DNA methylation in AgRP neurons regulates voluntary exercise behavior in mice

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DNA methylation regulates cell type-specific gene expression. Here, in a transgenic mouse model, we show that deletion of the gene encoding DNA methyltransferase Dnmt3a in hypothalamic AgRP neurons causes a sedentary phenotype characterized by reduced voluntary exercise and increased adiposity. Whole-genome bisulfite sequencing (WGBS) and transcriptional profiling in neuronal nuclei from the arcuate nucleus of the hypothalamus (ARH) reveal differentially methylated genomic regions and reduced expression of AgRP neuron-associated genes in knockout mice. We use read-level analysis of WGBS data to infer putative ARH neural cell types affected by the knockout, and to localize promoter hypomethylation and increased expression of the growth factor Bmp7 to AgRP neurons, suggesting a role for aberrant TGF-β signaling in the development of this phenotype. Together, these data demonstrate that DNA methylation in AgRP neurons is required for their normal epigenetic development and neuron-specific gene expression profiles, and regulates voluntary exercise behavior.
Homeostatic regulation of body weight involves a balance between energy intake and expenditure. The expenditure side of the equation is dominated by basal metabolism in sedentary individuals, but also includes voluntary exercise and other forms of physical activity. The roots of the obesity epidemic are multifactorial, but physical inactivity is a significant factor and itself a substantial contributor to worldwide morbidity and mortality. Understanding the origin of interindividual differences in physical activity is therefore essential. Whereas sociocultural environment and genetic variation clearly play an important role, mouse models of developmental programming show that environmental influences during critical ontogenic periods also have a substantial impact on adult voluntary physical activity.

Our current understanding of physiological mechanisms regulating voluntary physical activity is rudimentary. Recent studies, however, suggest a link between hormonal signals of energy balance such as leptin and locomotor activity. In particular, AgRP neurons in the hypothalamic arcuate nucleus (ARH), conventionally seen as the orexigenic side of the melanocortin system, also influence locomotor activity and restoration of leptin receptor activity in the ARH is sufficient to rescue home cage locomotor activity in Lepr KO mice.

The epigenetic mechanism DNA methylation, established in neurons during the perinatal period by the de novo DNA methyltransferase Dnmt3a, plays a fundamental role in neuronal differentiation. We previously reported widespread increases in CpG methylation in mouse hypothalamic neurons from postnatal day 0 (P0) to P21—particularly at neurodevelopmental genes—suggesting that DNA methylation plays an active role in hypothalamic development by epigenetically silencing genes at developmentally appropriate timepoints. Accordingly, we hypothesized that perturbing the establishment of DNA methylation in the hypothalamus will disrupt cellular differentiation and energy homeostasis. Previous studies of hypothalamic neuroepigenetics, using similar logic, provide evidence that DNA methylation plays a role in sexual differentiation, circadian rhythms, and food intake. As those studies relied on local injection of methyltransferase inhibitors or non-cell type-specific knockout of Dnmt3a, it remains unclear to what extent epigenetic changes within specific neuronal cell types can affect energy balance. Indeed, a major current limitation in neuroepigenetics is the difficulty of linking cell type-specific epigenetic changes to specific adult phenotypes.

Here we show that AgRP neuron-specific knockout of Dnmt3a leads to cell type-specific disruption of DNA methylation and developmental gene expression, culminating in a lower physical activity set point. Moreover, our epigenomic analyses indicate that AgRP neuron-specific changes in DNA methylation at Bmp7 increase the expression of this paracrine signaling molecule, leading to widespread effects on TGF-β signaling in the arcuate nucleus. Our findings demonstrate a crucial role for DNA methylation in the normal development of the hypothalamic energy balance circuitry and indicate that epigenetic mechanisms established early in life regulate individual proclivity for physical activity.

Results

Dnmt3a regulates DNA methylation in AgRP neurons. Because de novo DNA methylation in neurons is regulated by Dnmt3a, we first studied the dynamics of Dnmt3a expression in the wild-type mouse ARH. In line with findings in other brain regions, Dnmt3a expression in the postnatal ARH reached a peak at P12 and declined substantially by P21. We next studied Dnmt3a expression by immunofluorescent labeling of Dnmt3a in AgRP/NPY cells identified by the NPY-hrGFP transgene and found substantial co-localization at P10, confirming that AgRP neurons express Dnmt3a during postnatal life. To assess the importance of Dnmt3a expression in establishing DNA methylation patterns within AgRP neurons, we generated AgRP neuron-specific Dnmt3a knockout mice (AgRP-IRESCre+/Dnmt3aCre+/F/F, hereafter referred to as F/F mice) by crossing Agrptm1(cre)Lowl/J mice (see Methods; Supplementary Fig. 1A) with mice harboring loxP sites flanking exon 18 of Dnmt3a (see Methods; Supplementary Fig. 1B, C). (AgRP-IRESCre+/Dnmt3aCre+/+ mice carrying the wild-type Dnmt3a allele were used as controls—hereafter referred to as +/+ mice). Abrogation of Dnmt3a expression did not alter the number of AgRP neurons (Fig. 1c), but did significantly reduce levels of 5-methylcytosine (Fig. 1d). Bisulfite treatment-based sequencing approaches cannot differentiate 5-methylcytosine and the product of TET-mediated demethylation 5-hydroxymethylcytosine. We used immunofluorescent labeling and found that 5-hydroxymethylcytosine was also reduced in putative AgRP neurons (Supplementary Fig. 1D), consistent with the reduction in 5-methylcytosine. These data indicate that Dnmt3a helps establish DNA methylation in AgRP neurons.

Sedentary phenotype in mice lacking Dnmt3a in AgRP neurons. Given the central role of AgRP neurons in energy balance homeostasis, we were surprised that there was merely a non-significant trend toward higher body weight in F/F adults of both sexes (Fig. 2a; Supplementary Fig. 1G). This was not attributable to a difference in lean body mass (Supplementary Fig. 1E, F); however, F/F mice of both sexes did exhibit significantly increased body fat (Fig. 2b; Supplementary Fig. 1H). To probe the cause of...
the increased adiposity, we performed indirect calorimetry in adult male mice and found that lean- and fat mass-adjusted food intake was unchanged (Fig. 2c) but lean- and fat mass-adjusted energy expenditure was reduced in F/F mice (Fig. 2d). Since resting metabolic rate did not differ between genotypes (Supplementary Fig. 1I), this deficit is specific to the non-resting component (Supplementary Fig. 1J), consistent with reduced home cage activity in F/F mice (Fig. 2e). The cages used for indirect calorimetry offer limited space for physical activity, so we next offered an independent cohort of adult mice free access to running wheels for 8 weeks. After a few weeks of acclimating to the wheels, male F/F mice ran approximately half the daily distance of +/+ mice (Fig. 2f) and accordingly lost significantly less fat (Fig. 2g) while consuming the same amount of food per day (Supplementary Fig. 1K). To explore the possibility of reduced physical capability as the cause of reduced physical activity, we conducted metabolic treadmill testing in a new cohort of mice not previously exposed to running wheels. Adult F/F mice exhibited no deficit in either maximum rate of oxygen consumption (VO2max) or standardized endurance run time (Fig. 2h, i). Taken together, these results suggest that the increased adiposity of F/F mice is attributable to a reduced tendency for voluntary exercise.

**Paradoxically increased methylation in F/F ARH neurons.** We next explored the molecular basis of this behavior change. Early
postnatal life is a critical period for establishment of both de novo DNA methylation^16 and neural projections^21 in hypothalamic neurons. We chose to study ARH neurons from mice at P12, coinciding with the peak in Dnmt3a expression in the ARH (Fig. 1a) but before any body weight difference in F/F mice (Supplementary Fig. 1L). We FACS-purified NeuN-immunopositive nuclei from microdissected ARH samples (Fig. 3a, b, Supplementary Fig. 2A–C, Supplementary Table 1) and measured their DNA methylation by whole-genome bisulphite sequencing (WGBS) and gene expression by RNA-seq. Expression of the neuron marker Snhg11 and the astroglia marker Gfap were approximately tenfold enriched and depleted, respectively, in NeuN+ vs. NeuN− fractions (Supplementary Fig. 2C) confirming effective isolation of neuronal nuclei. Our WGBS data showed that knocking out Dnmt3a in AgRP neurons led, paradoxically, to increased average genomic CpG methylation in ARH neurons with no change in methylation at non-CpG cytosines (i.e. CHG and CHH methylation—H corresponds to A, T, or C; Supplementary Fig. 2D). Since only CpG sites show significant differential methylation, we focused our analysis on CpG differentially methylated regions (DMRs), finding many more that were hypermethylated (i.e. $F/F > +/+)$ (58,963) than hypomethylated (16,476) (Supplementary Fig. 2E, Supplementary Data 1). Both DMR types showed similar distributions of length and CpG density (Supplementary Fig. 2F, G). We validated several DMRs by quantitative bisulphite pyrosequencing in an independent cohort of P12 and P35 mice and found excellent agreement with our WGBS analysis (Supplementary Fig. 3A) providing as a Source Data file.

Fig. 3 Widespread changes in DNA methylation and gene expression point to disruptions in melanocortin and GABAergic neural signaling. a) Illustration of experimental strategy to limit cellular heterogeneity by microdissection of ARH at P12 followed by NeuN immunolabelling to isolate neuronal nuclei by FACS. b) Representative FACS plot showing gating strategy used to isolate neural (NeuN+) nuclei (see also Supplementary Fig. 3A). c) Violin plot showing genomic localization and methylation levels of hypomethylated and hypermethylated DMRs in ARH neurons as determined by whole-genome bisulphite sequencing. Hypermethylated DMRs predominate in F/F mice except in CpG island, 5′-UTR and enhancer regions. d) Proportional genomic distribution of hypo- and hypermethylated DMRs. e) Gene ontology (GO) function analysis of GREAT-defined cis-regulatory region-associated DMRs shows significant enrichment of genes associated with melanocortin signaling, GABAergic neurotransmission, and TGF-β signaling among hypomethylated DMRs (left). Genes belonging to the melanocortin signaling GO term tend to be downregulated in F/F mice, while genes associated with GABAergic neurotransmission tend to be upregulated in F/F mice (right). f) Correlogram showing Pearson correlation coefficients (R) between DMR methylation level in each genomic region relative to other regions in the same gene, and expression of the associated gene. g) Significant changes in the expression of GABA synthesis genes (Gad1), receptor subunits (Gabra2, 4, 5), and transporters (Slc6a1, Slc6a11) in ARH neurons relative to a background list of all genes with significant differential expression. p-Values represent results of $\chi^2$ test against background list of significantly up- and downregulated genes. Values reflect mean ± SEM; n = 3–5, *p < 0.05, **p < 0.001. Source data for g provided as a Source Data file.
associate DMRs with genes, and performed a gene ontology (GO) function analysis. Hypomethylated DMRs were associated with GO terms related to melanocortin signaling, TGF-β signaling, and GABAergic neurotransmission (Fig. 3e). Hypermethylated DMRs, on the other hand, were associated with GO terms with no obvious links to hypothalamic function, implying a less organized cellular origin (Fig. 3e). Genes in DMR-associated GO terms did not show a consistent tendency toward up- or downregulation (Fig. 3e).

Disrupted melanocortin gene expression in F/F mice. Using RNA isolated from the same ARH neuronal nuclei samples that were studied by WGBS, we profiled changes in gene expression by RNA-seq. We found widespread expression changes in F/F mice, with 1681 up- and 2063 downregulated genes (log2-fold change > 1 and FDR < 0.01, Supplementary Fig. 2H, Supplementary Data 2). GO function terms related to synaptic function and neurotransmission were associated with both up- and downregulated genes (Supplementary Fig. 2I). DNA methylation in most gene-associated regions was weakly and negatively correlated with expression of the associated gene, apart from the 3' region which showed a weak positive correlation with expression23 (Fig. 3f, Supplementary Fig. 2J). Surprisingly, there was negligible overlap in GO terms observed for differential methylation (Fig. 3e) and differential expression (Supplementary Fig. 2I). This is likely related to the fact that DMR methylation does not have a consistent relation to differential gene expression (Fig. 3e). Notably, GO terms related to GABA signaling were enriched in both the analysis of downregulated genes (Supplementary Fig. 2I) and hypomethylated DMRs (Fig. 3e). Specifically, Gad1, which encodes the primary enzyme involved in GABA synthesis, was upregulated in F/F neurons, whereas three of five GABA receptor subunit genes (Gabra2, Gabra4, and Gabra5) were downregulated (Fig. 3g). To help dissociate primary, cell autonomous effects in AgRP neurons from secondary effects in other cell types, we exploited a published single-cell RNA-Seq dataset on ARH neurons24 to assess differential expression among genes characteristically associated with each ARH neuronal cell type. Strikingly, genes associated with two AgRP neuronal cell types (AgRP-Sst and AgRP-Gm8773) were almost universally downregulated (Fig. 3h). Since the number of AgRP neurons is unchanged in F/F mice (Fig. 1c), these data indicate downregulation of their characteristic genes, attesting to the essential role of cell autonomous DNA methylation in differentiation and cell type-specific gene expression.

Read-level analysis identifies methylation changes in AgRP neurons. The potential for communication between neural cell types in the ARH complicates analysis of DNA methylation. While the loss of 5-mC in AgRP neurons (Fig. 1d) and coherence of our hypomethylation-associated GO terms (Fig. 3e) are consistent with our expectations, the preponderance of hypermethylation in F/F ARH neurons (Supplementary Fig. 2E) is more difficult to account for. One possibility is that these hypermethylated regions represent secondary effects in non-AgRP cell types. Numerous methods exist for deconvolving DNA methylation data from mixed samples25,26. These methods share the common goal of estimating and potentially controlling for cell type fractions in mixed samples. Our application is fundamentally different from this, as we are concerned with estimating the cellular origin of differential methylation and separating instances where increases and decreases in methylation overlap in the genome. Since each read in a WGBS library is the clonal derivative of a DNA molecule originating from a single cell, cell-specific information is inherent in WGBS data27, as it is in clonal BS-seq. The use of read-level bisulfite sequencing data to infer cell type- and allele-specific methylation patterns is well established in the literature28,29, though until now it has not yet been deployed on a genome-wide scale.

To pursue this, we divided the genome into 100 bp bins and grouped reads with identical methylation patterns (see Methods), and used these as the basis for identifying cell type-specific differential methylation. We prototyped this approach using a published WGBS dataset from sorted neurons and glia30 (Supplementary Fig. 4) providing clear evidence that unique clusters of reads with identical patterns are associated with neural cell type39. To draw inferences specific to AgRP neurons in our mice, we sought bins in which a group of reads with substantial CpG methylation (>55%) is missing in F/F mice, and is replaced by a new group of reads with lower methylation (<45%) (Fig. 4a). We identified 3651 such 100 bp bins, which we refer to as 'sector 2' in two-dimensional Euclidian space (Fig. 4b). The opposing sector ('sector 4') contains 5222 bins with a unique substantially methylated cluster (and loss of a less-methylated cluster) specifically in F/F mice, which likely originate from non-AgRP neurons. When this procedure is carried out on randomly permuted reads, we observe substantially fewer bins in all sectors with estimated false positive rates of 4.36% and 3.2% for sectors 2 and 4, respectively (Supplementary Fig. 4D, E). Sector 2 and sector 4 bins were similarly represented in most genomic regions (Supplementary Fig. 5A) including promoters (Fig. 4c, Supplementary Fig. 5B), but sector 2 bins were enriched in CpG islands (Supplementary Fig. 5A, C; Supplementary Data 3). Although it is possible in principle for a single bin to be in both sectors 2 and 4, this occurred only 1% of the time (Fig. 4c). We found minimal overlap between hypermethylated DMRs and sector 4 bins, and similarly minimal overlap between hypomethylated DMRs and sector 2 bins (Supplementary Fig. 5D, E, respectively), indicating that this read-clustering method provides a different level of information beyond that of DMRs.

We next asked if read-level differential methylation could be linked to a specific ARH neuronal cell type. We plotted the distribution of promoter bins for differentially expressed genes associated with each ARH neuronal cell type (Supplementary Fig. 6)24. Several non-AgRP neuron types show significant enrichment of differentially expressed genes with promoter bins in sector 4 (Fig. 4d), including Arx/Nr5a2 (Fig. 4e), suggesting that these cells may be the primary drivers of the hypermethylation observed in F/F mice. To further explore putative secondary methylation changes induced in non-AgRP neurons, we used GREAT22 to associate sector 4 bins with genes, finding a number of GO function terms related to TGF-β signaling and 1-SMAD binding (Fig. 5a). We used InterPro to search for common protein domains among genes associated with sector 4 bins; remarkably, the SMAD/FHA domain was the top hit (Fig. 5b). In line with this, there was significant enrichment of SMAD2 and SMAD3 binding motifs among hypermethylated DMRs (Fig. 5c). Together these data indicate that SMAD binding is a common feature of regions of hypermethylation in F/F mice. Since the SMAD family of transcription factors are the main transducers of TGF-β signaling31, we next sought out potential upstream TGF-β ligands. We focused our attention on bone morphogenetic protein (Bmp) signaling31. Expression of both Bmp5 and Bmp7 was increased in F/F ARH neurons, while that of the Bmp receptor Bmpr2 was decreased (Fig. 5d). Since neither Bmp5 nor Bmp7 are known to be expressed by ARH neuronal cell types24, we used read-level analysis of WGBS data to infer the cellular origin of this increased expression. In the case of Bmp7, we identified a sector 2 bin located in the promoter region of this gene (chr2:172938700–172938800) containing two unique hypomethylated read clusters in F/F neurons (Fig. 5e). Importantly, the Bmp7 promoter region does not contain any DMRs, nor does...
average methylation differ between the genotypes (Supplementary Fig. 5F). To test whether the hypomethylated read clusters in this bin are a signature of reduced methylation of Bmp7 specifically in AgRP neurons, we used immunohistochemical labeling of SynTom followed by laser capture microdissection (LCM) to capture the AgRP neuron-enriched mediobasal ARH (Fig. 5G). Indeed, quantitative bisulfite pyrosequencing overlapping the Bmp7 promoter region we identified (Fig. 5E) indicated decreased methylation in F/F AgRP neurons relative to +/+ (Fig. 5G). We next used dual fluorescence in situ hybridization to localize Bmp7 expression changes within the ARH and found increased levels of Bmp7 expression specifically in AgRP neurons of F/F mice (Fig. 5H, I), consistent with the AgRP neuron-specific decreases in promoter methylation. Together, these findings support the utility of our novel read-level analysis to draw cell type-specific inferences from WGBS data, and provide mechanistic insights linking primary (cell autonomous) and secondary epigenetic alterations potentially contributing to the physical activity phenotype in this model.

Discussion

Environmental influences during critical ontogenic periods can permanently shift an individual’s energy balance. Diverse models of such developmental programming of energy balance are characterized by alterations in energy expenditure and physical activity but not food intake4, leading us to propose that physical activity may be the most plastic or ‘programmable’ component of energy balance regulation32. At present the existence of a neural ‘activity-stat’33 regulating individual physical activity remains hypothetical and with no definitive neurobiological substrate3. Given the influence of central leptin signaling on physical activity7,13,34, such a system would likely be coupled to energy balance circuitry, and be programmable by early-life nutrition. Our discovery that DNA methylation in AgRP neurons governs voluntary physical activity meets both of those criteria, pointing to a role for epigenetic modification of hypothalamic neurons in setting the physical activity set point. This phenomenon likely depends on a range of downstream neurobiological changes, the full elaboration of which exceeds our present scope. However, several lines of evidence point to alterations in GABAergic signaling and synaptic development in ARH neurons, consistent with the established role of DNA methylation in synapse formation35 and the primarily GABAergic nature of AgRP neurons36. Given that AgRP neurons are GABAergic36, simultaneous disruption of GABA synthetic enzyme and receptor subunit expression could point toward broader, network-level changes in neural signaling, potentially brought about by disrupted differentiation of AgRP neurons in F/F mice.

Fig. 4 Read-level analysis of WGBS data points to epigenetic dysregulation in cell type-specific genes. a, b Illustration of strategy for pinpointing cell type-specific loss of methylation from read-level WGBS data. Searching for differentially methylated read clusters in 100 bp genomic bins enables inferences about changes taking place in individual cell types. Clusters comprise at least 4 reads (see Methods); single reads are shown here for simplicity. Hypomethylated read clusters specific to F/F mice likely originate from AgRP neurons, whereas substantially methylated read clusters specific to F/F mice may arise from secondary effects in other cell types. b For each bin with both a cluster unique to F/F and a cluster unique to +/+ mice, average methylation levels of the unique clusters are shown in a density plot. Bins in ‘sector 2’ are defined by the simultaneous absence of a substantially methylated (≥ 55%) read cluster in +/+ mice and appearance of a novel hypomethylated (≤ 45%) read cluster in +/+ mice. ‘Sector 4’ is defined conversely. c Circos plot showing density of sectors 2 and 4 bins located in promoter regions of genes. Right: Venn diagram shows minimal overlap of sectors 2 and 4 promoter bins. d, e Promoter bins for differentially expressed genes specific to the molecularly defined ARH neural cell types Gm8773/Tac1, Arx/Nr5a2, Tbx19, Unassigned2, Slc17a6/Ttrh, and Trh/Leff22 are enriched in sector 4 relative to sector 2, suggesting that these cell types contribute to the observed hypermethylation in F/F ARH neurons as a whole. Bars represent –log10(p) of χ² tests comparing cell type-specific sectors 2 and 4 bins relative to a background list of all sectors 2 and 4 bins (p < 0.05). Numbers in parentheses represent ratio of sector 2:sector 4 bin counts. e Plot showing the location of promoter bins for differentially expressed genes specific to Arx/Nr5a2 ARH neurons. Plot coordinates follow the same convention as b.
Reduced Bmp7 methylation and increased expression in AgRP neurons lacking Dnmt3a. a Bins in sector 4 were associated with GO terms related to TGF-β and SMAD signaling. b Genes associated with sector 4 bins were enriched for proteins containing SMAD/FHA domains. c MEME-ChIP analysis shows that a SMAD2 and SMAD3 transcription factor binding motif is enriched among hypermethylated DMRs, suggesting that establishment of hypermethylation in F/F mice is downstream of TGF-β signaling. d Increased expression of bone morphogenetic protein (Bmp) signalers Bmp5 and Bmp7, as well as downregulation of Bmpr1a and Bmpr2 receptor subunits and altered expression of Smad2, Smad4, and Smad5. Values represent mean ± SEM. e Gene diagram showing location of sector 2 bin in promoter region of Bmp7. Tanghulu plots of read-level CpG methylation data show clusters of hypomethylated reads unique to F/F neurons. Inset: plot showing location of Bmp7 promoter bin in sector 2. f Representative photomicrograph showing immunohistochemical localization and laser capture microdissection of AgRP neuron-enriched mediobasal ARH. Arrows indicate AgRP neurons (identified by SynTom immunostaining). White dashed lines indicate AgRP neuron-rich mediobasal ARH boundaries. Black dashed lines indicate ARH neuroanatomical boundaries. Scale bars indicate 100 μm. g Quantitative bisulfite pyrosequencing of the Bmp7 promoter region identified in j (CpG positions 1–4) plus 100 bp downstream (CpG positions 5–9). Linear mixed model analysis indicates significantly decreased Bmp7 methylation in the AgRP neuron-enriched mediobasal ARH (+/+ − F/F = −37.1 ± 4.49, df = 18.3, p = 0.0002). Linear mixed models were calculated using animal (n = 6–8) as a random factor. h Fluorescent in situ hybridization analysis of Aggr and Bmp7 co-expression. Scale bars = 10 μm. i Linear mixed model analysis of fluorescent in situ hybridization data shows increased Bmp7 expression in AgRP neurons (+/+ − F/F = −17.55 ± 6.68, df = 10.07, p = 0.025) but not in non-AgRP cells (+/+ − F/F = −6.38 ± 6.66, df = 9.89, p = 0.36). Linear mixed models were calculated using animal (n = 3) as a random factor with n = 90–111 cells per condition; *p < 0.05, ***p < 0.001. Source data for d and g are provided as a Source Data file.

Our use of AgRP-IRES-Cre mice means that the onset of the Dnmt3a knockout coincides with the onset of Aggr expression. In mice, this takes place in the late embryonic and early postnatal period, during which hypothalamic neurons gain significant amounts of DNA methylation. DNA methylation in the brain regulates gene expression, but is itself established secondary to gene expression during development. This, and the fact that epigenetic modifications affect gene expression potential, is consistent with our observations of an imperfect correspondence between differential methylation and expression, though the two were broadly correlated. These points do leave open numerous questions regarding the exact timing of de novo DNA methylation and demethylation and their role in dynamic epigenetic and gene expression changes in mature cells. Future studies using inducible Dnmt3a knockdown/reinstatement would be ideally suited for such questions.

Physical activity is an important component of the energy balance equation, though it has a complicated relationship with energy expenditure and body composition. Since neither wheel running nor home cage locomotion are resistance exercises, they do not increase muscle mass. Running wheel access does reliably increase rates of physical activity and total energy expenditure, though its effects on body weight and composition depend on potential compensatory increases in food intake. The fundamental cause of the reduced exercise in F/F mice remains mysterious. Although our metabolic treadmill results suggest a volitional origin, further study using larger samples sizes and complementary measures of volition will be required to confirm this.

Having perturbed a fundamental mechanism of transcriptional regulation in AgRP neurons, a major challenge lies in determining relevant downstream mediators of the observed phenotype. This...
imperative exists for every study of cell type-specific genetic disruption, because the resulting phenotype depends not only on the loss of function in one cell type, but also on how other cells in the system respond. Compensatory effects abound in the energy balance circuitry; indeed, mice can survive early-life ablation of AgRP neurons with only a subtle adult phenotype. Similar compensatory mechanisms may underlie the contrast between the numerous transcriptional and epigenomic changes we found at P12 and the comparatively mild adult adiposity phenotype we observed.

To begin to understand downstream, cell type-specific effects, we implemented a read-level analysis of our WGBS data, analogous to previously described approaches using ultra-deep BS-seq to identify minor cell populations in pituitary tissue. This led us to conclude that TGF-β1/β2/β3, a TGF-β ligand31 implicated in hypothalamic development46,47, is upregulated specifically in AgRP neurons of F1/F mice. Bmp7 influences axon guidance during hypothalamic neuroendocrine cells18, and deletion of Bmpra1a in Olig1 hypothalamic progenitors significantly decreases AgRP and POMC neuronal projections to the PVH48. Overexpression of Bmp7 (caused by AgRP neuron-specific promoter hypomethylation) may thus explain both cell autonomous developmental effects within AgRP neurons and, by virtue of being a secreted factor, effects on methylation in neighboring neurons. Still, Bmp7 was one of thousands of differentially expressed genes and while numerous lines of evidence suggest its involvement, it is unlikely a solitary actor—which will complicate the interpretation of potential directed overexpression follow-up experiments. While further studies will be needed to fully investigate the role of Bmp7 and potential other players, and to conclusively link developmental epigenetics in the melanocortin system to adult physical activity, our work provides a novel insight that neuroepigenetic development is a key determinant of voluntary exercise behavior.

Methods

Animals

This study was approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine, Childrens Hospital of Los Angeles, and Vanderbilt University, and animals were maintained in accordance with federal guidelines. Mice were housed in same-sex groups of 2–4 for most experiments, and maintained on a 12 h light/dark cycle (lights on at 6:00 AM) at 22 °C with ad libitum access to food and water in glass bottles. NPY-hrGFP21 mice (Jackson Laboratories, RRID:IMSR_JAX:004017) express humanized thalamic neuroendocrine cells48, and deletion of Bmpr1a in Olig1 hypothalamic progenitors significantly decreases AgRP and POMC neuronal projections to the PVH. Overexpression of Bmp7 (caused by AgRP neuron-specific promoter hypomethylation) may thus explain both cell autonomous developmental effects within AgRP neurons and, by virtue of being a secreted factor, effects on methylation in neighboring neurons. Still, Bmp7 was one of thousands of differentially expressed genes and while numerous lines of evidence suggest its involvement, it is unlikely a solitary actor—which will complicate the interpretation of potential directed overexpression follow-up experiments. While further studies will be needed to fully investigate the role of Bmp7 and potential other players, and to conclusively link developmental epigenetics in the melanocortin system to adult physical activity, our work provides a novel insight that neuroepigenetic development is a key determinant of voluntary exercise behavior.
antibody. The next day, sections were washed and developed using Alexa488-conjugated donkey anti-rabbit IgG (Thermo Fisher A-21206, 1:500). After washing, sections were incubated overnight in biotinylated anti-RFP (Abcam ab343771, 1:1000) at room temperature. The next day, sections were developed using Alexa647-streptavidin conjugate (Thermo Fisher S32357, 1:500) for 1 h at room temperature. 5-hydroxymethylcytosine -5-hmC staining was carried out similarly to -5-mC staining, with the exception of an antigen retrieval step (10 mM Sodium citrate, 6.0 for 20 min at 95 °C) prior to primary antibody incubation in rabbit anti-5-hmC (Abcam ab214278, 1:2000) took place overnight at room temperature. This combination of steps is needed to enable 5-hmC staining, but also renders RFP/SynTom labeling ineffective.

Confocal microscopy and image analysis. Immunofluorescence images were captured using a Leica DMi8 laser scanning confocal microscope. For -5-mC and -5-hmC staining, Z-stacks were captured at 0.5 μm intervals through at least three ARH sections (~1.46 mm to ~2.06 mm relative to Bregma) per animal at 10× magnification. -5-mC was quantitated by mean fluorescence intensity in AGRP neurons based on co-localization with SynTom-labeled cell bodies. To estimate whether loss of Dnmt3a lead to loss of AGRP neurons, we also compared the number of SynTom-labeled cell bodies in anatomically matched sections between the two genotypes. Because the antigen retrieval process for -5-hmC rendered RFP/SynTom labeling ineffective, we analyzed the intensity of -5-hmC labeling in neurons located in the mediobasal region of the ARH, which contains the highest density of AGRP neurons. Mean fluorescence intensity of these cells was compared to that of cells located in the dorsolateral ARH, a region that lacks AGRP neurons, to obtain a rough estimate of altered 5-hydroxymethylation in AGRP neurons.

Tissue preparation. Brains from -/+ and F/F mice (n = 5 per genotype) were collected at P12 following sacrifice by decapitation. Brains were removed and placed into ice-cold PBS then flash-frozen whole on powdered dry ice. AGRP microdissections were performed using the micromanipulator, and -5-mC microarrays were performed using 200-μm coronal vibratome sections from each brain under a dissecting microscope. Triangular-shaped cuts were made along the morphological boundaries of the nucleus (Fig. 3a). ARH microdissections were flash-frozen on dry ice and stored at -80 °C. ARH microdissections were dissociated by Dounce homo- genization, and nuclei were purified by ultracentrifugation against a 1.8 M sucrose column at 100,000 rpm. Purified nuclei were stained using rabbit-anti-NeuN (Millipore ABN78; 1:4000) followed by Alexa488-conjugated goat anti-rabbit IgG (Thermo Fisher A-11008; 1:2000) and the nucleic acid dye TO-PRO-3 as a counterstain (Thermo Fisher, 1:1000). Sorting was performed on a Sony SH800 Cell Sorter (Sony Biotechnology). FSC and SSC gates were established to eliminate debris and non-nuclear material (Supplementary Fig. 3A), and TO-PRO-3 gating was used to collect single nuclei only. NeuN− and NeuN+ populations were sorted on the basis of antibody staining in the sortier’s Pirity mode18. Nuclei were immediately frozen on dry ice and stored at -80 °C. ARH NeuN− nuclei were homogenized in lysis buffer, and DNA was extracted using the Affymetrix GeneChip® Human mRNA Micro Array (QIAGEN) according to the manufacturer’s instructions. DNA was eluted from columns using two rounds of 50 μl nuclease-free H2O, pH 8.0, dried in a SpeedVac (Eppendorf), and resuspended in 12 μl of TE buffer (pH 8.0). DNA was quantitated using the PicoGreen assay (Thermo Fisher) as directed by the manufacturer. DNA was prepared from low-throughput of 100 μl of DNA STAT-60 (Tel-Test Inc.) according to the manufacturer’s instructions and resuspended in 12 μl of nuclease-free H2O. RNA was quantitated by NanoDrop spectrophotometry (Thermo Fisher).

qPCR. RNA was extracted from ARH microdissections from neonatal C57BL/6 mice at P2, P10, and P12 using RNA STAT-60 (Tel-Test Inc.) according to the manufacturer’s instructions and resuspended in 12 μl of nuclease-free H2O. RNA was quantitated by NanoDrop spectrophotometry (Thermo Fisher). First-strand cDNA synthesis was performed using M-MLV reverse transcriptase (Promega) and random oligo primers (Thermo Fisher) according to the manufacturer’s directions. qPCR for β-actin and Dnmt3a was performed using TaqMan assays (Mm00432881_m1, respectively) according to the manufacturer’s instructions.

Bisulfite sequencing. Tagmentation-based whole-genome bisulfite library preparation was performed on genomic DNA extracted from ARH NeuN+ nuclei35. Adapters were pre-annealed and transposomes were assembled according to the protocol described in Wang et al.17; 10–30 ng of mouse genomic DNA was tagged by adding the assembled transposome and incubating at 55 °C for 8 min. Following purification using SPRI beads, oligonucleotide replacement and gap repair were performed using Ampliplok and T4 DNA Polymerase. The product was purified using SPRI beads and followed by bisulfite treatment using the EZ DNA Methylation Gold kit (Zymo Research). Subsequently libraries were generated by PCR amplification using KAPA 2G robust hotstart ready mix. After purification using SPRI beads, libraries were diluted and sequenced on an Illumina HiSeq2500 Sequencer in 150 bp paired-end mode.

RNA sequencing. Libraries for RNA sequencing were prepared using the SMART-Seq v4 Ultra Low Input kit (Takara Clontech) according to the manufacturer’s instructions for purified RNA was incubated with bisulfite buffer for 5 min. First-strand cDNA synthesis was performed using the included 3-SMART-seq CDS primer II and V oligonucleotide. cDNA was amplified using PCR Primer II A, and subsequently purified using Ampure XP beads (Beckman). Illumina libraries were prepared using Nextera XT DNA library preparation kit (Illumina), and sequenced on the Illumina HiSeq 2500 platform generating 100 bp paired-end reads.

WGBS analysis. Fastq reads were quality trimmed using TrimGalore (v0.4.3) with a minimum quality score of 20 and a minimum post-trimming read length of 50. Read quality was analyzed before and after trimming using Fastqc (v0.11.5). After trimming, the reads were aligned to the mouse genome (mm10) using Bismark (v0.18.1) set to paired-end mode and using all other default settings. Read counts corresponding to each individual were generated by use of the featureCounts algorithm with Picard (v2.10.10). ‘AddOrReplaceReadGroups’ BAM files were query-name sorted using Picard ‘SortSam’, and then deduplicated using Picard ‘MarkDuplicates’. One library was removed from the analysis due to high PCR duplicate rate (85%) and low coverage (1.2× average coverage). Coverage for the remaining libraries totalled 45× for +/+ and 47× for F/F. Differentially methylated CpG loci (DMs) were identified by DSS36 using the general experimental design with an FDR cutoff of 0.05. Only CpGs with at least five reads per library were considered. DMs were subsequently identified by DSS with a p-value threshold of 0.05 and an absolute delta cutoff of 10% between the genotypes. DMs were annotated to UCSC gene features, with promoter regions defined as ±2.5 kb from the canonical transcription start site, enhancers were identified by a combination of DNAse sensitivity and H3K27ac enrichment (ENCODE Encyclopedia, Version 3 http://zlab-annotations.umassmed.edu/encainers/). Intergenic DMs were defined as any DMR not overlapping any gene body, promoter, or 3’ region. Hyp and hypermethylated DMs were defined as DMRs in regions by VISTA > GREAT v3.09, and the top eight enriched GO process terms for each were reported22. Hypermethylated DMs were analyzed by MEME-Chip using the default settings37.

RNA-seq analysis. Fastq reads were quality trimmed using TrimGalore (v0.4.4) with a minimum quality score of 20 and a minimum read length of 50. Read quality was analyzed using fastqc (v0.11.5). Quality-trimmed reads were aligned to the mouse genome (mm10) using HISAT2 (v2.1.0).Mapped reads were quantitated and counted using featureCounts (v1.5.3) along with the genodice comprehensive genomic annotation (gencode vM15 gtf file). Differential expression testing was performed using DESeq2 (v1.16.1). Genes showing significant (FDR q value <0.01, absolute log, fold change ≥2) between genotypes were used for GO analysis in separate lists (www.geneontology.org). The top eight GO process terms (sorted by enrichment, FDR < 0.001) for both up-and downregulated genes were reported. Expression and gene-associated DNA methylation were correlated using Pearson’s product moment correlation coefficient with values collapsed over genotype. To plot our gene expression results against ARH cell-type-specific enrichment, we first selected the top 100 genes for enrichment in each of the neural cell types defined by the Campbell et al.34. For each of these lists, we plotted the ratio of highly (FDR q value <0.01, absolute log, fold change ≥2) up- and downregulated genes in our RNA-Seq results. We used 2 × 2 to analyze these ratios, comparing the number of up- and downregulated genes in each cell type class to the overall number of significi- cantly up- and downregulated genes detected. p-Values were adjusted for multiple testing by Benferroni correction.

Read-level WGBS analysis. The mouse genome was divided into non-overlapping 100 bp windows. Mapped BAM files were query- name sorted and deduplicated using Picard (v2.10.10) and coordinate sorted using SAMtools (v1.9). For each 100 bp window, all mapped reads were extracted from coordinate-sorted BAM files. The methylation state of each CpG site was extracted for every read. To ensure proper mapping, reads originating from the same DNA strand were removed. If the same read was mapped twice, we discarded overlapping bases originating from the same read pair. When possible, the remaining bases on the trimmed read were stitched onto the first read, maximizing the amount of information obtained. We excluded any reads not fully covering all CpGs within the 100 bp window, and the remaining read data was used to create a m × n matrix associated with a hypermethylation pattern, with m representing the number of reads and n representing the number of CpG sites. The matrix was populated with values of 0 and 1 representing unmethylated and methylated CpGs, respectively.

A read-methylation matrix was generated for both +/+ and F/F samples. If both matrices had at least 10 reads, the matrices were combined. Reads containing identical methylation patterns were grouped together using the DBSCAN method from the scikit-learn package38. Any group with <4 reads was dropped. The methylation pattern, methylation level, number of reads, and genomic location of each group was reported from the remaining groups. This method was carried out for each 100 bp window in the read-methylation matrix, with n representing the number of reads and m representing the number of CpG sites. The matrix was reported with values of 0 and 1 representing unmethylated and methylated CpGs, respectively.

To estimate the rate of spurious read cluster detection, we performed a permutation test. For each 100 bp bin, the genotype identification of each read was
randomly re-assigned. Re-assigned reads were labeled either A or B, and read clustering was then performed as described above. This process was repeated over 10 iterations. False-positive rates were calculated as the average number of bins detected in each sector of permuted data divided by the number detected in the unpermuted data.

**Bisulﬁte pyrosequencing.** A subset of DMRs were validated in a separate cohort of ARH neurons from mice sacriﬁced at P12 and P35 by bisulﬁte pyrosequencing using a Biomek Pyromark Q96 pyrosequencing system60. Prior to sequencing, pyrosequencing assays were assessed for sensitivity and linearity by running standard curves consisting of known mixtures of fully methylated and unmethylated mouse DNA (0, 25, 50, 75, and 100% methylation)39. Assay primers are provided in Supplementary Table 3.

**ISH analysis.** Two-color ﬂuorescent in-situ hybridization was performed on 15 μm ﬁxed frozen slices from P12 male +/+ and F/F mice mounted on SuperFrost Plus slides (Fisher Scientiﬁc) using probes and directions provided by Advanced Cell Diagnostics (ACD Technical Notes #320535 for tissue prep, and #320295 for Fluorescent Multiplex kit, http://www.acdbio.com/technical-support/downloads). A channel 2 probe against Agrp was used to identify AgRP neurons (Mm-Agrp-C2, formed as previously described60, using cell volumes from maximum intensity pyrosequencing and in situ hybridization was performed using a linear mixed system, were analyzed using repeated measures ANOVA. Analysis of bisulﬁte pyrosequencing data, including cell volumes from maximum intensity pyrosequencing, was assessed for sensitivity and linearity by running standard curves consisting of known mixtures of fully methylated and unmethylated mouse DNA (0, 25, 50, 75, and 100% methylation)39. Assay primers are provided in Supplementary Table 3.

**Laser capture microdissection.** LCM of AgRP neuron-enriched ARH samples was performed using ﬁxed frozen 40-μm corional tissue sections from male and female P12 +/+ and F/F SynTom mice. Sections were incubated overnight at room temperature in blocking buffer containing the rabbit anti-AFP (Abcam ab233862, 1:1000) primary antibody. Following this, sections were washed and developed using the rabbit-speciﬁc HRP/DAB detection kit (Abcam ab64261) according to the manufacturer’s directions. Sections were mounted on PEN membrane slides (ThermoFisher) prior to LCM. LCM was performed on an ArcturusXT LCM system (ThermoFisher) using the dual laser mode to micro-dissect and retrieve the AgRP neuron-enriched mediobasal ARH with immunohistochemical guidance. Microdissections were collected onto Capped Sure LC caps (ThermoFisher) and DNA was extracted, bisulﬁte converted, and pyrosequenced as described above.

**Statistics.** SPSS 17 (IBM) and R 3.4.0 (R Foundation for Statistical Computing) were used for all statistical analysis. To minimize the inﬂuence of litter effects in these studies, mice from at least three separate litters were used for all experiments. Except where noted, the experimental unit of analysis was individual mice. Genotype effects were compared using two-tailed independent samples t-tests. With genotype, age, sex, CpG position, and cell type serving as ﬁxed factors where appropriate, and animal serving as a random factor. Post-hoc analysis of ﬁxed effects was performed using linear contrasts. Statistical analysis of and RNA-Seq and WGBS data are explained in their respective sections.

**Data availability**

All described sequencing data (WGS and RNA-Seq) have been deposited in the Gene Expression Omnibus (GEO) (Accession number GSE12405). Processed and normalized data including DMR locations, RNA-Seq expression values, and regions identiﬁed by read-level analysis are available in Supplementary Data 1, 2, and 3, respectively. The source data underlying Figs. 1a, c, d, 2a–i, 3g, 5d, g, Supplementary Figs. 1D–L, 2C, and 3A–L are provided as a Source Data file.

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**References**

1. Garland, T. et al. The biological control of voluntary exercise, spontaneous physical activity and daily energy expenditure in relation to obesity: human and rodent perspectives. J. Exp. Biol. 214, 206–229 (2011).

2. Kohl, H. W. et al. The pandemic of physical inactivity: global action for public health. Lancet 380, 294–305 (2012).

3. Lightfoot, J. T. et al. Biological/genetic regulation of physical activity level: consolidated from GenBioPAC; Cell Metab. 50, 863–873 (2018).

4. Eclarinal, J. D. et al. Maternal exercise during pregnancy promotes physical activity in adult offspring. FASEB J. 30, 2541–2548 (2016).

5. Baker, M. S., Li, G., Kohorst, J. I. & Waterland, Ra Fetal growth restriction promotes physical inactivity and obesity in female mice. Int. J. Obes. 39, 98–104 (2015).

6. Li, G. et al. Early postnatal nutrition determines adult physical activity and energy expenditure in female mice. Diabetes 62, 2773–2783 (2013).

7. Fernandes, M. F. A. et al. Leptin suppresses the rewarding effects of eating via STAT3 signaling in dopamine neurons. Cell Metab. 22, 741–749 (2015).

8. Cone, R. D. et al. The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. Int. J. Obs. Relat. Metab. Disord. 25, S63–S67 (2001).

9. Huang, H. et al. ROCK1 in AgRP neurons regulates energy expenditure and locomotor activity in male mice. Endocrinology 154, 3660–3670 (2013).

10. Dietrich, M. O. et al. AgRP neurons regulate development of dopamine neuronal plasticity and non-food-associated behaviors. Nat. Neurosci. 15, 1108–1110 (2012).

11. Menaros, A. et al. Activation of Stat3 signaling in AgRP neurons promotes locomotor activity. Cell Metab. 7, 236–248 (2008).

12. He, Z. et al. The hypothalamic arcuate nucleus: a key site for mediating leptin’s effects on glucose homeostasis and locomotor activity. Cell Metab. 1, 71–76 (2005).

13. Feng, J., Chang, H., Li, E. & Fan, G. Dynamic expression of de novo DNA methyltransferases Dnmt3a and Dnmt3b in the central nervous system. J. Neurosci. Res. 79, 734–746 (2005).

14. Mo, A. et al. Epigenomic signatures of neuronal diversity in the mammalian brain. Neuron 86, 1369–1384 (2015).

15. Li, G. et al. Major epigenetic development distinguishing neuronal and non-neuronal cells occurs postnatally in the murine hypothalamus. Hum. Mol. Genet. 23, 1579–1590 (2014).

16. Nigent, B. M. et al. Brain feminization requires active repression of masculinization via DNA methylation. Nat. Neurosci. 18, 690–697 (2015).

17. Azizi, A. et al. Circadian behavior is light-reprogrammed by plastic DNA methylation. Nat. Neurosci. 17, 377–382 (2014).

18. Kohno, D. et al. Dnmt3a in Simi1 neurons is necessary for normal energy homeostasis. J. Neurosci. 34, 15288–15296 (2014).

19. Santiago, M., Antunes, C., Guedes, M., Sousa, N. & Marques, C. J. T. Epigenetic enzymes and DNA hydroxymethylation in neural development and function - How critical are they? Epigenetics 10, 344–349 (2015).

20. Kanitakahara, A., Bouyer, K., Wang, C. H. & Simmerly, R. A critical period for the trophic actions of leptin on AgRP neurons in the arcuate nucleus of the hypothalamus. J. Comp. Neurol. 526, 133–145 (2018).

21. McLean, C. Y. et al. GREAT improves functional interpretation of cis-regulatory regions. Nat. Biotechnol. 28, 495–501 (2010).

22. Yu, D.-H. et al. Developmentally programmed 3′ CpG island methylation confers tissue- and cell-type-specific transcriptional activation. Mol. Cell. Biol. 33, 1845–1858 (2013).

23. Campbell, J. N. et al. A molecular census of arcuate hypothalamic and median eminence cell types. Nat. Neurosci. 20, 484–496 (2017).

24. Newman, A. M. et al. Robust enumeration of cell subsets from tissue expression proﬁles. Nat. Methods 12, 453–457 (2015).

25. Houseman, E. A. et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics 13, 86 (2012).

26. Guo, S. et al. Identiﬁcation of methylation haplotype blocks aids in deconvolution of heterogeneous tissue samples and tumor-tissue-of-origin deconvolution from plasma DNA. Nat. Genet. 49, 635–642 (2017).

27. Harris, R. A. et al. Comparison of sequencing-based methods to proﬁle DNA methylation and identiﬁcation of monoallelic epigenetic modiﬁcations. Nat. Biotechnol. 28, 1097–1105 (2010).

28. Arai, Y. et al. Ultra-deep bisulﬁte sequencing to detect specific DNA methylation patterns of minor cell types in heterogeneous cell populations: an example of the pituitary tissue. PLoS ONE 11, e0146948 (2016).

29. Lister, R. et al. Global epigenomic reconfiguration during mammalian brain development. Science 341, 1237905 (2013).

30. Shi, Y. & Massague, J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113, 685–700 (2003).

31. Housman, D. et al. Eclarinal, J., Baker, M. S., Li, G. & Waterland, Ra D. Developmental programming of energy balance regulation: Is physical activity more ‘programmable’ than food intake? Proc. Nutr. Soc. 75, 73–77 (2016).
33. Rowland, T. W. The biological basis of physical activity. *Med. Sci. Sports Exerc.* 30, 392–399 (1998).

34. Pelleymounter, M. A. et al. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 269, 540–543 (1995).

35. Fung, J. et al. Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nat. Neurosci.* 13, 423–430 (2010).

36. Tong, Q., Ye, C.-P., Jones, J. E., Elmquist, J. K. & Lowell, B. B. Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance. *Nat. Neurosci.* 11, 998–1000 (2008).

37. Nilsson, I., Johansen, J. E., Schalling, M., Hökfelt, T. & Fetissov, S. O. Maturation of the hypothalamic arcuate agouti-related protein system during postnatal development in the mouse. *Brain Res. Dev. Brain Res.* 155, 147–154 (2005).

38. Sutcliffe, S. H. et al. Early-life gene expression in neurons modulates lasting epigenetic states. *Cell* 171, 1151–1154.e16 (2017).

39. Sharma, A., Klein, S. S., Barboza, L., Lohdi, N. & Toth, M. Principles governing DNA methylation during neuronal lineage and subtype specification. *J. Neurosci.* 36, 1711–1722 (2016).

40. Luo, C., Hajkova, P. & Ecker, J. R. Dynamic DNA methylation: In the right place at the right time. *Science* 316, 1336–1340 (2012).

41. Azzi, A. et al. Network dynamics mediate circadian clock plasticity. *Neuron* 93, 441–450 (2017).

42. Allen, D. L. et al. Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse. *J. Appl. Physiol.* (1985) 90, 1900–1908 (2001).

43. Novak, C. M., Burghardt, P. R. & Levine, J. A. The use of a running wheel to measure activity in rodents: relationship to energy balance, general activity, and reward. *Neurosci. Biobehav. Rev.* 36, 1001–1014 (2012).

44. Luquet, S., Perez, Fa, Hnasko, T. S. & Palmiter, R. D. NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science* 310, 683–685 (2005).

45. Tzan, K., Knight, Z. A. & Friedman, J. M. Ablation of AgRP neurons impairs adaption to restricted feeding. *Mol. Metab.* 3, 694–704 (2014).

46. Dale, J. K. et al. Cooperation of BMP7 and SHH in the induction of forebrain ventral midline cells by prechordal mesoderm. *Cell* 90, 257–269 (1997).

47. Ohyama, K., Das, R. & Placzek, M. Temporal progression of hypothalamic patterning by a dual action of BMP. *Development* 135, 3325–3331 (2008).

48. Liu, F. & Placzek, M. Axon guidance effects of classical morphogens Shh and BMP7 in the hypothalmo-pituitary system. *Neurosci. Lett.* 562, 108–113 (2014).

49. Peng, C.-Y., Mukhopadhyay, A., Jarrett, J. C., Yoshikawa, K. & Kessler, J. BMP receptor 1A regulates development of hypothalamic circuits critical for feeding behavior. *J. Neurosci.* 32, 17211–17224 (2012).

50. van den Pol, a.N et al. Neuromedin B and gastrin-releasing peptide excite arcuate nucleus neuropeptide Y neurons in a novel transgenic mouse expressing strong renilla green fluorescent protein in NPY neurons. *J. Neurosci.* 29, 4622–4639 (2009).

51. Fukuda, M. et al. Monitoring FoxO1 localization in chemically identified neurons. *J. Neurosci.* 28, 13640–13648 (2008).

52. Kaneda, M. et al. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* 429, 900–903 (2004).

53. Harmo, E., Cottrell, E. C. & White, A. Metabolic pitfalls of CNS cre-based technology. *Cell Metab.* 18, 21–28 (2013).

54. Marosi, K. et al. Metabolic and molecular framework for the enhancement of endurance by intermittent food deprivation. *FASEB J.* 32, 1–15 (2018).

55. Wang, Q. et al. Tagmentation-based whole-genome bisulfite sequencing. *Nat. Protoc.* 8, 2022–2032 (2013).

56. Park, Y. & Wu, H. Differential methylation analysis for BS-seq data under general experimental design. *Bioinformatics* 32, 1446–1453 (2016).

57. Machnik, P. & Bailey, T. L. MEME-ChIP: motif analysis of large DNA datasets. *Bioinformatics* 27, 1696–1697 (2011). PMC3106185.

58. Pedregosa, F. et al. Scikit-learn: machine learning in Python. *J. Mach. Learn. Res.* 1, 1–2825–2830 (2011).

59. Shen, L., Guo, Y., Chen, X., Ahmed, S. & Isa, J. P. J. Optimizing annealing temperature overcomes bias in bisulfite PCR methylation analysis. *BioTechniques* 42, 48–58 (2007).

60. Henry, F. E., Sugino, K., Tozer, A., Branco, T. & Sternson, S. M. Cell type-specific transcriptomics of hypothalamic energy-sensing neuron responses to weight-loss. *Elife* 4, 1–30 (2015).

61. Kuhnen, P. et al. Interindividual variation in DNA methylation at a putative POMC metastable epiallele is associated with obesity. *Cell Metab.* 24, 502–509 (2016).

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**Author contributions**

R.A.W., R.B.S., and H.M. conceived and designed the experiment. H.M. carried out all animal studies with assistance from M.S.B. and E.L. H.M. performed tissue microdissection, FACS, DNA/RNA isolation, qPCR, LCM, and all histology with the exception of immunofluorescent analysis of Dnmt3a/NPY-Gfp colocalization, which was performed by A.E.E. Library preparation and sequencing was performed by Y.L. and R.C. Analysis of sequencing data was performed by H.M., C.A.S., and J.D.D. under the supervision of R.A.W. and C.C. Read-level WGBS analysis was conceived by R.A.W. and implemented by C.A.S. Bisulfite pyrosequencing was performed by M.S.B. and E.L. Guidance on evaluation of locomotor behavior was provided by T.G. and M.L.F. The manuscript was drafted by H.M. and R.A.W. with input from all coauthors.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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