of **ELS**. In this respect, certain parallels may be drawn between the mechanisms underlying **ELS** and **Rett syndrome**; the latter, caused by mutations in Mecp2, also presents with altered cognitive, mood and **HPA axis** function [36].

> Together with other recent work[3],[10], our results suggest that adverse events in early life can leave persistent epigenetic marks on specific genes that may prime susceptibility to neuroendocrine and behavioral dysfunction.

Focusing on **DNA methylation**, our results provide evidence for postmitotic epigenetic modifications in neuronal function; such modifications can serve to facilitate (or disfavor) physiological and behavioral adaptations [3],[37]. These marks and their initiators, mediators and readers (for example, **MeCP2**) provide new inroads for understanding the molecular basis of stress-related disorders of the brain.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

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

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AUTHOR CONTRIBUTIONS

The study was conceived and designed by D.S. and O.F.X.A. C.M. and D.S. designed and interpreted the molecular studies that were carried out by C.M., Y.W., Y.B. and D.F., A.V.P. and O.F.X.A. were responsible for the neuroendocrine studies and A.V.P. and V.M. carried out the behavioral experiments under the guidance of C.T.W. C.M., A.V.P., F.H., O.F.X.A. and D.S. wrote the paper, with input from all of the other authors.

ONLINE METHODS

ELS

Maternal separation stress [11,15] was used to induce **ELS**. Briefly, pups delivered (postnatal day 0 (PO) on day of birth) by timed-pregnant C57BL/6N mice (Charles River) were placed, as individual litters, in a clean cage (with heating pad) for 3 h each day on P1-10, having no physical contact with their mothers. Control (non-ELS) pups remained undisturbed in the maternal nest throughout. Pups remained with their mothers until weaning (P21), when they were housed in sex-matched groups (3-5 mice per cage); only males were used for analyses. Standard laboratory animal housing conditions were maintained throughout, with 12-h daily illumination (lights on at 06:00). All procedures were approved by the Regierung von Oberbayern and were in accordance with European Union Directive 86/609/EEC.

Behavioral phenotyping

At 3 months of age, control and **ELS** mice were housed singly and randomly assigned to the following long-term treatment groups: naive (no injections), vehicle (5% DMSO (vol/vol), 5% Chremophor EL (vol/vol), saline, intraperitoneal injection), and SSR149415 (20 mg per kg of body weight per d). Treatments started 4 weeks before behavioral phenotyping and continued for the duration of the experiments; injections were administered 1 h before behavioral testing. An investigator who was blind to the treatments carried out the behavioral analyses; an interval of 2 d was allowed between each test procedure. Anxiety-like behavior was assessed in the elevated plus-maze[38], light-dark avoidance [39] and novelty-induced hypophagia [40] tests. The forced swim test was used to evaluate passive stress coping behavior and was performed essentially as described previously[11]. Briefly, each mouse was placed into a glass beaker (51×23.5×16.5 cm) that was filled with water (25±1◦C) up to a height of 15 cm, for 6 min. Floating (immobility) was scored during the last 4 min of the exposure. A mouse was considered to be immobile when it floated passively in an upright position, making only small movements to keep its head above water surface. Memory was evaluated using the step-down avoidance learning test. Training sessions involved placing mice onto a platform (2.5×10×10 cm3) and administering a scrambled electric foot shock (0.7 mA) when they stepped off the platform onto a metal grid; mice were thereafter immediately returned to their home cages. Passive avoidance memory was tested by placing mice back onto the platform 24 h later and step-down latencies (four-paws criterion)

were measured in three consecutive trials. Trials were terminated after 5 min in cases of failure to step down; a step-down latency of 301 s was ascribed to the trial. The mean of the three trials served as a measure of memory performance.

Tissue preparation and hormone assays

Serum **corticosterone** was measured in adulthood by radioimmunoassay in blood samples. Samples were collected at 6 p.m. (peak) and 30 min following application of a previously described acute psychological stressor (9−11 a.m.)42. At the age of 3 months, mice received an intraperitoneal injection of dexamethasone (10 µg per 100 g) at 12 a.m. noon and blood was collected at 6 p.m. for determination of **corticosterone**; the latter measurements were compared to values obtained at the nocturnal sampling on the previous day. At various ages, mice were killed by cervical dislocation and tissues (brain, pituitary, thymus and adrenal) were collected. Brains for ISH and micro-punching were cryosectioned (10 µm) at the level of the rostral PVN (bregma -0.75 to-0.85) and the hippocampus (bregma-1.70 to-1.90). Punches of the PVN were obtained by in loco microdissection under histological control.

ISH

Avp and Crh transcripts were detected with 48/50-mer 35S-labeled antisense probes, complementary to the murine **Avp** (bases 1,493-1,540, accession number M88354) and Crh (bases 1,685-1,732, accession number AY128673) genes, respectively. Nr3cl and Mecp2 transcripts were measured using ribonucleotide probes (Nr3c1, bases 81-528, accession number M14053; Mecp2, bases 612-1,604, accession number NM010788) and previously published protocols [42]. **Avp**, Crh and Mecp2 transcript signal intensities were measured in the ventromedial compartment of the PVN, representing the parvocellular division. Nr3c1 hybridization signals were measured in the PVN and hippocampal subfields CA1-3 and dentate gyrus.

Bisulfite sequencing

Genomic DNA (200-400 ng) isolated from PVN tissue punches was digested with EcoRI, sodium bisulfite converted (Qiagen **DNA methylation** kit), aliquoted and used for PCR reactions. Primers used are listed in Supplementary Table 1. Products were cloned into PGEM-T vector; at least 20 independent recombinant clones per PCR and mouse were analyzed on an ABI Prism 3700 capillary sequencer. Overall methylation levels (Fig. 3d) were calculated for the entire enhancer region from mean levels of individual CpG residues.

RT-PCR and RNA extractions

Primer sequences are listed in Supplementary Table 2. The expression levels of the housekeeping genes Hprt and Gapdh were used for normalization. Total RNA was extracted with Trizol (Invitrogen) and reverse-transcription reactions were performed on 1 µg of total cell culture-extracted RNA or 100 ng of tissue-derived RNA with SuperscriptII (Invitrogen) and poly-dT primer. qPCR was carried out on a LightCycler (Roche) using LightCycler FastStart DNA Master Plus SYBR Green (Roche).

In vitro methylation

Vectors were methylated with Sssl and S-adenosylmethionine (New England Biolabs). For site-specific methylation, an 888-bp fragment containing CpGs 10-25 in the **Avp** enhancer was excised by digestion with Eco811; the vector was further digested with Xbal and BamHI to

prevent re-ligation. Methylated or control unmethylated digests were ligated into the dephosphorylated reporter construct, cleaved with Eco811. Completeness of in vitro methylation and maintenance (until cell harvesting) was confirmed by bisulfite sequencing.

Recombinant proteins and EMSA

GST- or His-**MeCP2** fusion proteins were grown in DH50, purified and quantified as described previously [43]. For in vitro DNA-binding assays (Fig. 4j and Supplementary Fig. 8), recombinant **MeCP2** (0.5 µg) was incubated with 20,000 cpm of double-stranded 32P end-labeled naive or in vitro methylated oligonucleotides[27]. Reactions were fractionated on 8% polyacrylamide gels. Although GST protein itself does not recognize methylated CpG10, inclusion of a GST antibody abolished **MeCP2** binding, verifying the identity of the shifted complex (Fig. 4j). Dissociation constants (KD) were deduced by Scatchard analysis of saturation binding isotherms [43].

Plasmids

The AVP expression vectors (kindly gifted by H. Gainer and R.L. Fields, US National Institutes of Health) were modified by exchanging the egfp reporter gene in the third exon of the **Avp** gene [44], with a cDNA for Gaussia luciferase KDEL encoding intracellular Gaussia luciferase (Targeting Systems) (see Fig. 4c). The parent **Avp**-Gaussia construct contained 288 bp of the promoter region, all exons (numbered) and introns, and the entire 2.1-kb enhancer. The AvpAenhancer construct has the entire enhancer sequence removed, whereas the AvpA10-25 was generated by deletion of an 888-bp fragment of the enhancer containing CpGs1-25 by digestion with Eco811 and subsequent vector religation.

Cell culture and transfection experiments

Mouse hypothalamic cells (N6 line) [26] were grown using standard conditions (DMEM supplemented with 10% fetal calf serum, vol/vol). Cells (105) were treated for 5 consecutive days with different concentrations of 5-azacytidine (Calbiochem), which was replenished in fresh growing medium every other day. N6 cells were transfected using Lipofectamine 2000 (Invitrogen). Briefly, 8×105 cells were seeded 24 h earlier in 6-well plates. DNA was mixed with 4 µl of Lipofectamine, incubated at 25◦C for 20 min and then added to the cells, which were grown for 18 h. Epithelial kidney cells (LLC-PK1, ATTC CL-101) were transfected by electroporation as described previously [49]. For cotransfection, we used 0.1 µg of the Camk2a expression constructs, 1 µg of the PRK7-Flag Mecp2 constructs and 1 µg of the Mbd1, Mbd2 and Mbd3 expression vectors. Luciferase values were normalized against -galactosidase values [42].

Immunohistochemistry, immunoblots and ChIP experiments

Brains were extracted from microwave-fixed heads, placed overnight in 4% paraformaldehyde (wt/vol), cryo-preserved and sectioned (20 µm) at the level of the PVN before immunostaining. Immunoblot analysis was carried out on whole-cell extracts (50 µg) after fractionation by PAGE gel electrophoresis [43]. For ChIP experiments, chromatin from N6 cells or mouse PVN punches (individual pools formed from groups of three or five mice for 6-week-old and 10-d-old mice, respectively) was cross-linked[50], disrupted by sonification (Diagenode Bioruptor, and purified with the Magna ChIP G kit (Millipore). The ChIP primers that we used for qPCR are listed in Supplementary Table 3.

Antibodies

The antibodies that we used are listed in Supplementary Table 4. The polyclonal antibody to **MeCP2**, which recognizes **MeCP2** (accession number GI:123122664) irrespective of its phosphorylation status, was generated by injecting New Zealand White rabbits with the KLH-conjugated peptide NH2-CSMPRPNREEPVDSRTPV-CONH2, corresponding to amino acids 480-496. The antiserum was purified by affinity chromatography on a column containing coupled **MeCP2** 480-496 peptide and the affinity-purified antibody to **MeCP2** was eluted. The polyclonal antibody to phosphor-S438-**MeCP2** (MeCP2-pS438) was generated by injecting New Zealand White rabbits with the KLH-conjugated peptide NH2-CMPRGGpSLES-CONH2. The antiserum was purified by affinity chromatography on a column containing coupled nonphosphorylated MeCP2-S438 peptide. The flow through was then passed over a second column containing coupled phosphorylated MeCP2-S438 peptide and the affinity-purified antibody to MeCP2-pS438 was eluted.

Statistical analysis

Numerical data were analyzed by tests or ANOVA, followed by Newman-Keuls post hoc test. In all cases, the nominal level of significance was $P \leq 0.05$. Correlations between AVP expression and CpG methylation were tested by Pearson's correlation coefficient.

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