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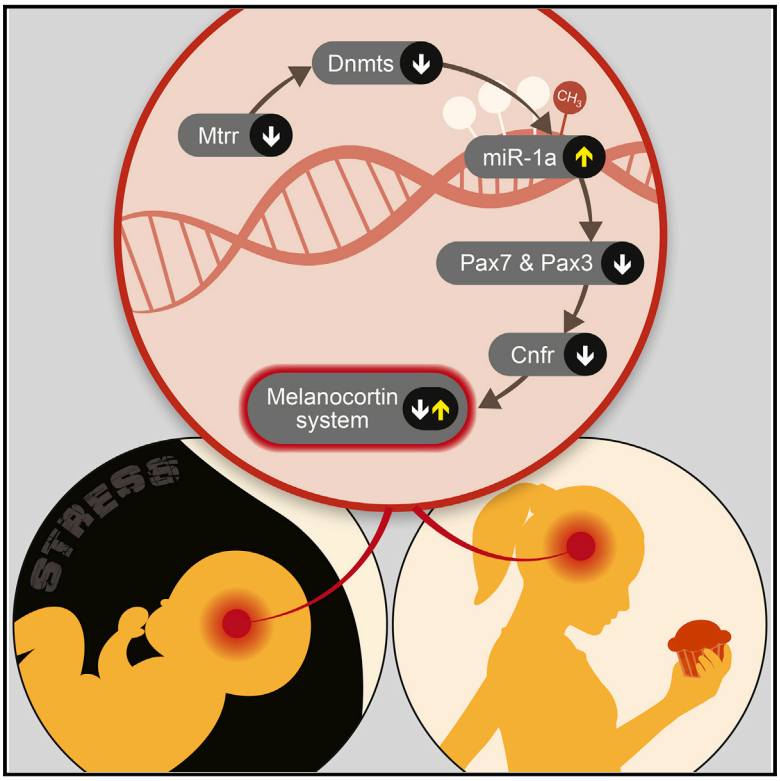
Cell Metabolism

A Methyl-Balanced Diet Prevents CRF-Induced Prenatal Stress-Triggered Predisposition to Binge Eating-like Phenotype

Graphical Abstract



Authors

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In Brief

Binge eating seems to mostly affect females. Schroeder et al. show that that late gestation prenatal stress rewires neural circuits in female mice, leading to binge-like behavior, if triggered during adolescence. Remarkably, this predisposition to binge eating can be prevented by a balanced, methyl-rich diet.

Highlights

- Prenatal stress predisposes females to binge-eating-like behavior
- Binge eating stems from hypothalamic programming at multiple epigenetic levels
- BE can be prevented by a balanced diet in adolescence

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A Methyl-Balanced Diet Prevents CRF-Induced Prenatal Stress-Triggered Predisposition to Binge Eating-like Phenotype

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SUMMARY

Binge eating (BE) is a common aberrant form of eating behavior, characterized by overconsumption of food in a brief period of time. Recurrent episodes of BE constitute the BE disorder, which mostly affects females and is associated with early-life adversities. Here, we show that corticotropin releasing factor (CRF)-induced prenatal stress (PNS) in late gestation predisposes female offspring to BE-like behavior that coincides with hypomethylation of hypothalamic miR-1a and downstream dysregulation of the melanocortin system through Pax7/Pax3. Moreover, exposing the offspring to a methyl-balanced diet during adolescence prevents the dysregulation and predisposition from being triggered. We demonstrate that gestational programming, per se, will not lead to BE-like behavior, but pre-existing alterations due to prenatal programming are revealed only when challenged during adolescence. We provide experimental evidence for long-term epigenetic abnormalities stemming from PNS in predisposing female offspring to BE disorder as well as a potential non-invasive prevention strategy.

INTRODUCTION

Binge eating (BE) is a common form of aberrant eating behavior (with a lifetime prevalence in women of 1.9%–3.6%; Smink et al., 2012) characterized by the compulsive consumption of large amounts of high fat/carbohydrate/sugar food in a brief time period. Frequent episodes of BE constitute the binge eating disorder (BED), which is 2.5 times more common in women than men (https://www.nimh.nih.gov). BED was recently added to the DSM-V as an independent diagnosis (Brewin et al., 2014). Like other eating disorders (EDs), BED typically has its onset in late adolescence (Smink et al., 2012) and has been associated with early-life stress, such as childhood trauma (Groleau et al., 2012) and obstetric complications (Favaro et al., 2006). Indeed, early-life stress increases the probability of developing an ED by 3–4 times (Rayworth et al., 2004).

It is widely acknowledged that perinatal stress impacts the developing fetus and increases susceptibility for various psychiatric and metabolic disorders (Bale et al., 2010). Intriguingly, a suboptimal prenatal or postnatal environment may predispose males and females to different diseases (Davis and Pfaff, 2014).

In this study, we aimed at exploring the mechanistic link between a CRF-induced model of gestational stress and predisposition to BE-like phenotype in female mice offspring. We used a well-established protocol to induce BE-like phenotype in mice that does not require food deprivation or stressors (Czyzyk et al., 2010) and is characterized by repeated and robust BE episodes and weight gain, similarly to the human condition (Brewin et al., 2014). We examined the mechanism underlying the stress-induced predisposition to BE-like phenotype, focusing on hypothalamic adaptations, and then explored a non-invasive approach of rescuing the offspring's BE-like phenotype.

To our knowledge, no other study has attempted to elucidate the central adaptations resulting from prenatal stress (PNS) that predispose the offspring to develop BE-like phenotype. We report that PNS, triggered by overexpression (OE) of maternal corticotropin releasing factor ($_{\text{CRF-induced}}$PNS), during late gestation affects the hypothalamus of female offspring, altering the expression levels of genes responsible for DNA methylation and predisposes adolescent female offspring to BE-like phenotype. By providing predisposed female offspring in adolescence with a diet balanced in methyl donors (MDs), we were able to rescue the BE-like phenotype. We further suggest that the BE-like phenotype is caused by abnormalities in the melanocortin system and a further cascade of hypothalamic events originating in abnormal methylation patterns that can be mimicked in vitro by global reduction in DNA methylation enzymes. As such, we have revealed a potential mechanism of gestational programming of BE-like phenotype and a non-invasive prevention strategy for adolescent females epigenetically predisposed to BED.

RESULTS

CRF-Induced PNS Prompts BE-like Behavior in Female Offspring

As a model of gestational stress, we manipulated CRF in the brain of pregnant females using a previously established

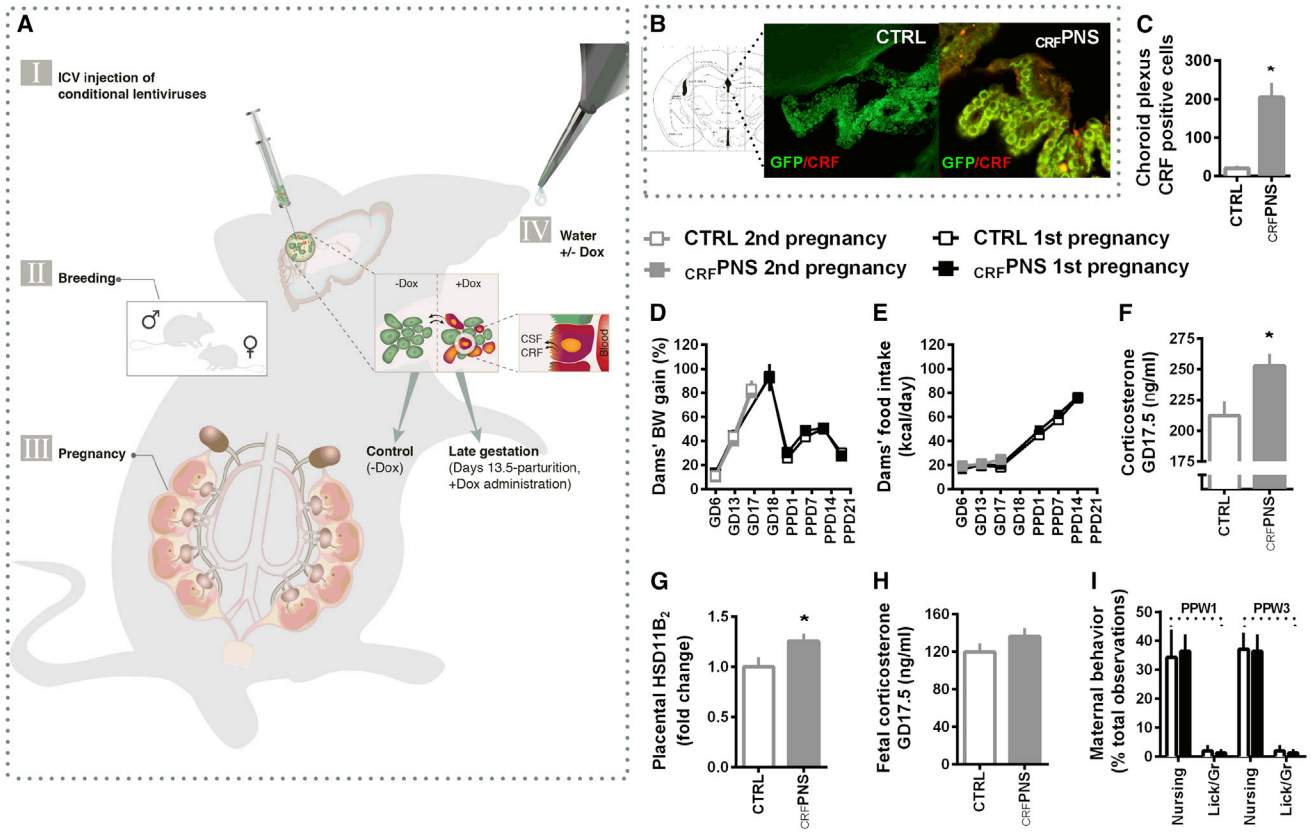

Figure 1. CRF-OE during Pregnancy Induces PNS without Affecting the Dam's Postnatal Phenotype

(A) Experimental design.

(B) Representative picture of the infected choroid plexus with and without Dox.

(C) Dox increased the number of CRF-positive cells in the choroid plexus of the pregnant dams ($t_{(4)} = 4.887$, $p < 0.01$, n = 3 per group).

(D and E) Dam's body weight gain (D) and caloric intake (E) were similar between the groups during pregnancy and lactation.

(F) Dams' circulating corticosterone levels on GD17.5 were higher in the $_{CRF\text{-}induced}$PNS group compared to CTRLs ($t_{(11)} = 2.682$, $p < 0.05$).

(G) Placental 11βHSD$_2$ was increased in $_{CRF\text{-}induced}$PNS pregnancies ($t_{(22)} = 2.101$, $p < 0.05$, n = 12 CTRLs, n = 12 CRF-OE).

(H) Fetal corticosterone levels were similar between CTRL and $_{CRF\text{-}induced}$PNS fetuses on GD17.5.

(I) Maternal behavior was not affected by CRF-OE. Dams: n = 6–7.

PPD, postpartum day; GD, gestation day; PPW, postpartum week; CTRL, control. Data are presented as mean ± SEM. *$p < 0.05$.

inducible genetic system. After being treated with the inducer, mice with specific CRF-OE in the choroid plexus display increased levels of anxiety-like behavior (Regev et al., 2010). Here, we provided the inducer Dox at a non-toxic dose (Moutier et al., 2003) during late pregnancy (days 13.5 to parturition, Figure 1A). Induction of CRF-OE increased CRF secretion in the choroid plexus (Figures 1B and 1C), an effect that lasts for at least 12 hr following Dox administration (Figure S1A). We chose late gestation since it is a critical time window for stress reactivity (Bale, 2015). The females were mated with the same male and exposed to the same treatment twice, producing two consecutive litters: the first litters were born and subjected to adult testing; the second litters were sacrificed at gestation day 17.5 (GD17.5) for tissue collection. Body weight and food intake of the dams during pregnancy were similar for both groups (Figures 1D and 1E). As expected, circulating corticosterone levels were higher in CRF-OE dams (Figure 1F). Placental *Hsd11B$_2$*, a glucocorticoid-inactivating enzyme, was increased to protect the fetuses (Figure 1G) (Vaughan et al., 2012), which showed normal corticosterone levels (Figure 1H) but a dysregulated CRF system (Figure S1B). Increased corticosterone levels in pregnant mice has been shown to reduce the amino acid supply and density of blood vessels in the placenta, an outcome that directly affects the fetal environment and its development (Vaughan et al., 2012). Maternal behavior did not differ between groups (Figure 1I). Further parameters, such as gestation length, pup number, sex ratio, and placental and fetal weight, were also unaffected between groups (Figure S1C).

On postpartum week (PPW) 5, the female offspring of stressed ($_{CRF\text{-}induced}$PNS) and control (CTRL) dams were exposed to a modified version of an established "limited access" model for the development of BE-like phenotype (Corwin and Buda-Levin, 2004; Czyzyk et al., 2010), which combines the punctual access to highly caloric foods (Kales, 1990), in this case western diet (WD) in parallel to ad libitum standard chow access. This "nutritional challenge" was designed to mimic the recurrent dietary self-restrictions (i.e., limiting consumption of palatable food) that adolescent girls frequently engage in and are an integral part of

all EDs. Specifically, the offspring were habituated to WD for 5 days, switched back to chow, and then allowed access to WD for 2 hr a day, 3 days a week for a period of 4 weeks (a total of 12 BE sessions) (Figure 2A). In order to control for the effects of the exposure to WD on the parameters examined, an additional group of females was exposed to chronic WD access. Thus, from every CTRL and $_{CRF\text{-}induced}$PNS litter, randomly chosen females were allocated to the CTRL chow (CH), WD, or BE group. In the first week after weaning, all female pups displayed similar body weight and consumed similar amounts of food (Figures 2B, 2C, and S2A). The "limited access" protocol induced BE-like phenotype in all adolescent females. However, exaggerated eating in $_{CRF\text{-}induced}$PNS offspring subjected to the BE protocol developed instantly, as reflected by the amount of WD consumed during the habituation period (Figure 2D). This overconsumption in the $_{CRF\text{-}induced}$PNS group continued into the following weeks of the manipulation, while CTRLs showed a gradual increase in total food intake across the weeks (Figure 2E) that resulted from greater WD intake (Figure 2F). To examine whether BE-like phenotype became a chronic state, we exposed all BE females to a further 2 hr BE re-exposure session a month after the end of the protocol, when intake of chow had returned to normal (Figure 2G) and body weight between the groups was also similar (Figures 2H and S2A). During this re-exposure, both groups that had previously been exposed to the BE protocol displayed robust hyperphagia compared to females on chow or on chronic WD, despite having ad libitum chow access. However, $_{CRF\text{-}induced}$PNS BE offspring showed an even greater occurrence of BE-like phenotype compared to the CTRL BE females (Figure 2I).

Sensitivity to BE-like Phenotype Stems from Abnormalities at the DNA Methylation Level In Utero

In order to find possible mediators of the link between $_{CRF\text{-}induced}$PNS and BE-like phenotype, we focused on hypothalamic DNA methylation. Tissue-specific alterations in DNA methylation have been recurrently reported in previous PNS studies, pointing at an adverse gestational environment as a contributing factor in the development of various psychiatric disorders (Booij et al., 2013; Vinkers et al., 2015). We focused on the hypothalamus given its function in the regulation of energy balance through integration of central and peripheral signals that control food intake and metabolism. We examined the expression levels of the methyltransferases responsible for de novo methylation, *Dnmt3a* and *Dnmt3b*, and the *maintenance* methyltransferase *Dnmt1*, all of which are essential for normal mammalian development and function (Li et al., 1992; Moore et al., 2013). $_{CRF\text{-}induced}$PNS reduced all three *Dnmts* in the hypothalamus of the exposed fetuses (Figures 2J–2L). To confirm the stress-related impact of our $_{CRF\text{-}induced}$PNS model on the fetal *Dnmts* downregulation, we examined the genes' hypothalamic expression in a parallel set of fetuses, which were exposed to chronic variable stress (CVS) protocol during the same developmental period. Similarly to $_{CRF\text{-}induced}$PNS, all *Dnmts* were downregulated (Figures 2J–2M and S2B). Remarkably, the Dnmts recovered and were similar to CTRLs in adult $_{CRF\text{-}induced}$PNS female offspring on chow and chronic WD. In contrast, exposure to the BE protocol induced downregulation of all *Dnmts* in $_{CRF\text{-}induced}$PNS BE females (Figures 2J–2L), exposing the programmed vulnerability of this mechanism to the nutritional challenge. To examine this further, we measured the expression levels of methionine synthase reductase (*Mtrr*), an enzyme responsible for the activation of methionine synthase that is required for normal progression of the folate and methionine cycles. *Mtrr* deficiency causes abnormal folate metabolism in mice, increases homocysteine (Hcy), and induces DNA hypomethylation (Padmanabhan et al., 2013). Hcy, a sulfur-containing non-protein-forming amino acid, occurs naturally in plasma and is biosynthesized as an intermediate in the one-carbon pathway from methionine. It is produced as a byproduct of the methyltransferase reaction and can therefore reflect alterations in DNA methylation (Mandaviya et al., 2014). Moreover, increased levels of plasma Hcy reflect abnormalities in folate metabolism (Padmanabhan et al., 2013). Strikingly, $_{CRF\text{-}induced}$PNS reduced *Mtrr* in the fetuses' hypothalami, similarly to CVS (Figures 2M and S2B). *Mtrr* levels were normal in adult $_{CRF\text{-}induced}$PNS females exposed to chow or WD, but $_{CRF\text{-}induced}$PNS BE females on chow displayed reduced *Mtrr* compared to CTRLs (Figure 2M). We next examined plasma Hcy levels in the adult offspring and found high circulating levels in $_{CRF\text{-}induced}$PNS BE compared to CTRL BE females on chow. In unchallenged females on chow or WD, Hcy levels were normal (Figure 2N). Finally, we examined global hypothalamic DNA methylation and found no differences between CTRL and $_{CRF\text{-}induced}$PNS fetuses (Figure 2O), suggesting that changes in methylation resulting from the reduction in all *Dnmts* may be specific to particular genes. Thus, sensitivity to BE-like phenotype appears to stem from abnormalities at the DNA methylation level in utero (or potentially histone methylation), inducing a cascade of events that may pave the way for the different response during adolescence.

A Methyl-Balanced Diet during Adolescence Normalizes the Expression of DNA Methylation Enzymes and Prevents BE-like Behavior

To investigate whether alterations in DNA methylation were the origin of the adaptations leading to sensitivity to BE-like phenotype, we designed a follow-up study in which we aimed at changing DNA methylation by manipulating the availability of MDs through the animals' diet. Dietary MDs are necessary for the one-carbon metabolic pathway that produces S-adenosyl-methionine, the universal MD essential for the DNA methylation process. The effects of reducing/increasing the availability of certain MDs in the maternal diet during gestation are known to affect the offspring in the long term by changing DNA methylation and *Dnmts* levels (Cordero et al., 2013). However, a few studies have recently suggested that MDs can have also considerable effects when provided to animals after weaning (Bermingham et al., 2013; Dahlhoff et al., 2014). With this objective, we challenged CTRL and $_{CRF\text{-}induced}$PNS BE-like phenotype female offspring using a different background diet other than standard chow during the BE protocol (Figure 3A). Standard chow contains high levels of vitamin B12, B1, B2, B6, folic acid, methionine, and zinc according to the recommended values for laboratory animals (National Research Council, 1995; Ritskes-Hoitinga, 2004; Table S1), all of which play key roles in the folate/methionine metabolic pathway (Su et al., 2012). These amounts of MDs and co-factors may affect DNA methylation in animals that were previously sensitized in utero. Therefore, we chose a background diet instead of the

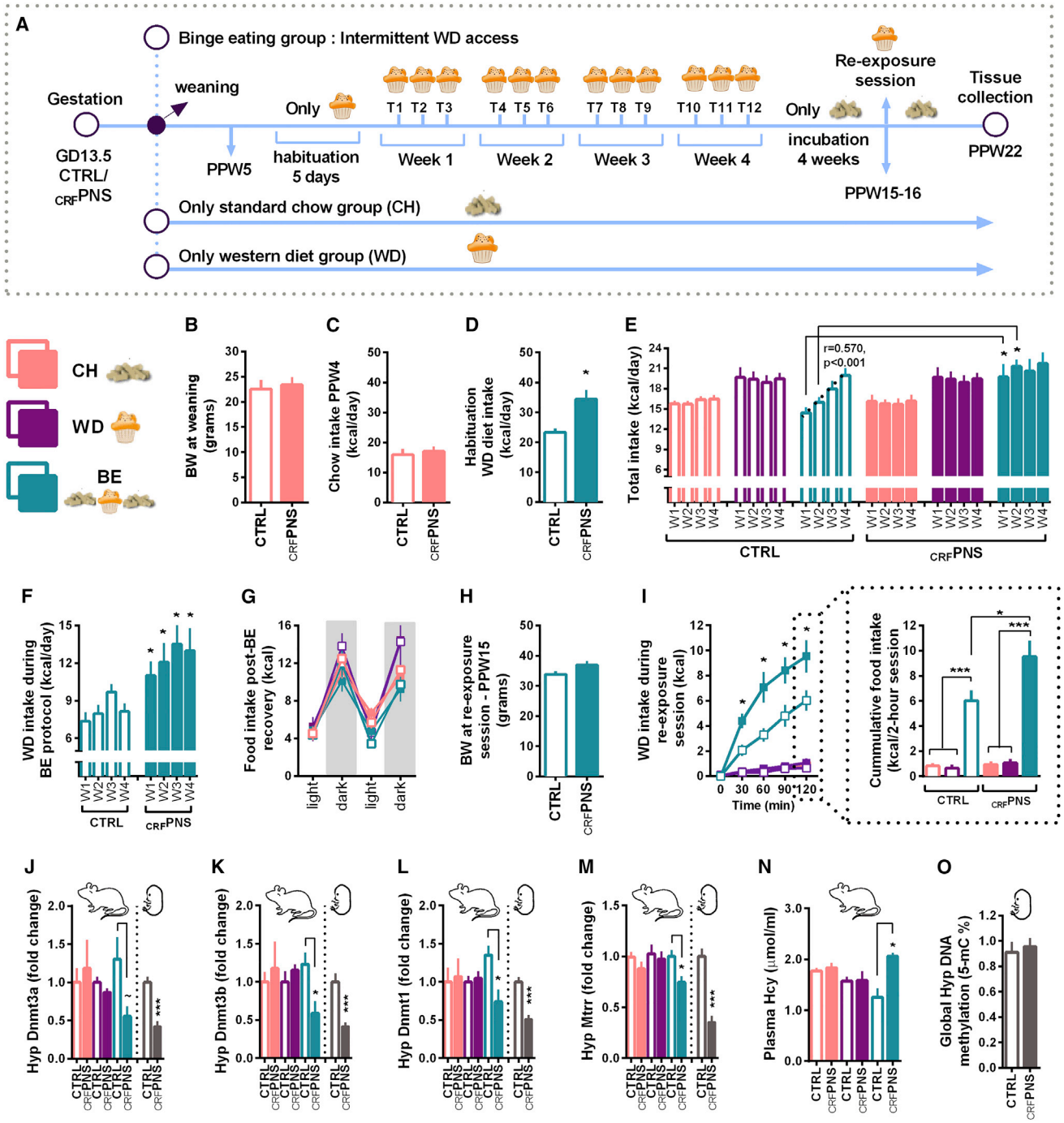

Figure 2. CRF-Induced PNS Predisposes Female Offspring to BE-like Behavior and Dysregulates the Hypothalamic DNA Methylation Enzymes

(A) Research outline.

(B and C) BW at weaning (B) and food intake (C) were similar between CTRL and PNS ($_{CRF\text{-}induced}$PNS) groups during the first week after weaning.

(D) Intake of western diet (WD) was higher in $_{CRF\text{-}induced}$PNS females during the habituation period ($t_{(20)} = 3.193$, $p < 0.01$).

(E) Repeated-measures MANOVA showed that total caloric intake during the 4 follow-up weeks was similar between CTRLs and $_{CRF\text{-}induced}$PNS females on chow or chronic WD but was gradually increased in CTRL BE females and was constant and high in $_{CRF\text{-}induced}$PNS females ($F_{int(6,106)} = 2.74$, $p < 0.05$, $n = 10$).

(F) Repeated-measures ANOVA showed that WD intake was increased in BE females subjected to $_{CRF\text{-}induced}$PNS ($F_{(1,18)} = 7.19$, $p < 0.05$, $n = 10$, each time point compared individually by post hoc t tests).

(G and H) Food intake (%BW) (G) and BW (H) were similar among the groups 4 weeks after the end of the BE protocol and previous to the re-exposure session.

(legend continued on next page)

standard chow that provided the animals with the necessary amounts of MDs and vitamins for them to be healthy, with *no excess* and *no deficiency* ("balanced methyl diet," D10012G). Thus, in this second experiment, we exposed all CTRL and $_{CRF\text{-}induced}$PNS females to the BE protocol but provided some with standard chow and some with the "balanced" diet as the background food (Figure 3A). We then allowed them to recover, tested them again in a "relapse" session, and sacrificed them 2 months later in order to examine the DNA methylation enzymes. While $_{CRF\text{-}induced}$PNS females on chow showed a similar pattern of expression as in the first experiment, $_{CRF\text{-}induced}$PNS BE females fed the "balanced" methyl diet showed normal expression levels of all three *Dnmts* (Figures 3B–3D) and *Mtrr* (Figure 3E), which reflects normal progression of the hypothalamic folate and methionine cycles and the methyltransferase reaction. Accordingly, plasma Hcy was also normalized (Figure 3F), further reflecting the improvement in folate/methionine metabolism. We next explored whether these changes had any effect on feeding behavior under the BE protocol. $_{CRF\text{-}induced}$PNS females fed the "balanced" methyl diet showed a gradual reduction in food consumption across the weeks, reaching caloric intake levels similar to those of the CTRL BE group at the beginning of the protocol (Figure 3G). In addition, while WD intake in the relapse session was high in all groups, reflecting the effectiveness of the protocol, the overconsumption observed in $_{CRF\text{-}induced}$PNS BE females on chow was abolished with the "balanced" diet (Figure 3H). These differences may result from the amounts of MDs and co-factors consumed by females in the chow group, which were greater than the amounts consumed by females in the "balanced" group. In particular, $_{CRF\text{-}induced}$PNS BE females on chow consumed more methionine and choline compared to CTRLs, while in the "balanced" groups both $_{CRF\text{-}induced}$PNS and CTRL females consumed similar amounts of MDs (Table 1).

BE-like Behavior Coincides with Upregulation of Hypothalamic miR-1a and Downstream Dysregulation of the Melanocortin System through Pax7/Pax3 and Cnfr

To further examine the hypothalamic adaptations stemming from abnormal DNA methylation that lead to BE-like phenotype, we focused on the hypothalamic melanocortin system, which plays a central role in body weight and food intake through regulation of appetite and energy expenditure and includes a number of neuropeptides and receptors responsible for the central integration of peripheral signals (Williams and Elmquist, 2012). Abnormalities in this system have been reported for all EDs (Lu, 2001; Helder and Collier, 2011), making it a recognizable mechanistic candidate. We found that $_{CRF\text{-}induced}$PNS increased hypothalamic *AgRP* (Figure 4A), reduced *Pomc* and *MC$_4$R* (Figures 4B and 4D), and did not affect *MC$_3$R* (Figure 4C), *Npy*, or *Cart* (Figure S3A). Expression levels of all examined genes were unaffected in the adult unchallenged $_{CRF\text{-}induced}$PNS offspring, but the exposure to BE protocol in the chow group ($_{CRF\text{-}induced}$PNS BE chow) was associated with a dramatic reduction in *Pomc* and *MC$_3$R* compared to CTRL BE females on chow. In contrast, $_{CRF\text{-}induced}$PNS BE "balanced" females displayed normal levels of all the melanocortin system genes examined (Figures 4A–4D and S3A). Thus, the abnormalities observed in this system are programmed in utero as a result of maternal CRF-OE and can resurface with the BE protocol, potentially underlying the defective coping of these females with the nutritional challenge. However, it was surprising that most of the genes were downregulated in $_{CRF\text{-}induced}$PNS BE chow females, given the observed reduction in the methylation enzymes (Figures 2 and 3), suggesting that the effects on this system were likely mediated indirectly through a more complex process as opposed to direct methylation of *Pomc* as previously described (Cho et al., 2013). This downregulation in *Pomc* lead us to investigate the ciliary neurotrophic factor receptor (*Cnfr*). *Cnf* is a pluripotent neurotrophic factor involved in the development and maintenance of the nervous system. Centrally administered *Cnf* induces neuronal proliferation in the hypothalamus and sustained weight loss long after treatment is terminated (Kokoeva et al., 2005). *Cnfr* is mainly expressed in the Arcuate nucleus (ARC) and paraventricular nucleus of the hypothalamus (PVN), and together with *Cnf*, they translocate to the cell nucleus of *Pomc* neurons, directly initiating *Pomc* transcription and modulating energy homeostasis (Couvreur et al., 2012). Here, we found that *Cnfr* shows a similar pattern of expression as *Pomc*. It is downregulated in $_{CRF\text{-}induced}$PNS fetuses, which likely inhibited *Pomc* transcription, it was normalized in adult unchallenged females, downregulated again by BE protocol on chow, and rescued by the balanced diet (Figure 4E). To further explore potential developmental aspects leading to dysregulation of *Pomc* and *Cnfr*, we next focused on the developmental transcription factor *Pax7*.

Pax7 is a member of the Pax family that specifically targets genes involved in myogenesis, neurogenesis, and neural activity (Mansouri et al., 1994). *Pax7* is essential for CNS development (White and Ziman, 2008), and it is specifically expressed in the developing PVN and the caudo-basal hypothalamus in different species starting on gestation day 13 (Stoykova and Gruss, 1994) (see also http://developingmouse.brain-map.org). More specifically, Pax7 targets the *Cnfr* in the mouse brain (White and Ziman, 2008). Given its function as a regulator of transcription by remodeling chromatin and allowing binding of transcription enhancers (Budry et al., 2012), targeting among others, genes involved

(I) Both CTRL and $_{CRF\text{-}induced}$PNS BE females consumed larger amounts of WD during the 2 hr re-exposure session compared to chow and WD groups. Within the BE group, $_{CRF\text{-}induced}$PNS females consumed more WD than CTRLs, about 50% of their daily caloric intake (main effect $F_{(1,18)} = 8.11$, $p < 0.05$, n = 10, significance between the groups based on Tukey's multiple comparisons test).

(J–M) *Dnmt3a* (J), *Dnmt3b* (K), *Dnmt1* (L), and methionine synthase reductase (*Mtrr*) (M) mRNA were downregulated by $_{CRF\text{-}induced}$PNS in the hypothalamus of female fetuses (main effect of prenatal treatment, $F_{(4,7)} = 6.39$, $p < 0.05$). Adults females exposed to $_{CRF\text{-}induced}$PNS recovered and displayed normal gene expression levels when on chow or WD, but all three *Dnmts* and *Mtrr* reverted to the fetal condition once challenged with the BE protocol as adolescents (MANOVA diet × prenatal treatment interaction; $F_{(8,60)} = 2.30$, $p < 0.05$, n = 6–7).

(N) Plasma homocysteine (Hcy) was similar between CTRLs and $_{CRF\text{-}induced}$PNS on chow and chronic WD but was increased by BE in $_{CRF\text{-}induced}$PNS adults (diet × prenatal treatment interaction; $F_{(2,35)} = 3.50$, $p < 0.05$, n = 6).

(O) Global methylation of the hypothalamus was not affected by $_{CRF\text{-}induced}$PNS in female fetuses (n = 6). Data are presented as mean ± SEM.

PPW, postpartum week; CH, standard chow; WD, western diet; BE, binge eating; W, week; BW, body weight; Hyp, hypothalamus; CTRL, control. *$p < 0.05$, ***$p < 0.001$.

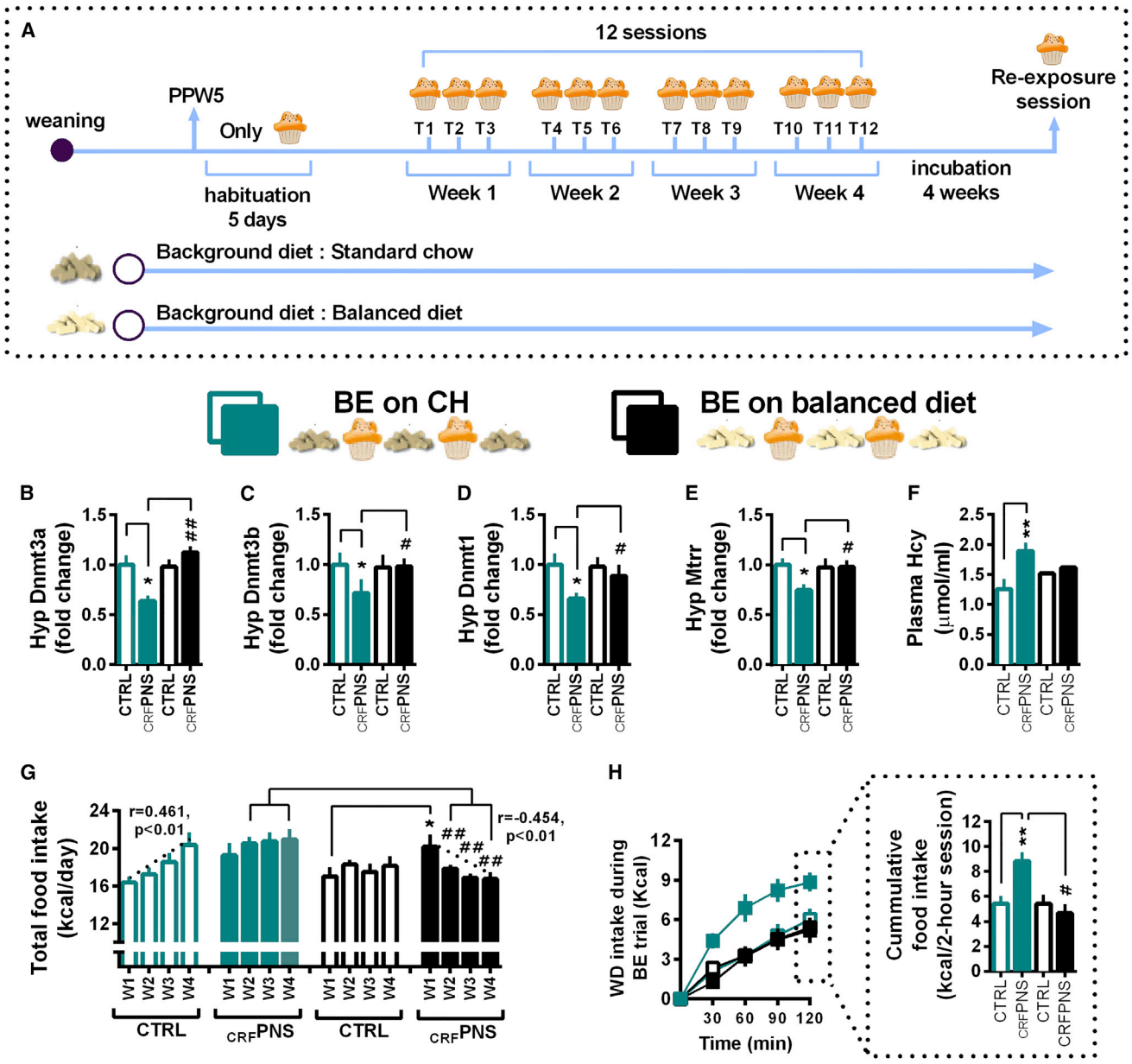

Figure 3. CRF-Induced PNS BE Females on "Balanced" Methyl Donor Diet Displayed Normal Eating Behavior and Hypothalamic Gene Expression

(A) Experimental design. Female offspring of CTRL and $_{CRF\text{-}induced}$PNS dams were exposed to the BE protocol on either standard chow or a balanced methyl donor diet from weaning until sacrifice.

(B–E) Hypothalamic *Dnmt3a* (B), *Dnmt3*b (C), *Dnmt1* (D), and *Mtrr* (E) were normalized in $_{CRF\text{-}induced}$PNS BE "balanced" females and were higher compared to $_{CRF\text{-}induced}$PNS BE chow group (MANOVA, prenatal treatment × diet; $F_{int(4,17)} = 4.31$, $p < 0.05$, n = 6).

(F) Total plasma homocysteine (Hcy) concentration was higher in $_{CRF\text{-}induced}$PNS BE chow females compared to CTRLs but was normalized in the "balanced" diet group (two-way ANOVA, prenatal environment × diet interaction; $F_{(1,23)} = 5.81$, $p < 0.05$, n = 6, with Tukey post hocs).

(G) Repeated-measures two-way ANOVA ($F_{int(3,32)} = 3.05$, $p < 0.05$, n = 8–10) revealed that total caloric intake was gradually increased in CTRLs BE on chow ($r = 0.461$, $p < 0.01$) and was constant and high in $_{CRF\text{-}induced}$PNS BE females on chow. CTRL BE females on "balanced" diet did not develop explicit BE and $_{CRF\text{-}induced}$PNS "balanced" females gradually decreased the caloric intake during the protocol ($r = -454$, $p < 0.01$), reaching CTRL BE-like levels.

(H) The 2 hr re-exposure session 4 weeks after the end of the protocol showed high levels of consumption in all groups. However, in the $_{CRF\text{-}induced}$PNS BE group, "balanced" females consumed less western diet than chow females and similar amounts to CTRLs ($F_{(3,36)} = 7.01$, $p < 0.001$, n = 8–10).

CH, standard chow; CTRL, control. *#$p < 0.05$, **##$p < 0.01$. Data are presented as mean ± SEM.

Table 1. 4-Week Total Intake of MDs Differed according to Diet and Prenatal Treatment

Total 4-week Intake	Control Chow	Control Balanced	CRF-OE Chow	CRF-OE Balanced
Vitamin B12 [μg]	10.00 ± 0.02	2.09 ± 0.01[a]	11.02 ± 0.02	2.23 ± 0.00[a]
Folate [mg]	0.53 ± 0.00	0.19 ± 0.00[a]	0.60 ± 0.00	0.20 ± 0.00[a]
Methionine [g]	0.66 ± 0.00	0.54 ± 0.00	0.79 ± 0.00[b]	0.56 ± 0.00[a]
Cystine [g]	0.37 ± 0.00	0.23 ± 0.00[a]	0.40 ± 0.00	0.25 ± 0.00[a]
Choline chloride [g]	0.19 ± 0.00	0.12 ± 0.00	0.22 ± 0.00[b]	0.12 ± 0.00[a]
Vitamin B6 [mg]	2.35 ± 0.00	0.67 ± 0.00[a]	2.63 ± 0.01	0.70 ± 0.00[a]

Overall, females fed the "balanced" diet consumed less MDs than chow-fed females ($F_{(6,27)} = 292.62$, $p < 0.001$). $_{CRF\text{-}induced}$PNS females on chow consumed more methionine and choline compared to CTRL BE females on chow (post hoc Duncan's). Data are presented as mean ± SEM. *$p < 0.05$ for balanced versus chow diet within prenatal treatment and #$p < 0.05$ for CTRL versus $_{CRF\text{-}induced}$PNS on chow diet.

[a]$p < 0.05$ compared to chow diet within prenatal treatment.

[b]$p < 0.05$ for control chow versus CRF-OE chow.

in metabolic regulation, we reasoned that changes in *Pax7* through *Cnfr* could underlie downregulation of *Pomc* and the consequent alterations in the hypothalamic melanocortin system in $_{CRF\text{-}induced}$PNS females. We found that *Pax7* mRNA was downregulated in $_{CRF\text{-}induced}$PNS fetuses and in adult $_{CRF\text{-}induced}$PNS females on chow, with or without BE protocol (Figure 4F). Thus, this gene appeared to be affected by $_{CRF\text{-}induced}$PNS, an effect that lasted until adulthood. However, unchallenged $_{CRF\text{-}induced}$PNS mice showed normal *Pomc* levels, suggesting that another protein may compensate for the reduction in *Pax7* levels. The natural candidate was the *Pax7* homolog, *Pax3*. Both members of the Pax family are expressed in the developing hypothalamus (Stoykova and Gruss, 1994; White and Ziman, 2008), share the same structure, and overlap in some functions (Lang et al., 2007). Specifically, there is evidence that cross-talk between *Pax3* and *Pax7* may enable a tight, reciprocal regulation of both gene products, resulting in functional compensation when one of the proteins is dysfunctional (Agoston et al., 2012). Pax3, like Pax7, was downregulated in fetuses both at the mRNA and protein levels (Figures 4G and 4H). Remarkably, hypothalamic *Pax3* mRNA was upregulated in unchallenged $_{CRF\text{-}induced}$PNS adults, suggesting a possible compensatory mechanism for the loss of *Pax7*. In contrast, this *Pax3* compensation was not observed in $_{CRF\text{-}induced}$PNS BE females (Figure 4H), resulting in reduced levels of *both* Pax genes in parallel to the reduction of *Cnfr* and *Pomc*. In accordance with the expression levels of *Cnfr* and *Pomc* in $_{CRF\text{-}induced}$PNS BE "balanced" females, both *Pax7* and *Pax3* were normalized (Figures 4F–4H). Thus, *Pax7* and *Pax3*, both involved in chromatin formation (Budry et al., 2012; Bulut-Karslioglu et al., 2012) and remodeling (Bulut-Karslioglu et al., 2012), are affected by PNS, an effect that resurfaced in the presence of nutritional stress (BE) during adolescence.

To investigate the cause of reduced expression of *Pax3* and *Pax7* by $_{CRF\text{-}induced}$PNS and BE protocol, in parallel to the reduction in the Dnmt reaction, we examined the 3′ untranslated regions (3′ UTRs) of both genes in search for evolutionarily conserved 7-nt matches to microRNA (miR) seed regions. Both *Pax7* (Chen et al., 2010) and *Pax3* (Goljanek-Whysall et al., 2011) have been previously validated as targets of miR-1a and miR-206, presenting 4 and 2 conserved seeds, respectively (www.targetscan.org), for the family of miRs-1ab/206. Although expression of these miRs has been reported to be restricted to skeletal muscle, we found them both in the hypothalamus. In accordance with the low levels of both Paxes, both miRs were upregulated following $_{CRF\text{-}induced}$PNS in fetuses (Figures 4I and 4J). MiR-1a recovered in unchallenged $_{CRF\text{-}induced}$PNS adults, became upregulated again by BE protocol in chow females, and was normalized by the balanced diet, mirroring the gene expression patterns of *Pax7* and *Pax3* (Figures 4I). To explore the link between high expression of miR-1a and Pax7 further, we infected N42 embryonic hypothalamic cells (mHypoE-N42) with lentiviruses overexpressing miR-1a or with a scrambled CTRL. The infection led to increased levels of the miR, which in turn reduced the protein level of *Pax7*, an effect that coincided with reduced *Cnfr* and *Pomc* mRNA (Figure S3B).

Altogether, this suggests that in $_{CRF\text{-}induced}$PNS females, the BE nutritional challenge induces miR-1a upregulation in the hypothalamus, which inhibits *Pax3* to compensate for the downregulation of *Pax7* induced by maternal CRF-OE. MiR-206, on the other hand, is constitutively overexpressed in both adult groups on chow once it is activated by $_{CRF\text{-}induced}$PNS (Figures 4J), probably keeping Pax7 levels down. In addition, it does not present nearby CpG islands, suggesting that it may not be directly modulated by DNA methylation, but rather by chromatin level regulation as previously described (Saccone et al., 2014). Both miRs were normalized in $_{CRF\text{-}induced}$PNS BE adults on the balanced diet, suggesting that their upregulation was linked to the abnormalities observed at the DNA methylation level.

CRF-Induced PNS and BE-like Predisposition Are Linked to Alterations Both at the DNA and Histone Methylation Levels in the miR-1a Locus

To examine the link between miR-1a upregulation and abnormalities in methylation, we focused on the regulation of miR-1, which belongs to a specific group of miRs regulated by DNA methylation (Datta et al., 2008; Das et al., 2013). In the hypothalamus, it is specifically expressed in the ARC (Figure 4K). The miR-1a-2 transcript, located in an intron of the *Mib1* gene, presents a nearby CpG island supporting potential regulation by DNA methylation. The second transcript, miR-1a-1, is located in an intron of the *Gm6307* gene and does not present a nearby CpG island. In addition, since we found that *Mib1* is about 1,000 times more abundant in the hypothalamus than *Gm6307* (CT21 and 31, respectively), we focused on the miR-1a-2 transcript. Interestingly, levels of miR-1b and miR-133a-1, which are located in the same genomic cluster with miR-1a-2, were also upregulated

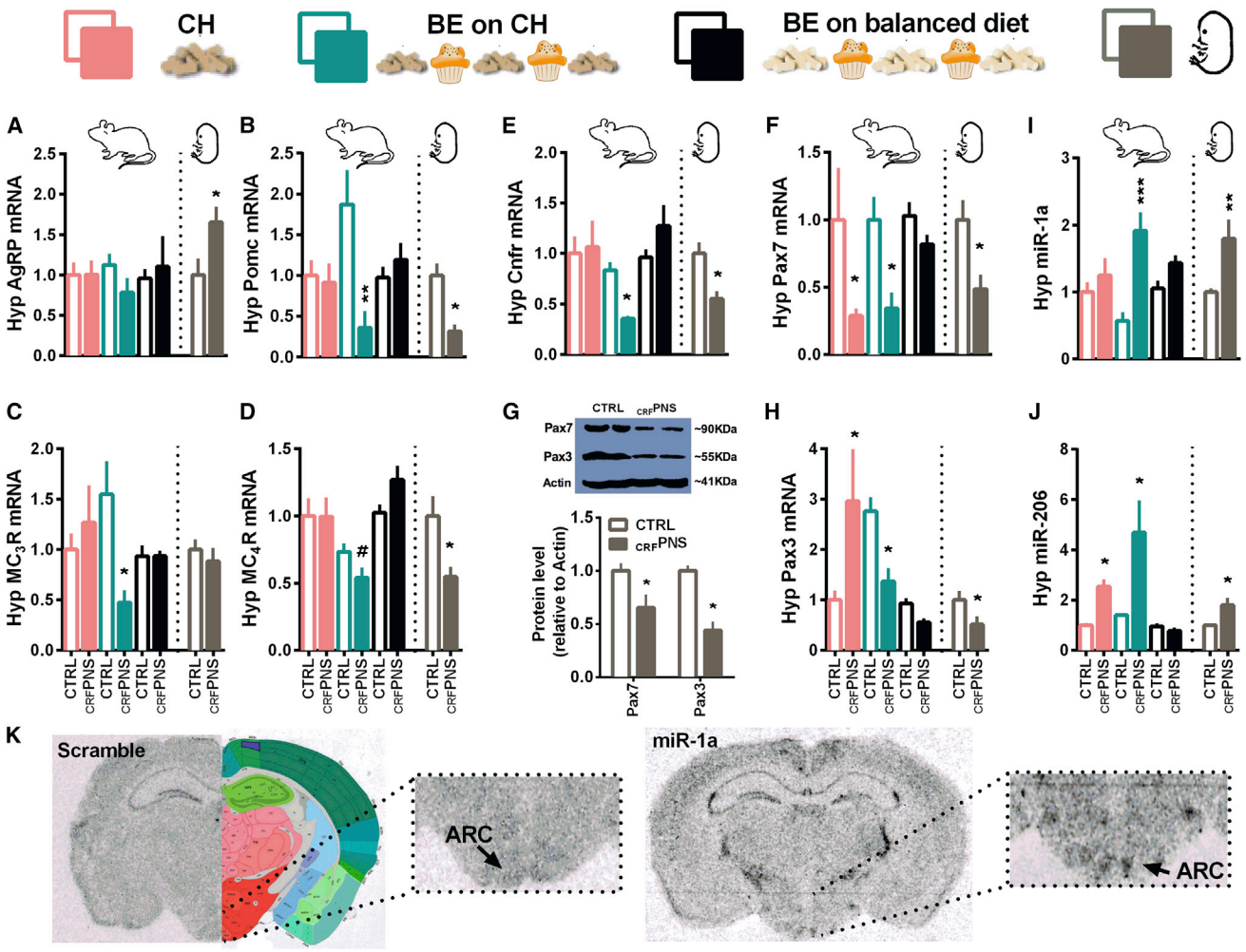

Figure 4. CRF-Induced PNS Leads to Dysregulated Gene Expression in the Melanocortin System, *Cnfr*, *Pax7/3* and miR-1a, an Effect that Can Be Re-exposed by Binge Eating and Normalized by a Balanced Methyl Donor Diet

Hypothalamic gene expression was affected by the prenatal and adolescent nutritional manipulation in adults ($F_{int(18,46)} = 1.99$, $p < 0.05$) and by prenatal treatment in fetuses ($F_{(9,2)} = 4.26$, $p < 0.05$) as reflected by MANOVA.

(A–D) Within the melanocortin system, selective genes showed dysregulation by $_{CRF\text{-}induced}$PNS in the fetuses. These females recovered as adults when unchallenged, but the fetal pattern resurfaced when facing the BE protocol in adolescence. Providing the females with a balanced diet during the BE protocol prevented this dysregulation. Fetal *AgRP* ($F_{(1,\ 11)} = 8.03$, $p < 0.05$) (A). *Pomc* levels in fetuses ($F_{(1,11)} = 17.71$, $p < 0.01$) and adults ($F_{(5,36)} = 3.85$, $p < 0.01$) (B). Adult *MC$_3$R* ($F_{(5,36)} = 2.97$, $p < 0.05$) (C). *MC$_4$R* levels in fetuses ($F_{(1,11)} = 7.67$, $p < 0.05$) and in adults ($F_{(5,35)} = 6.24$, $p < 0.01$) (D).

(E) *Cnfr* expression was reduced in $_{CRF\text{-}induced}$PNS fetuses ($F_{(1,11)} = 12.29$, $p < 0.01$) and $_{CRF\text{-}induced}$PNS BE adults but recovered by the balanced diet ($F_{(5,37)} = 3.54$, $p < 0.05$).

(F) $_{CRF\text{-}induced}$PNS permanently reduced the expression of *Pax7* in all chow adults but was recovered by the balanced diet ($F_{(1,11)} = 13.41$, $p < 0.01$ for fetuses and $F_{(5,37)} = 4.40$, $p < 0.01$ for adults).

(G) Female $_{CRF\text{-}induced}$PNS fetuses showed downregulation in protein levels of Pax7 ($F_{(1,11)} = 5.98$, $p < 0.05$) and Pax3 ($F_{(1,11)} = 35.41$, $p < 0.001$).

(H) *Pax3* mRNA was downregulated by $_{CRF\text{-}induced}$PNS in fetuses ($F_{(1,11)} = 7.24$, $p < 0.05$), upregulated in unchallenged adults, downregulated again by BE, and rescued by the balanced diet ($F_{(5,37)} = 4.15$, $p < 0.01$).

(I and J) MiR-1a and miR-206 were upregulated by $_{CRF\text{-}induced}$PNS in fetuses ($F_{(1,11)} = 12.23$, $p < 0.01$ and in adults ($F_{(1,11)} = 11.85$, $p < 0.01$). MiR-1a recovered in unchallenged adults but was upregulated when exposed to BE and was rescued by the balanced diet ($F_{(5,35)} = 5.85$, $p < 0.001$) (I). MiR-206 remained high in both unchallenged and BE females exposed to $_{CRF\text{-}induced}$PNS but was normalized by the balanced diet ($F_{(5,35)} = 5.86$, $p < 0.001$) (J). CH, standard chow. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ for comparisons between prenatal treatments within each manipulation (chow, BE on chow or BE on balanced diet) and #$p < 0.05$ for comparisons between $_{CRF\text{-}induced}$PNS chow and $_{CRF\text{-}induced}$PNS BE chow. n = 6–7 based on Bonferroni post hocs.

(K) In situ hybridization of miR-1a (right) and a scramble CTRL (left) showed that, within the hypothalamus, miR-1a is expressed in the Arcuate nucleus (ARC). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for comparisons between CTRL and $_{CRF\text{-}induced}$PNS within each diet. #$p < 0.05$ between CH and BE on CH in the $_{CRF\text{-}induced}$PNS group.

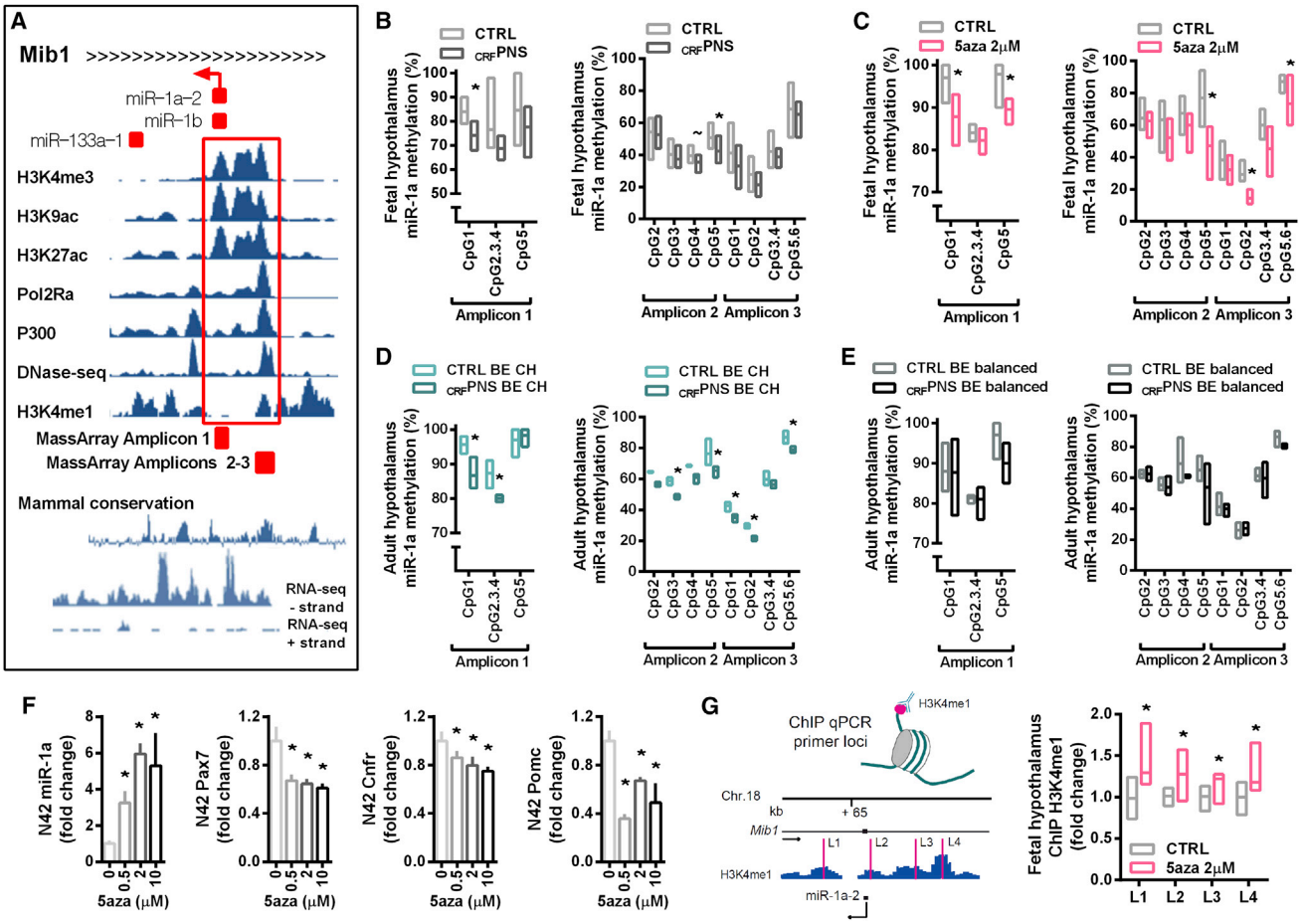

Figure 5. CRF-Induced PNS Prompted Fetal and BE-Induced Hypomethylation in the Hypothalamic miR-1a Locus Can Be Mimicked by 5-Aza Exposure and Is Normalized by the Balanced Methyl Donor Diet

(A) The putative miR-1a promoter presents chromatin marks associated with active promoters (H3K4me3 [ENCFF001KHV], H3K9ac [ENCFF001KGM], H3K27ac [ENCFF001KGR]), active enhancers (H3K4me1 [ENCFF001KHP]), open accessible chromatin (DNase hypersensitivity regions [ENCFF001PMH]), and transcription factor binding sites (POL2RA [ENCFF001LKL], P300 [ENCFF001LKH]) (http://genome.ucsc.edu and ENCODE Data Coordination Center).

(B) MassArray analysis showed hypomethylation in female fetuses exposed to $_{\text{CRF-induced}}$PNS in Amplicon 1 (Mann-Whitney U = −2.52, p = 0.012, left) and a tendency to hypomethylation in Amplicons 2 and 3 (Mann-Whitney U = −1.650, p = 0.099, right, n = 6 per group).

(C) MassArray analysis of female fetuses exposed to 5-Aza showed hypomethylation in Amplicon 1 (Mann-Whitney U = −2.115, p = 0.034, left) and in Amplicons 2 and 3 (Mann-Whitney U = −2.670, p = 0.008, right, n = 4–5).

(D) MassArray analysis of adult BE females on chow showed selective hypomethylation in $_{\text{CRF-induced}}$PNS across the putative miR-1a promoter area and a tendency to global hypomethylation in Amplicon 1 (Mann-Whitney U = −1. 727, p = 0.084) and Amplicons 2 and 3 (Mann-Whitney U = −1. 764, p = 0.078, n = 3 per group).

(E) Adult $_{\text{CRF-induced}}$PNS BE females on a balanced diet showed normal methylation of the miR-1a cluster (n = 3 per group). Data are presented as median and range.

(F) 5-Aza treatment of N42 cells at different concentrations increased miR-1a levels ($F_{(3,15)} = 4.88$, $p < 0.05$), while *Pax7* ($F_{(3,15)} = 6.64$, $p < 0.01$), *Cnfr* ($F_{(3,15)} = 3.997$, $p < 0.05$), and *Pomc* ($F_{(3,15)} = 8.50$, $p < 0.01$) were decreased by the manipulation. *p < 0.05 compared to untreated cells based on Bonferroni post hocs. Data are presented as mean ± SEM. n = 4 for all groups.

(G) Chromatin immunoprecipitation (ChIP) of the hypothalamus of female fetuses exposed to 5-Aza in utero showed higher occupation of H3K4me1 in the miR-1a putative promoter region (Mann-Whitney U = −4.774, p < 0.0001, n = 6 per group). L, locus. Data are presented as median and range.

*p < 0.05 comparing CTRL and $_{\text{CRF-induced}}$PNS or 5-Aza, accordingly.

in the hypothalamus of $_{\text{CRF-induced}}$PNS fetuses (Figure S4A), suggesting that the cluster as a whole is regulated by methylation. Specifically, the *Mib1* locus carries chromatin marks associated with active promoters (H3K4me3, H3K9ac, and H3K27ac), active enhancers (H3K4me1), actively transcribed regions (H3K36me3), open accessible chromatin (DNase hypersensitivity regions), and transcription factor binding sites (POL2RA and P300) around the transcription start site (TSS) region of *Mib1*, indicative of the regulatory region of *Mib1* (Kent et al., 2002) (Figure S5). Methylation analysis of this region demonstrated moderate hypermethylation rather than the expected hypomethylation if it were to regulate miR-1a, which was in accordance with the reduced *Mib1* mRNA levels following $_{\text{CRF-induced}}$PNS (Figure S5). A second region of active transcription of regulatory marks is specifically found at the miR-1a-2/miR-1b/miR-133a-1 cluster, indicating that the transcription of this cluster may be regulated via its own promoter independently of the *Mib1* promoter. This second putative promoter is characterized by broad POL2RA and P300

binding and high chromatin accessibility, indicating potential independent high regulatory capacity of the region. We focused on two particular sequences of this area for analysis of CpG methylation, including the area immediately before miR-1a (Amplicon 1) and the area with the most regulatory markers (Amplicons 2 and 3) (Figure 5A). We found that the area nearby the miR-1a-2/miR-1b/miR-133a-1 cluster, including five CpGs, was hypomethylated in $_{CRF\text{-}induced}$PNS fetal hypothalamic samples (Figure 5B) while the more distanced area with the large numbers of regulatory markers only tended to be hypomethylated (Figure 5B). These results suggest that, as a consequence of $_{CRF\text{-}induced}$PNS and the resulting reduction in the *Dnmts*, the miR-1a cluster is selectively hypomethylated, resulting in miR-1a overexpression. To examine this effect further, we next treated pregnant ICR females with the DNA methyltransferase inhibitor 5-aza-2′-cytidine (5-Aza) on GD17.5 to reduce the expression of all Dnmts and mimic the effects of $_{CRF\text{-}induced}$PNS in the fetal hypothalamus in vivo. 5-Aza treatment induced hypomethylation in the miR-1a cluster in the fetal hypothalamus in a similar manner to in utero CRF-OE and with the strongest effects on Amplicon 1 (Figure 5C). In the adults exposed to BE protocol, methylation of the miR-1a cluster was reduced in $_{CRF\text{-}induced}$PNS females on chow (Figure 5D) and was normalized by the balanced methyl donor diet (Figure 5E). Finally, we treated N42 cells with 5-Aza to further assess the causality of the cascade of hypothalamic adaptations explored so far. As expected, miR-1a levels increased after 5-Aza treatment (Figure 5F), as opposed to other unrelated miRs (Figure S4B), and methylation at miR-1a cluster was dramatically downregulated (Figure S4C). Upregulation of miR-1a in these cells coincided with decreased *Pax7*, *Cnfr*, and *Pomc* (Figure 5F), mimicking the apparent cascade of adaptations induced by $_{CRF\text{-}induced}$PNS in fetuses and in $_{CRF\text{-}induced}$PNS females exposed to BE protocol on chow. Next, we explored histone methylation by chromatin immunoprecipitation (ChiP) against the H3K4me1 enhancer, since active enhancers ubiquitously feature hypomethylation of their target DNA (Stadler et al., 2011). We found that after 5-Aza treatment, both in the fetal hypothalamus (Figure 5G) and N42 cells (Figure S4C), the occupation of the DNA of H3K4me1 was increased, which is indicative of open chromatin allowing increased gene expression of miR-1a. Altogether, the results suggest that the BE-like phenotype predisposition may, at least partially, be linked to alterations both at the DNA and histone methylation levels in the miR-1a locus.

Finally, to further support the stress-related origin of CRF-OE in inducing the hypothalamic adaptations leading to programming of BE, we examined the expression of miR-1a and the suggested downstream cascade in the hypothalami of fetuses exposed to CVS in the third trimester of gestation. In accordance with the $_{CRF\text{-}induced}$PNS results, the reduction in the *Dnmts* and *Mtrr* by CVS coincided with upregulation of miR-1a, low levels of *Pax7*, *Pax3*, and *Cnfr*, and dysregulation of the melanocortin system (Figure S3C).

Thus, in summary, exposure to $_{CRF\text{-}induced}$PNS produced a cascade of hypothalamic adaptations prompted by abnormal DNA methylation (Figure 6A) that rendered fetuses vulnerable to BE-like phenotype in adolescence. These females recovered when left untouched (Figure 6B), but the BE challenge re-exposed the vulnerability reverting hypothalamic gene expression to the fetal profile under stress (Figure 6C). Given that the susceptibility to BE appears to be rooted in abnormal methyltransferase metabolism, providing these predisposed females with a balanced methyl donor background diet was enough to prevent the predisposition from being triggered (Figure 6D).

DISCUSSION

We have shown that exposure of female fetuses to $_{CRF\text{-}induced}$PNS elicits a chain of epigenetic adaptations in the hypothalamus that generated a permanent epigenetic change that left female offspring vulnerable to a nutritionally oriented environmental challenge after weaning. It is broadly accepted that gestational abnormalities predispose the offspring to disease, but the mechanisms through which it takes place are largely unclear. In general, it appears that PNS affects the offspring in a sex-dependent manner, rendering males less reactive and females more reactive to stress (Tibu et al., 2014) and posing distinctive advantages and disadvantages for each sex. It has been further suggested that male exposure to early adversity threatens their viability, efficiently culling the weak and creating a surviving cohort of the fittest. In contrast, females adjust more successfully to early adversity but display increased vulnerability expressed later in development (Sandman et al., 2013). Once such an epigenetic defect is generated, it may never completely revert back to the original state (Padmanabhan et al., 2013; Reynolds, 2013). Thus, a further stressor during a developmentally critical time window, such as adolescence, can re-expose the epigenetic defect resulting from gestational programming. This would lead to widespread dysregulation at the DNA methylation level consequently affecting the downstream cascade of hypothalamic alterations, leading to the distinctive phenotype of these females.

Finally, we showed that while this stress-related epigenetic predisposition can increase the vulnerability to disease in these females, it can be prevented from being triggered. Given the multilevel and complex modifications that underlie the predisposition to BE and the plasticity of the developing adolescent brain, we chose a dietary "preventive" approach that has proven effective in previous research (Bermingham et al., 2013; Dahlhoff et al., 2014) and also grants clear translational potential. We chose to alter the availability of nutrients related to one-carbon metabolism, such as methionine, choline, and folic acid, all involved in the maintenance of genomic DNA methylation, which can affect DNA methylation and *Dnmts* expression in a more global manner. Despite the general belief that excess of dietary MDs are beneficial to health, recent studies suggest that both excess *and* deficiency may have negative implications (Herrmann and Obeid, 2011; Christensen et al., 2015), particularly in vulnerable individuals. Therefore, our findings have important implications for psychiatric disease prevention. While a recent advance was achieved by targeting the maternal diet during gestation as means of prevention of mental illness (O'Neil et al., 2014), we show that an adequate diet during adolescence can significantly ameliorate the repercussion of PNS by blocking the acquired epigenetic defect in the offspring.

Overall, we have identified a mechanistic pathway through which increased maternal CRF levels during late gestation (mimicking exposure to chronic stress) predispose female offspring to develop BE-like behavior in response to a nutritional challenge during adolescence. These data are consistent with

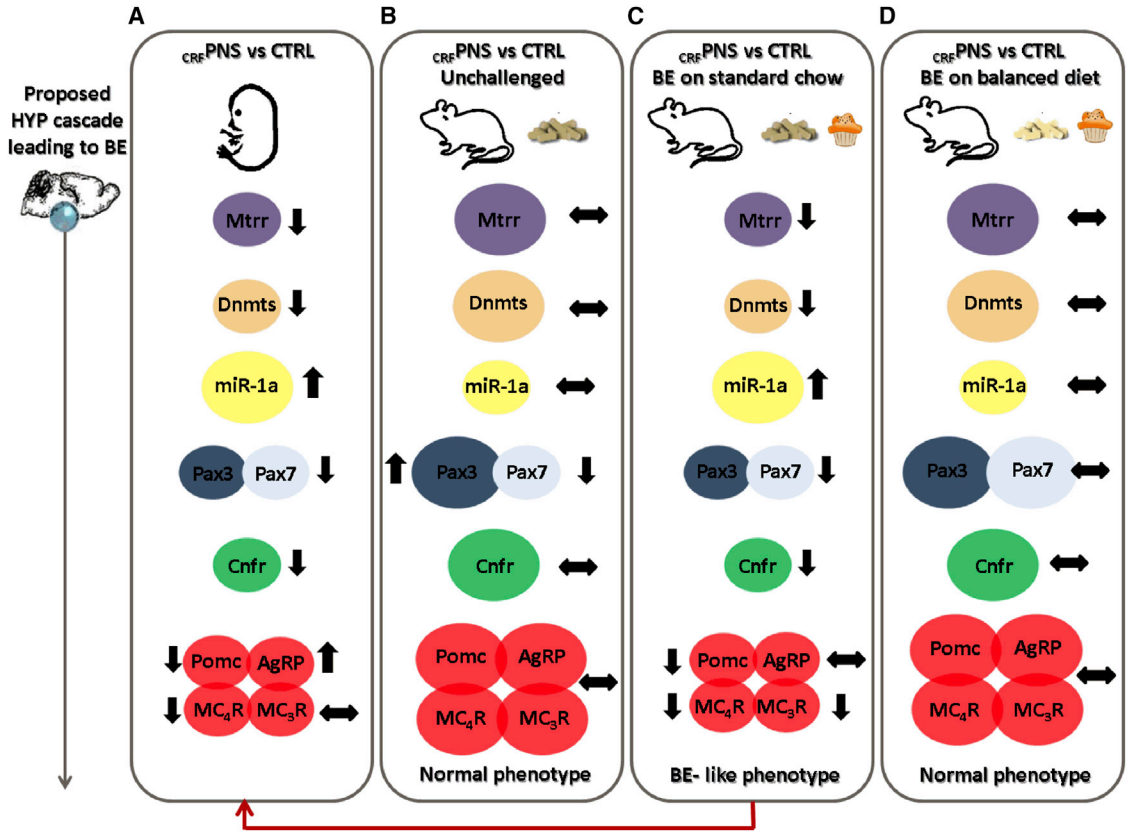

Figure 6. Step-by-Step Diagram of Hypothalamic Gene Expression in Females Exposed to CRF-Induced PNS and after Different Dietary Manipulations

(A) $_{CRF\text{-}induced}$PNS dysregulated fetal gene expression of DNA methylation enzymes that results in upregulation of miR-1a, which targets *Pax7/3*, which in turn downregulate *Cnfr*, leading to reduction in *Pomc* and disruption of the melanocortin system.

(B) Adult unchallenged females exposed to $_{CRF\text{-}induced}$PNS present a normal phenotype and hypothalamic gene expression compared to CTRLs despite the low level of *Pax7* resulting from the manipulation, which is compensated by overexpression of *Pax3*.

(C) $_{CRF\text{-}induced}$PNS females exposed to the BE protocol on standard chow rapidly develop BE compared to CTRLs. BE in these females is underlie by hypothalamic abnormalities similar to fetuses exposed to $_{CRF\text{-}induced}$PNS. Specifically, DNA methylation enzymes are downregulated, miR-1a is upregulated, and both *Pax7* and *3* are downregulated, which causes the consequent downregulation of *Cnfr*, leading to reduced *Pomc* and dysregulation of the melanocortin system.

(D) Exposure of $_{CRF\text{-}induced}$PNS females to the BE protocol on "balanced" methyl donor diet prevented BE through normalization of the DNA methylation enzymes and the downstream cascade of hypothalamic genes. Large circle, high expression levels; small circle, low expression levels. ↑, upregulation; ↓, downregulation; ↑↓, no change in gene expression.

the developmental origin of disease hypothesis (Barker, 1990) and provide mechanistic insight into the early life origins of BED and potential prevention strategies. While from a survival perspective these adaptations may help females thrive in a food-deprived environment, the wide availability of food rich in fat and carbohydrates combined with the frequent self-imposed diet constraint by adolescent girls (turning palatable food into "forbidden") may trigger BED when an epigenetically induced predisposition is present. We showed that the development of BED can be prevented by providing a balanced diet during adolescence, which stabilizes eating behavior even when a nutritional challenge is present. Our findings directly link, for the first time, abnormalities in the prenatal environment with sensitivity for BED in adolescent female mice and propose a non-invasive nutritional approach as an effective form of prevention. This has important consequences for understanding and treating BED in women, exposing the beneficial impact of dietary habits during adolescence as a means of prevention in epigenetically prone individuals.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2017.05.001.

AUTHOR CONTRIBUTIONS

M.S. designed and conducted most of the experiments. T.P. and M.L. assisted with the behavioral experiments, dietary manipulations, and RNA, DNA, and protein extractions. T.P. and Y.D. sacrificed the animals. M.L. assisted with the in vitro experiments and performed the lentiviral injections. M.J. performed the methylation analysis, and M.J., S.B.-D., and M.E. performed the bioinformatic predictions and analysis. A.C. supervised the project. M.S. and A.C. wrote the manuscript.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
α-GFP	Abcam	Cat# ab6658; RRID: AB_305631
α-CRF	Gift from the lab of Dr. Wylie Vale	N/A
Secondary antibody: Streptavidin conjugated Cy2 anti-rabbit	Jackson ImmunoResearch Laboratories	N/A
α-H3K4me1	Abcam	Cat#ab8895; RRID: AB_306847
α-Actin	Santa Cruz Biotechnology	sc-1616; RRID: AB_630836
α-Pax3	Sigma-Aldrich	SAB2502032
α-Pax7	Sigma-Aldrich	AV32742; RRID: AB_1855005
Bacterial and Virus Strains		
Lentiviral vector to overexpress miR-1a	GeneCopoeia	LPP-MmiR3314MR03
Scrambled control	GeneCopoeia	LPP-CmiR0001MR03
Chemicals, Peptides, and Recombinant Proteins		
5-aza-2′-deoxycytidine	Sigma-Aldrich	Cat# A3656
Tri Reagent	Molecular Research Center	Cat# TR118
Nitrotetrazolium blue chloride 2	Sigma-Aldrich	Cat# N6876
RIPA buffer	Thermo Fisher Scientific	Cat# 89900
Pierce ECL Western Blotting Substrate	Thermo Fisher Scientific	Cat# 32106
FastStart Essential DNA Green Master	Roche Applied Science, Roche Diagnostics	Cat# 06402712001
Chromatin immunoprecipitation (ChIP) dilution buffer	EMD Millipore	Cat# 20-153
proteinase K	Sigma-Aldrich	Cat# P2308
MNase	Thermo Fisher Scientific	Cat# 88216
Critical Commercial Assays		
miRNeasy mini kit	QIAGEN	Cat# 217004
miScript II Reverse transcription kit	QIAGEN	Cat# 218160
QIAquick PCR purification kit	QIAGEN	Cat# 28104
MethylFlash Methylated DNA Quantification ELISA kit	EpiGentek	Cat# P-1035
AllPrep DNA/RNA/Protein Mini kit	QIAGEN	Cat# 80204
SYBRGreen PCR kit	QIAGEN	Cat# 218073
Homocysteine ELISA kit	BIOTREND	Cat#BMA-21715
Deposited Data		
ENCODE	UCSC	https://genome.ucsc.edu/ENCODE/
UCSC genome browser	UCSC	https://genome-euro.ucsc.edu/cgi-bin/hgGateway?redirect=manual&source=genome.ucsc.edu
Experimental Models: Cell Lines		
N42 immortalized hypothalamic cells	N/A	N/A
Experimental Models: Organisms/Strains		
Harlan ICR mice	N/A	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Sequence for the miR-1a promoter, Amplicon 1 - miR1a_T1 #2: Target length 244. CpGs: 5 ATGGAAGTCATCCTCCTGGAAAGTTTCCTTT AGCTTCTTCTTGGCGGACATTACCTACCCAAAAT ACATACTTCTTTACATTCCATAGCACTGAAT GTTCATATGGGTACATAAAGAAGTATGTGCTCTGA GTAGGCACTCCTGCGCCGGCCGATAGCTGTG AAAACATGAAGCAGCCCACCTCACCCAGCTGTTAG TAGGACATTTGGATTACTTTTCTGGAAGAG TAATGGATCCAGTGCGGAGATTAGCTTAT CAGAGACTGAATTTTACAACAGATTTACCT GATCCCATCAATGCCACTTCTGCTTGTG GTTCTTTTAAGCACTG	Varionostic	N/A
Sequence for the miR-1a promoter, Amplicon 2: miR1a_T2 #3: Target length 321. CpGs: 5 GTGGTAGATCTGGAGTGAAGCTACCTTGTTATAT CCAGAGGTCAAGTCTGCAAGCGAGGTCAGGACT CCCACATAGTCACCACAGAAGCCAACCTATGTTC TTCCCGACTCAGTGGCCACAGCCTAGGCTGCTC GAGCTTTATAGGCAGCTAAGCATTTGAAACATGC ACTCCCAGGCAAACAGACTCTCTTGTGCCTCTCT CTTCCCTGACCATATTTAGTCAGCCATAGCAAAA AATAGTCTCTCAGCTGTCGATTGTCGATCTAAA AGCTGAGGAGGATTCTATCTTGCTTTCTGCCT ATTGATTGTTCTTCCTGTGG	Varionostic	N/A
Sequence for the miR-1a promoter, Amplicon 3: miR1a_T2 #7: Target length 241. CpGs: 6 CTATTGATTGTTCTTCCTGTGGTCCCAACACTGC TTTGCCATTTGTCCTGCTATTTTTTTTTTAAGTAC TGCTAAATAGAGCCGTGTCTCATGTTTCCACTTACA TTTCCCAGATTCTCCCCGAGCTTGTGCTGAGACT AGGAAAACCTCACGCGGCTGTCACTATACTTAGAA GCCCTGAAGAGAAAAAGGGCGGCGGGGGTGGG GTGAGGTGGGGTGGGGCAACAAATTTGCCCT GGCT	Varionostic	N/A
ChIP: Mib1 Locus 1, to amplify mm9 chromosome 18, base pair 10,784,260-10,784,362: F-TTTAGTAGGTCCCTGGCAGC, R-ATGGCCTCTC CTTGTCCTGA	N/A	N/A
ChIP: Mib1 Locus 2, to amplify mm9 chromosome 18, base pair 10,785,597-10,785,721: F- CCACCTCACCCAGCTGTTAG, R- TGGCATTGA TGGGATCAGGT	N/A	N/A
ChIP: Mib1 Locus 3, to amplify mm9 chromosome 18, base pair 10,786,892-10,787,019: F- GTGTCCTGCCCCACTTACAG, R- GTGCTTACCT CACCCAGCTT	N/A	N/A
ChIP: Mib1 Locus 4, to amplify mm9 chromosome 18, base pair 10,787,652-10,787,765: F- AGTCACCACAGAAGCCAACC, R- GAGTCTGTT TGCCTGGGAGT	N/A	N/A
ChIP: Housekeeping gene B2m: F- GGGAAAGTCCCTTTGTAACCT, R- GCGCGCGCT CTTATATAGTT	N/A	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and Algorithms		
SPSS v.16.0	SPSS	N/A
GraphPad Prism 6	GraphPad Software	N/A
Other		
Proteome Protein G magnetic bead system	EMD Millipore	Cat# LSKMAGG02
Standard chow, Teklad global rodent diet 14% protein	Envigo	Cat# 2018S
Western diet	Research Diets	Cat# D12079Bi
Balanced diet	Research Diets	Cat# D10012G
LabMaster system	TSE-Systems	N/A
StepOnePlus Real time PCR system	Applied Biosystems, Thermo Fisher Scientific	Cat# 4376600
Bioanalyzer Agilent 2100	Agilent Technologies	Cat# G2939AA
Angle Two Stereotaxic Instrument – motorized nanoinjector	myNeurolab, Leica Biosystems	Cat# 39464610
Film	Fujifilm	N/A
miRNA primers	N/A	N/A
miR-1a-3p	miScript, QIAGEN	Cat# MS00023905
miR-1b	miScript, QIAGEN	Cat# MS00011004
miR-133a-2	miScript, QIAGEN	Cat# MS00032305
miR-206-3p	miScript, QIAGEN	Cat# MS00001869
Further RT-PCR primers presented in Table S2	N/A	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by Alon Chen (alon_chen@psych.mpg.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse Model

Mouse Strain

ICR (CD1) mice (Harlan Laboratories, Madison WI, USA) were maintained in a pathogen-free temperature-controlled (22 ± 1°C) mouse facility on a reverse 12-h light-dark cycle at the Weizmann Institute of Science, according to institutional guidelines. Food (Teklad global, Harlan Laboratories, Madison WI, USA) and water were given ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

Stereotactic ICV Injection

Eight-week-old ICR female mice (Harlan Laboratories, Madison WI, USA) were injected with a mixture of the two lentiviruses, the "Effector" and the "CRF-Target," into the lateral ventricle. A computer-guided stereotaxic instrument and a motorized nanoinjector (Angle Two Stereotaxic Instrument, myNeurolab, Leica Biosystems, Buffalo Grove, IL, USA) were used. Mice were placed on a stereostaxic apparatus under general anesthesia (1.5% Isofluorane). Two microliters of the lentiviral preparation were delivered into the lateral ventricle using a 2 μL Hamilton syringe connected to a motorized nanoinjector system at a rate of 0.5 μL per min (coordinates, relative to Bregma: AP = −0.22mm, LM = −1mm, DV = −2.7mm, based on a calibration study indicating these coordinates as leading to the ventricles in ICR strain). Mice were given a two-week period for recovery.

Breeding

Female ICR mice were mated at 11-13 weeks of age. Two or 3 females were housed with 1 male (minimum age 12 weeks) at the beginning of the dark period and were examined for the presence of a vaginal copulation plug at the end of the dark period. Presence of a copulation plug denoted day 0.5 of gestation. After breeding, the females were individually housed.

Offspring Physiological Measurements

Pregnant and lactating dams underwent weekly follow-ups of body weight and food intake. For pre-weaning weights, total litter weights were averaged (males and females separated). Post-weaning, pups were weighed individually and food intake was monitored weekly.

Experimental Groups

Females with plugs were randomly assigned to the treatment group to receive Dox-containing drinking water at a non-toxic dose (0.5 mg/mL Dox and 0.2% sucrose) (Moutier et al., 2003) during the third trimester of gestation (days 13.5-parturition), or to a CTRL group without Dox. From day 18.5 of gestation, females were checked twice a day for the presence of a litter (9:00-10:00, 17:00-18:00). Newborn litters found before 18:00 were designated as born on that day- postpartum day 0 (PPD 0). On PPD 1, pups were counted and litters were culled to 10 pups (with sex distribution kept as equal as possible in each litter). Litters with less than seven pups were excluded. On PPD 21-22, pups were weaned and female offspring were group housed until PPD30, where they were randomly assigned to 1 of 3 experimental groups: 1) Chow (CH) CTRL group, 2) Western Diet (WD, D12079Bi, Research Diets, New Brunswick, NJ, USA) group or 3) Binge eating group (BE). For the rescue experiment, the BE group was repeated and given either standard chow or a balanced methyl donor diet (D10012G, Research Diets, New Brunswick, NJ, USA) as background diets. A maximum of 2 female siblings were located in the same experimental group.

Binge Eating Protocol

BE was induced using a modified version of the ''limited access'' protocol (Czyzyk et al., 2010) and consisted of sporadic and limited access (for 2 hr a day, 3 times a week during the dark cycle) to Western diet (D12079Bi, Research Diets, New Brunswick, NJ, USA) for a period of four weeks, without food restriction. The protocol was preceded by a 5-day habituation to the new diet and started on PPD30, the pre-pubertal period equivalent to adolescence in humans.

Cell Cultures

N42 immortalized (male) hypothalamic cells (mHypoE-N42) were plated into 35mm plates with DMEM medium with 2 mM L-glutamine, fetal bovine serum (10%) and Penicillin Streptomycin Solution (10,000 units/ml Penicillin G Sodium Salt, 10mg/ml Streptomycin Sulfate) (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a 5% CO_2 humidified incubator.

METHOD DETAILS

Inducible Overexpression of CRF

In order to achieve stability of our inserted gene on the one hand, and inducibility and reversibility of its expression on the other hand, we genetically targeted the choroid plexus tissue and used the bacterial Tet-On transcriptional regulation system (Gossen and Bujard, 1992; Gossen et al., 1995; Regev et al., 2010). This system is composed of two complimentary lentiviral vectors. The ''Effector'' construct consists of a choroid plexus-specific promoter that drives the expression of reverse tetracycline trans activator (rtTA) protein and the reporter green fluorescent protein (GFP). The ''Target'' construct includes the tetracycline-responsive element (TRE) DNA sequence, upstream to the nucleotide coding sequence of the requested gene of interest, followed by the reporter red fluorescent protein (RFP). Transcription initiation of the gene of interest and the RFP is mediated only in the presence of the inducer, doxycycline (Dox). Dox was the inducer of choice for our purposes as it has been demonstrated to cross the blood-brain barrier (Mansuy et al., 1998).

Production of Lentiviral Vectors

Choroid plexus-specific Tet-On lentiviral vectors were constructed as described previously (Regev et al., 2010). Recombinant lentiviruses were produced by transient transfection in HEK293T cells, as described previously (Naldini et al., 1996). Briefly, infectious lentiviruses were harvested at 48 and 72 hs following transfection, filtered through 0.45 μm-pore cellulose acetate filters, concentrated by ultracentrifugation, re-dissolved in sterile HBSS, aliquoted and stored at −80°C. Vector concentrations were analyzed using eGFP fluorescence in HEK293T cells infected with serial dilutions of the recombinant lentivirus. The viral constructs were kindly provided by Dr. Inder, The Salk Institute for Biological Studies, La Jolla, CA.

Immunohistochemistry of Dam Brains

Dams were perfused on GD17.5. Fixed brains were serially sectioned and confirmation injection to the choroid plexus was done by double-immunostaining using biotinylated α-GFP antibody raised in goat as primary antibody (Abcam, Cambridge, UK) and α-CRF anti-rabbit (a gift from the lab of Dr. Wylie Vale). Streptavidin conjugated Cy2 anti-rabbit was used as secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA).

Embryo Tissue Collection

Immediately after cesarean section, embryos were weighed and decapitated, the brain was extracted and the hypothalamus was removed, immediately frozen on dry ice and stored at −80°C. A portion of the embryos' tail was removed for later DNA extraction and sex genotyping.

Genotyping

Sex of the embryo was determined by Sry genotyping (forward 5′-TCATGAGACTGCCAACCACAG-3′ and reverse 5′-CATGACCAC CACCACCACCAA-3′) (Petropoulos et al., 2011). Amplification product was detected by 2% gel electrophoresis.

Maternal Behavior

On days 6/7 and 17/18 postpartum, patterns of undisturbed nocturnal maternal behavior were observed during 160 min sessions. Each mother was observed every 15 min, for 1-3 s. This allowed the identification of the ongoing maternal behavior at the observation time. Various maternal and non-maternal behaviors were recorded in every observation. The score was "1" if the behavior occurred and "0" if it did not occur. Maternal Behavior measures were based on existing literature (Schroeder et al., 2006) and included both self-(grooming, eating) and pup-directed behaviors (nursing, licking/grooming) and activity measures.

Food Intake after Incubation Period

Food and water intake were measured using the LabMaster system (TSE-Systems, Bad Homburg, Germany). The LabMaster instrument consists of a combination of sensitive feeding and drinking sensors for automated online measurement. Data were collected for five consecutive days after 24 hr of adaptation to the apparatus.

Tissue Collection

After decapitation, the brain was extracted and the hypothalamus was removed and immediately stored at −80°C. Trunk blood was collected for plasma Homocysteine measurement with the Mouse Homocysteine (Hcy) ELISA kit (BIOTREND Chemikalien GmbH, Köln, Germany). Plasma samples were immediately centrifuged and stored at −80°C. Plasma samples from the pregnant dams were obtained from trunk blood from a separate set of pregnancies. Corticosterone levels were analyzed using radioimmunoassay (MP Biomedicals, Solon, Ohio, USA).

RNA Extraction and Real-Time PCR

Purification of total RNA containing miRs was isolated from frozen hypothalami using miRNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. RNA integrity was evaluated using a bioanalyzer (Agilent 2100; Agilent Technologies, Santa Clara, CA, USA). RNA preparations were reverse transcribed to generate cDNA using miScript Reverse transcription kit (QIAGEN, Hilden, Germany). Quantitative measurements of miR and mRNA expression was done using a SYBRGreen PCR kit (QIAGEN, Hilden, Germany) according to the manufacturer's guidelines and a StepOnePlus Real time PCR system (Applied Biosystems, Waltham, MA, USA), using specific primers. U6 snRNA was used as internal control for the miRs, Hprt was used as internal control for the hypothalamic samples.

For placental tissue, purification of total RNA containing miRs was done using Tri Reagent (MRC, Cincinnati, OH, USA) according to the manufacturer's recommendations. TBP was used as internal control for placental samples.

DNA and Protein Extraction

Purification of DNA and protein was done using AllPrep DNA/RNA/Protein Mini kit (QIAGEN, Hilden, Germany) from a separate set of samples according to the manufacturer's instructions.

Western Blot

Protein obtained from the AllPrep Mini kit was boiled and centrifuged for 10 min at 4°C. The supernatant was transferred and separated in a 12% polyacrylamide gel by electrophoresis. For transfer to membrane, a wet transfer was performed using an assembly of nitrocellulose membrane and Whatman paper. The transfer was performed at 100v, 350 mAmp for 1hr. After 4 washes with PBST (PBS + 20% Tween20), the membrane was blocked with 5% skim milk in PBST for 1h. The primary antibodies (anti-Pax3 and anti-Pax7 (Sigma-Aldrich, St. Louis, MO, USA) were added to PBST in 5% skim milk and placed on constant shaking at 4°C overnight. The primary antibody against Actin (Santa Cruz Biotechnology, Dallas, Texas, USA) was added for an incubation period of 1 hr the next day at RT. After several washes with PBST, the secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) were added for 1 hr incubation at RT. Finally, the membrane was scanned using ECL (Thermo Fisher Scientific, Waltham, MA, USA) and film (Fujifilm, Tokyo, Japan).

Global DNA Methylation

Global methylation of the hypothalamus was done using the MethylFlash Methylated DNA Quantification ELISA kit (EpiGentek Group, Farmingdale, NY, USA) according to the manufacturer's instructions.

Site-Specific DNA Methylation Analysis

DNA methylation analysis of the fetal hypothalamus was performed using bisulfite conversion and MassArray technology (Varionostic, Ulm, Germany). For the *Mib1* promoter, a total of 4 amplicons covering the whole CpG Island around the TSS site were used. For the putative miR-1a promoter analysis, 3 further amplicons on selected CpGs were run. The sequences for the putative miR-1a promoter are presented in the Key Resources Table.

Chromatin Immunoprecipitation (ChIP)

Homogenates of N42 cells and fetal hypothalami were sheared using digestion/nuclei permeabilization buffer containing MNase at 37°C. Samples were incubated in hypotonization buffer to release chromatin from swollen nuclei and centrifuged to remove cell

debris. The supernatant was used for each ChIP reaction, supplemented with ChIP dilution buffer and anti H3K4me1 antibody (Abcam, Cambridge, UK). After incubation for 16 hr, the DNA-protein-antibody complexes were captured with protein G magnetic beads (EMD Millipore, Billerica, MA, USA). Beads were washed and DNA-protein-antibody complexes were eluted, proteins were digested with proteinase K and ChIP-DNA was eluted using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). DNA from each input sample was extracted in parallel (Jakovcevski et al., 2015). For quantification by qPCR ChIP-DNA was diluted and run using the SYBR Green MasterMix FastStart Essential DNA Green Master (Roche Applied Science, Roche Diagnostics, Indianapolis, IN, USA) with specific primers listed in the Key Resources Table.

5-Aza Functionality Assay of Methylation

N42 immortalized hypothalamic cells were plated into 35mm plates and treated for four days with different concentrations (0, 0.5, 2, 10μM) of 5-aza-2′-deoxycytidine (5-Aza; Sigma-Aldrich, St. Louis, MO, USA). Every day, in the evening, medium was replaced with fresh growth medium containing fresh 5-Aza. Cells were then lysed and RNA was extracted using the miRNeasy mini kit (QIAGEN, Hilden, Germany). RNA was reverse transcribed to cDNA using miScript Reverse transcription kit (QIAGEN, Hilden, Germany).

5-Aza Treatment during Pregnancy

On day 17.5 of gestation, pregnant ICR mice were injected intraperitoneally (i.p.) with 10 mg/kg of 5-Aza and then euthanized 6 hr after treatment (Ueno et al., 2006). The fetuses were excised and the hypothalamus was removed and immediately frozen on dry ice and stored at −80°C. A portion of the embryos' tail was removed for later DNA extraction and sex genotyping.

In Situ Hybridization

Adult (12 weeks) ICR female brains were collected and immediately frozen in liquid nitrogen.20μm sections were hybridized with miR-1a and scramble control oligonucleotide probes (Invitrogen, Carlsbad, CA, USA and Dharmacon RNAi Technologies, Lafayette, CO, USA.) overnight at 37°C and developed with nitro blue tetrazolium chloride 2 (KODAK) (Thompson et al., 2007).

Chronic Variable Stress (CVS) Protocol

From GD13.5 until GD17.5, randomly chosen pregnant females were exposed to a chronic variable mild stress protocol, including 2 short manipulations during the dark phase and a further overnight (ON) manipulation during the light phase. A further set of females was left undisturbed and served as CTRLs. The chosen stressors did not induce pain and did not directly influence maternal food intake or weight gain. Fetal tissues were collected on GD17.5. The overnight manipulations included overnight illumination, saturated bedding (200ml of autoclaved water), novel object in cage (marbles), and overcrowding (15 females in cage). The shot manipulations included multiple cage changes, cage tilt (30 degrees for 2 hr), white noise 80 db for 2 hr, immobilization in tube for 30 min, no bedding or saturated bedding (with 200ml of autoclaved water) for 3 hr, swim stress in warm water for 15 min and standing in an elevated platform for 30 min.

Overexpression of miR-1a in N42 Cells

N42 immortalized hypothalamic cells were plated into 35mm plates and treated for 48 hr with lentiviral vectors to overexpress miR-1a (LPP-MmiR3314MR03 GeneCopoeia, Rockville, MD, USA) or a scrambled control (LPP-CmiR0001MR03, GeneCopoeia, Rockville, MD, USA). On the second day, fresh DMEM medium was added to each well. Cells were then lysed and RNA was extracted using the miRNeasy mini kit (QIAGEN, Hilden, Germany) or were lysed using RIPA buffer for protein extraction.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software, version 16.0 (SPSS, Chicago, IL, USA). Tests included repeated-measures ANOVA, MANOVA (multivariate analysis of variance) or one-way ANOVA with Tukey or Bonferroni corrections when relevant. Student t tests were used for all other measures between two groups. Linear regression and r values were determined in GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Non-parametric tests were used for DNA methylation analysis. All statistical parameters are outlined in the relevant figure legends.

DATA AND SOFTWARE AVAILABILITY

Statistical analyses were performed using SPSS version 16.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

Bioinformatics

Prediction of Mib1 regions as targets for bisulphite sequencing and MassArray was done using the UCSC genome browser (https://genome-euro.ucsc.edu/cgi-bin/hgGateway?redirect=manual&source=genome.ucsc.edu) (Kent et al., 2002) and the ENCODE Data Coordination Center (https://genome.ucsc.edu/ENCODE/) to define the genomic region of interest and prediction of potential methylation and transcription factor binding sites.