

Aberrant DNA methylation of hypothalamic angiotensin receptor in prenatal programmed hypertension

Fumiko Kawakami-Mori,^{1,2} Mitsuhiro Nishimoto,¹ Latapati Rehemani,³ Wakako Kawarazaki,¹ Nobuhiro Ayuzawa,¹ Kohei Ueda,¹ Daigoro Hirohama,¹ Daisuke Kohno,⁴ Shigeoyoshi Oba,¹ Tatsuo Shimosawa,³ Takeshi Marumo,¹ and Toshiro Fujita¹

¹Division of Clinical Epigenetics, Research Center for Advanced Science and Technology (RCAST), The University of Tokyo, Tokyo, Japan. ²Division of Endocrinology, Mitsui Memorial Hospital, Tokyo, Japan. ³Department of Clinical Laboratory, International University of Health and Welfare, School of Medicine, Narita Hospital IUHW, Tokyo, Japan. ⁴Advanced Scientific Research Leaders Development Unit, Gunma University, Gunma, Japan.

Maternal malnutrition, which causes prenatal exposure to excessive glucocorticoid, induces adverse metabolic programming, leading to hypertension in offspring. In offspring of pregnant rats receiving a low-protein diet or dexamethasone, a synthetic glucocorticoid, mRNA expression of angiotensin receptor type 1a (*Agtr1a*) in the paraventricular nucleus (PVN) of the hypothalamus was upregulated, concurrent with reduced expression of DNA methyltransferase 3a (*Dnmt3a*), reduced binding of DNMT3a to the *Agtr1a* gene, and DNA demethylation. Salt loading increased BP in both types of offspring, suggesting that elevated hypothalamic *Agtr1a* expression is epigenetically modulated by excessive glucocorticoid and leads to adult-onset salt-sensitive hypertension. Consistent with this, dexamethasone treatment of PVN cells upregulated *Agtr1a*, while downregulating *Dnmt3a*, and decreased DNMT3a binding and DNA demethylation at the *Agtr1a* locus. In addition, *Dnmt3a* knockdown upregulated *Agtr1a* independently of dexamethasone. Hypothalamic neuron-specific *Dnmt3a*-deficient mice exhibited upregulation of *Agtr1a* in the PVN and salt-induced BP elevation without dexamethasone treatment. By contrast, dexamethasone-treated *Agtr1a*-deficient mice failed to show salt-induced BP elevation, despite reduced expression of *Dnmt3a*. Thus, epigenetic modulation of hypothalamic angiotensin signaling contributes to salt-sensitive hypertension induced by prenatal glucocorticoid excess in offspring of mothers that are malnourished during pregnancy.

Introduction

The idea that cardiac and metabolic disease can have intrauterine origins, referred to as developmental origins of health and disease (DOHaD), was initially based on early epidemiological studies of prenatal nutrition and late-onset coronary heart disease published by Barker and colleagues in the late 1980s (1). Nowadays, late childbearing, stress, and a low-calorie diet during pregnancy are known to be high risk factors for utero-placental insufficiency (2), which is accompanied by impaired expression of 11- β -dehydrogenase type 2 (11 β -HSD2), the physiological fetoplacental barrier to maternal glucocorticoid, in the placenta (3). Exposure to excessive glucocorticoid during pregnancy reduces birth weight of offspring, leading to permanent hypertension, hyperglycemia, and hypothalamic-pituitary-adrenal axis aberrant activity in adult life. Once developed, prenatally programmed hypertension seems to persist indefinitely and to get progressively worse with age (4). Notably, the young offspring of dams either fed a low-protein diet (LP) or treated with dexamethasone, a synthetic glucocorticoid, during pregnancy do not yet show a significant increase in BP, but — when placed on a high-salt (HS) diet — they exhibit a rise in BP (i.e., salt-sensitive hypertension; refs. 5, 6). Although the precise mechanism linking neonatal glucocorticoid excess with adult-onset salt-sensitive hypertension is still unknown, there is a plausible hypothesis that the renin-angiotensin system (RAS) in the CNS plays an important role in the development of salt-sensitive hypertension (7–10) and prenatal programmed hypertension (11, 12) through increased sympathetic nerve activity. Supporting the hypothesis, malnutrition or glucocorticoid exposure induces high BP and a concomitant increase in the response of

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sympathetic activity to physical stress (13), and renal denervation may inhibit prenatal programming of hypertension (14). Moreover, either systemic or icv administration of RAS inhibitors reduces BP in protein-restricted rat offspring (12, 15) through the inhibition of sympathetic overactivity. Consistently, the direct injection of antisense oligodeoxynucleotides targeting angiotensin receptor type 1a (*Agtr1a*, encoding AT1a) into the paraventricular nucleus (PVN) of the hypothalamus prevents increased renin-angiotensin expression in a transgenic animal model, the mRen-2 rat, from developing salt-sensitive hypertension (16), and optogenetic stimulation of PVN *Agtr1a* neurons induces BP elevation (17), implying an important role of hypothalamic *Agtr1a* in the control of sympathetic nerve activity and BP (18, 19). Interestingly, the expression of AT1a in the regions of the brain involved in cardiovascular regulation is upregulated not only in dexamethasone-treated rats (20), but also in their offspring exposed to maternal protein restriction (15), suggesting that the increased expression of *Agtr1a* induced by neonatal exposure to excessive glucocorticoids continues into adulthood.

Prenatal exposure to excessive glucocorticoids or stress might induce adverse metabolic programming and lead to hypertension in the offspring. The mechanisms through which prenatal insults or overexposure to glucocorticoids cause permanent hypertension and sympathetic overactivity is currently unknown. However, there has been increasing interest in the role played by epigenetic dynamics — notably, changes in DNA methylation patterns and histone modifications. Epigenetic markers, which regulate chromatin structure and DNA accessibility, are attractive candidates because they can permanently modify gene expression without altering DNA sequence, and once set, they are persistent and irreversible (21). A recent study showed changes in the DNA methylation of hypothalamic obesity-related genes in offspring exposed to a low-fat parental diet (22). Among DNA methyltransferases (DNMTs), including DNMT1, DNMT3a, and DNMT3b, DNMT3a expression is specifically downregulated in the PVN of high-fat-fed mice, and PVN-specific deletion of *Dnmt3a* induces hyperphagia, glucose intolerance, and obesity (23). Given the increased neuronal response to Ang II in the hypothalamus of high-fat-fed mice and the induction of hyperphagia in mice with a PVN-specific deletion of *Agtr1a*, as well as a further increase in their adiposity and body mass when fed a high-fat diet and a significant reduction in their BP (18), the elevated Ang II responsiveness that accompanies obesity could serve as a negative feedback signal for the activation of neurons to alleviate weight gain, with the increased sympathetic activity, in turn, resulting in an increase in BP. Taken together, these findings led us to investigate the possible link between DNMT3a activity and *Agtr1a* mRNA expression in the control of BP, as well as in energy homeostasis. In this study, we tested the hypothesis that prenatal exposure to increased glucocorticoid levels induced by a LP or synthetic glucocorticoid administration might modulate DNA methylation by DNMT3a and that the subsequent permanent changes in the expression of specific genes, key among which is *Agtr1a*, would lead to adverse central nervous programming of angiotensin signaling and postnatal salt-sensitive hypertension.

Results

Salt-sensitive hypertension in offspring of pregnant mothers exposed to a LP and dexamethasone treatment. A LP during pregnancy in rats (Supplemental Figure 1; supplemental material available online with this article; <https://doi.org/10.1172/JCIInsight95625DS1>) was accompanied by reduced placental expression of *Hsd11b2* mRNA, encoding 11 β -HSD2, a key component of the physiological fetoplacental barrier to maternal glucocorticoid (Figure 1A). Body weight (BW) at week 1 was lower in the LP-treated offspring than in those born to mothers fed a normal-protein diet (NP); at week 12, however, BW was higher in LP-treated offspring (Figure 1B), with no increase in mRNA expression of leptin in adipose tissue at week 1 or serum leptin concentration at week 12 (Supplemental Figure 2).

Reduced 11 β -HSD2 activity in the placenta of LP-treated pregnant rodents results in excessive exposure of the fetus to corticosterone (3, 4). Accordingly, we administered 200 μ g/kg/day dexamethasone (20) to pregnant rats in the third trimester (F16–21) (Supplemental Figure 1). Consistent with the changes in BW of LP-treated offspring, dexamethasone-treated offspring had lower BW at 1 week and higher BW at 12 weeks than control offspring (Figure 1C).

We next investigated whether either a LP or dexamethasone treatment during the prenatal period can induce hypertension during adult life. At 12 weeks, systolic BP did not differ between LP-treated offspring and NP-treated controls. When we administered a HS diet for 7 days, BP (as measured by telemetry) increased significantly in LP-treated offspring, as previously reported (5, 6) — but not in NP-treated offspring (Figure 1D). At the end of salt loading, mean BP was significantly higher in LP-treated offspring

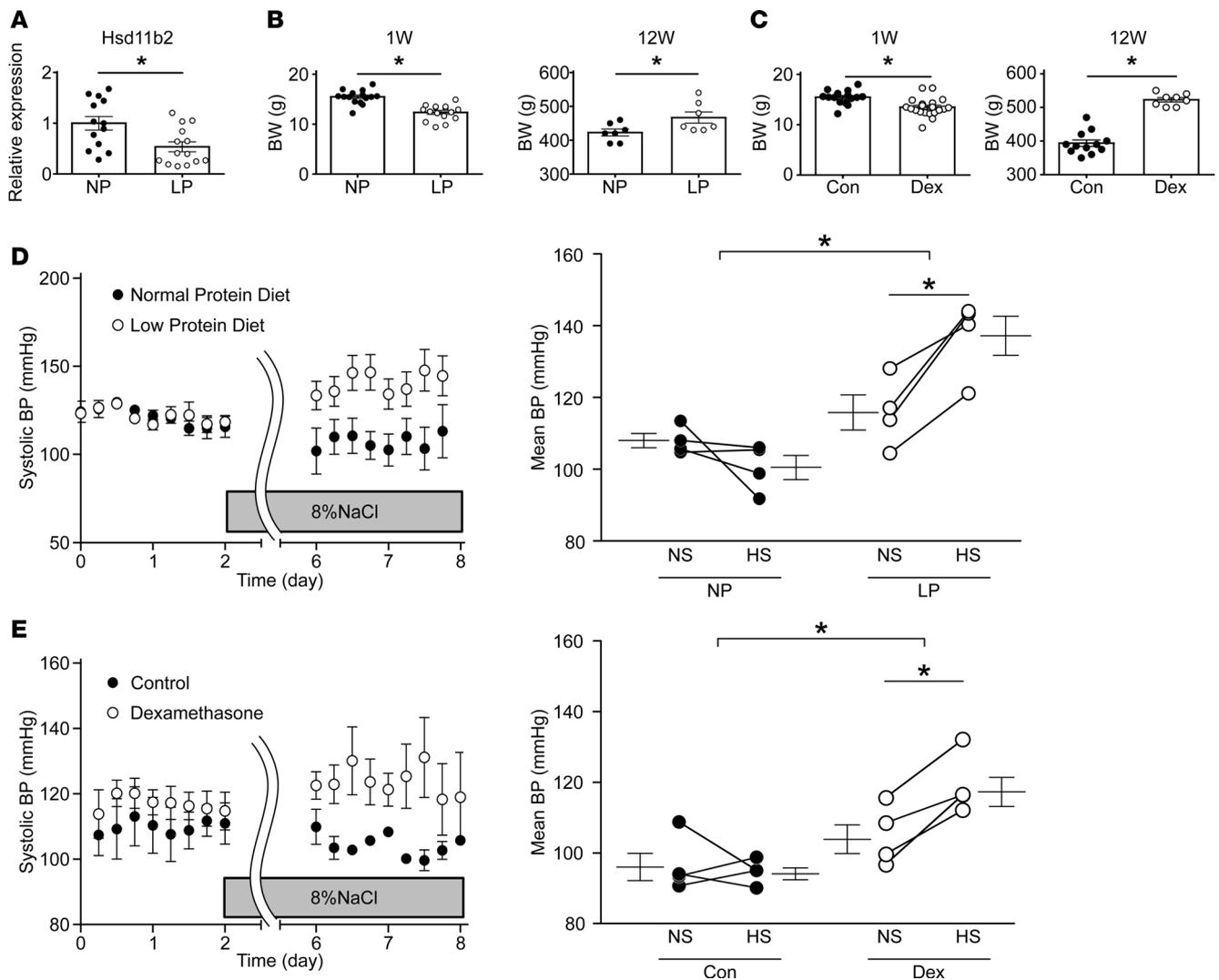


Figure 1. Comparison between offspring treated with a low-protein diet (LP) and those treated with dexamethasone (Dex). (A) Real-time PCR of *Hsd11b2* mRNA expression (normalized against *Actb*) in placenta at the end of the third trimester (F19–20) of pregnant rats receiving a normal-protein diet (NP) ($n = 13$) or a low-protein diet (LP) ($n = 14$). Filled circles, NP-treated rats; open circles, LP-treated rats. (B) Body weight (BW) at weeks 1 ($n = 16$) and 12 ($n = 7$) in male NP- and LP-treated offspring. (C) BW at weeks 1 and 12 in Dex-untreated (control) ($n = 16$ and 12) and Dex-treated ($n = 20$ and 8, respectively) offspring. Filled circles, control offspring; open circles, Dex-treated offspring. (D) Systolic BP measured by radiotelemetry in NP-treated and LP-treated offspring that received a 0.5% salt (NS) or 8% salt (HS) diet for 1 week ($n = 4$) (left panel). Mean arterial pressure by radiotelemetry before and after 1 week of HS in NP-treated and LP-treated offspring (right panel). Filled circles, NP group; open circles, LP group. (E) Systolic BP, measured by radiotelemetry, in control and Dex-treated offspring that received 0.5% salt (NS) or 8% salt (HS) diet for 1 week ($n = 4$) (left panel). Mean arterial pressure, measured by radiotelemetry, before and after 1 week of HS in control and Dex-treated offspring (right panel). Filled circles, control offspring; open circles, Dex-treated offspring. Throughout, data represent means \pm SEM. In A, B, and C, $*P < 0.05$ versus NP offspring or control offspring (t test). In D, $*P < 0.05$ in NP-treated group versus LP-treated group and in NS versus HS within each group. In E, $*P < 0.05$ in control versus Dex-treated offspring and in NS versus HS within each group (2-way repeated ANOVA, Bonferroni post hoc test).

than in NP-treated offspring (Figure 1D). Although BP was comparable between dexamethasone-treated and control rats at week 12, HS increased BP in the dexamethasone-treated offspring but not in the controls, similar to the pattern observed in LP-treated offspring (Figure 1E). In addition, mean BP at the end of salt loading was also higher in dexamethasone-treated rats than in control rats (Figure 1E).

These results led us to hypothesize that exposure to excess glucocorticoid induces prenatal programmed hypertension. To test this hypothesis, we first sought to determine which genes contribute to the hypertension phenotype. Using microarrays, we compared gene expression in the PVN of the hypothalamus between dexamethasone-treated and control groups at week 12. This approach identified 912 genes that were upregulated more than 2-fold in the dexamethasone-treated group relative to the controls; these genes were subjected to pathway analysis (Supplemental Table 1). *Agtr1a* was included

in several of the top ranked pathways, including calcium signaling, neuroactive ligand–receptor interaction, and aldosterone synthesis and interaction. Accordingly, we focused on AT1a as a potentially novel regulator of BP in the hypothalamus.

Epigenomic analysis of salt-sensitive hypertension in offspring of pregnant mothers exposed to a LP. Consistent with the overactivation of central RAS in prenatal programmed hypertension (12, 15, 24) and the results of the microarray analysis, expression of *Agtr1a* mRNA in the PVN of the hypothalamus was higher in 12-week-old LP offspring than in NP-treated controls. In addition, the LP-treated pups exhibited reduced expression of *Dnmt3a*, but no changes in *Agtr1b*, *Dnmt1*, *Dnmt3b*, Methyl-CpG binding protein 2 (*MeCP2*), or Ten-eleven translocation methylcytosine dioxygenase (*Tet*) 1, 2, 3 (Figure 2A; Supplemental Figure 3). DNA methylation of *Agtr1a* gene in the PVN, measured by methylated DNA immunoprecipitation (MeDIP) analysis (25), was significantly lower in LP-treated offspring than in NP controls (Figure 2B).

To more precisely assess DNA methylation status, we performed bisulfite sequence analysis of the DNA region from +114 to +214 bp of the *Agtr1a* transcription start site (TSS). Demethylation of DNA from –1,000 to +500 bp of a TSS is correlated with active gene expression (26). Figure 2C shows that CpG sites were demethylated to a greater extent in LP-treated offspring than in NP-treated offspring.

To determine which DNMTs were responsible for *Agtr1a* demethylation, we performed a ChIP assay. The results revealed that binding of DNMT3a, but not DNMT1, to the *Agtr1a* locus in the regions –97/+107 bp and +174/+366 bp relative to the TSS (Supplemental Figure 4A and Figure 2D) was reduced in the PVN of LP-treated offspring, suggesting that DNMT3a plays a key role in DNA demethylation and *Agtr1a* transcription. We also found that DNMT3a activity was reduced in the PVN of LP-treated offspring (Supplemental Figure 5). This result implies that expression of *Dnmt3a* would change methyltransferase activity, proving the role of DNMT3a-altering methylation on *Agtr1a*. Based upon the fact that salt-sensitive hypertension is induced by RAS overactivity (16) and salt-dependent BP elevation in prenatal programmed hypertension can be reversed by the RAS inhibitor (5), we suggest that persistent upregulation of hypothalamic *Agtr1a* mRNA expression, resulting from the DNA demethylation induced by excessive glucocorticoid, causes salt-sensitive hypertension, possibly via sympathetic overactivity (11, 27).

Epigenomic analysis of salt-sensitive hypertension in offspring of pregnant mothers treated with dexamethasone. Quantitative PCR (qPCR) analysis revealed that mRNA expression of *Agtr1a*, but not *Agtr1b*, in the PVN was significantly elevated in the dexamethasone-treated offspring, whereas expression of *Dnmt1* and *Dnmt3a* mRNA was reduced (Figure 3A).

Bisulfite sequencing analysis revealed demethylation of the CpG sites in the region +114/+214 bp relative to the TSS of *Agtr1a* in the PVN of dexamethasone-treated offspring relative to controls, similar to the results obtained in LP-treated offspring (Figure 3B). Moreover, a ChIP assay revealed that binding of DNMT3a, to the *Agtr1a* gene in regions –97/+107 bp and +174/+366 bp was weaker in the dexamethasone group than in the control group (Figure 3C). However, notwithstanding the demethylation of *Agtr1a*, binding of DNMT1 was elevated rather than reduced. Because the pattern of DNMT3a binding to the *Agtr1a* locus was shared between LP-treated and dexamethasone-treated offspring, we concluded that DNMT3a, rather than DNMT1, plays a key role in dexamethasone-induced DNA demethylation of *Agtr1a* during pregnancy.

Changes in DNA methylation with dexamethasone treatment in the PVN cell line. To evaluate the direct effect of dexamethasone on *Agtr1a* DNA methylation in PVN cells, we applied 10 and 100 nM dexamethasone to the mouse fetal PVN neuronal cell line, N39. Twenty-four-hour treatment with dexamethasone decreased expression of *Dnmt1* and *Dnmt3a* mRNA in a dose-dependent manner (Supplemental Figure 6) and 6-day treatment with 100 nM dexamethasone decreased expression of *Dnmt3a*, but not *Dnmt1*, concomitant with upregulation of *Agtr1a* (Figure 4A). Upon demethylation of DNA, presumably, *Agtr1a* mRNA did not change at the early phase but increased at the late phase (Supplemental Figure 6), as shown in the previous study indicating that the starting time of the increase in mRNA lag a few days behind the start of DNA demethylation (28). Accordingly, DNA methylation in *Agtr1a* decreased significantly in cells subjected to 6-day dexamethasone treatment (Figure 4B), as did binding of DNMT3a, but not DNMT1, to the *Agtr1a* locus (Figure 4C and Supplemental Figure 4B). To further evaluate the involvement of DNMT3a in dexamethasone-induced upregulation of *Agtr1a*, we transfected small interfering RNA against *Dnmt3a* (siDnmt3a) into a mouse hypothalamic cell line (Supplemental Figure 7) and observed a significant decrease in DNA methylation of *Agtr1a* (Figure 4D). Notably, silencing of the *Dnmt3a* gene increased *Agtr1a* mRNA expression to levels similar to those observed after dexamethasone

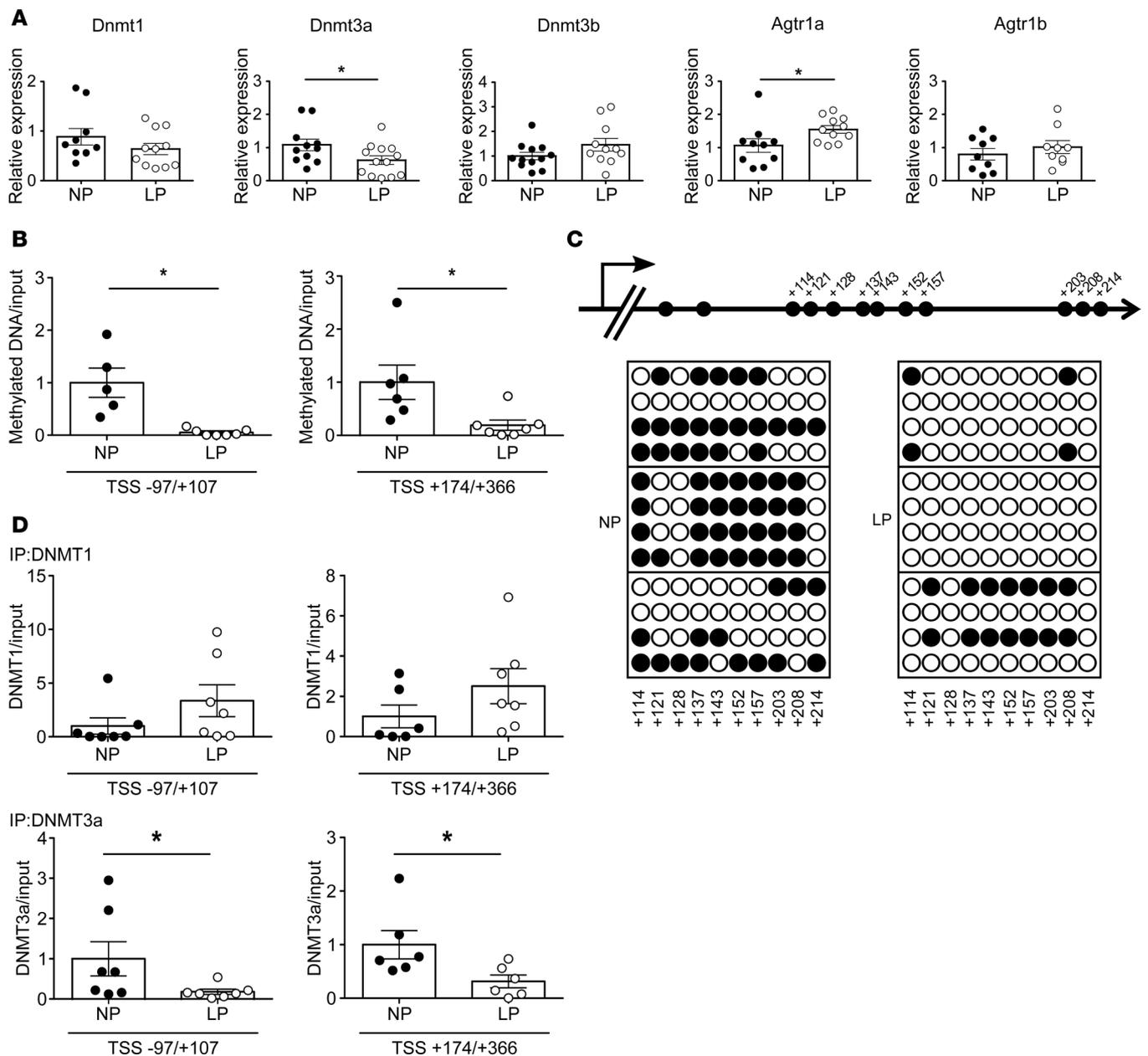


Figure 2. Offspring of pregnant rats treated with a low-protein diet (LP). (A) mRNA levels of *Dnmt1* ($n = 8-11$), *Dnmt3a* ($n = 11-13$), *Dnmt3b* ($n = 12$), *Agtr1a* ($n = 9$), and *Agtr1b* ($n = 10$) in NP-treated or LP-treated offspring. Filled circles, NP group; open circles, LP group. (B) Quantitation of DNA methylation (by MeDIP) at the *Agtr1a* locus in regions $-97/+107$ and $+174/+366$ relative to the transcription start site (TSS) in NP-treated ($n = 5$ for $-97/+107$; $n = 6$ for $+174/+366$) and LP-treated ($n = 7$) offspring. (C) Bisulfite sequence analysis of the *Agtr1a* locus in the PVN from 12-week-old NP-treated or LP-treated offspring. Top, schematic diagram of the *Agtr1a* locus. Dashes and numbers indicate the positions of the cytosine residues of CpG dinucleotides relative to the TSS (+1). Bottom, DNA methylation status of the CpG sites between +114 and +214 bp relative to the TSS. Filled circles, methylated CpG sites; open circles, demethylated CpG sites. (D) ChIP assays showing DNMT1 (top) and DNMT3a (bottom) binding to the regions $-97/+107$ ($n = 7$ and 7 for DNMT1; $n = 9$ and 11 for DNMT3a) and $+174/+366$ relative to the TSS ($n = 6$ and 7 for DNMT1; $n = 10$ and 8 for DNMT3a, NP and LP, respectively), in the PVN of NP-treated and LP-treated offspring. Filled circles, NP-treated offspring; open circles, LP-treated offspring. Throughout, data represent means \pm SEM. * $P < 0.05$ versus control offspring (t test).

treatment (Figure 4E). Moreover, the observation that upregulation of *Agtr1a* induced by *Dnmt3a* silencing was not further increased by additional treatment with dexamethasone (Figure 4E) implies that DNMT3a is involved in dexamethasone-induced upregulation of *Agtr1a* expression. Thus, dexamethasone decreases DNA methylation by releasing DNMT3a from the gene, and in turn, DNA demethylation promotes transcription of *Agtr1a*, suggesting the importance of DNMT3a in dexamethasone-induced DNA demethylation and the subsequent upregulation of *Agtr1a* mRNA in the hypothalamic cells.

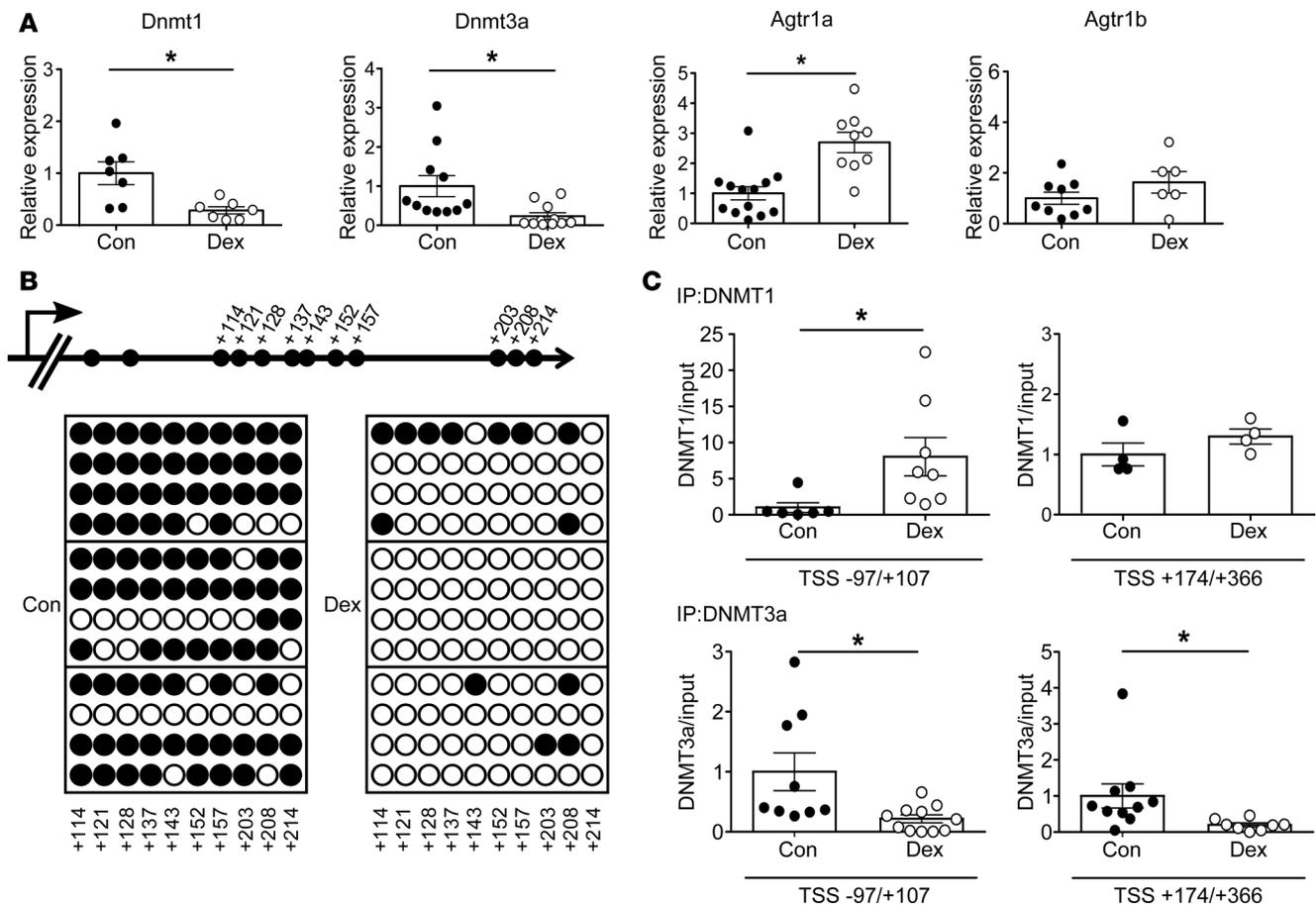


Figure 3. Offspring of pregnant rats treated with dexamethasone (Dex). (A) Levels of *Dnmt1* ($n = 7$) and *Dnmt3a* ($n = 11$) mRNA in control and Dex-treated offspring, and levels of *Agtr1a* and *Agtr1b* mRNA in control ($n = 13$ and 9 , respectively) and Dex-treated ($n = 9$ and 6 , respectively) offspring. (B) Bisulfite sequence analysis of the *Agtr1a* locus in the PVN from 12-week-old control and Dex-treated offspring. Top, schematic diagram of the *Agtr1a* locus. Dashes and numbers indicate the positions of the cytosine residues of CpG dinucleotides relative to the TSS (+1). Bottom, DNA methylation status of the CpG sites between +114 and +214 bp relative to the TSS. Filled circles, methylated CpG sites; open circles, demethylated CpG sites. (C) ChIP assays showing DNMT1 (top) and DNMT3a (bottom) binding to the sites -97 to +107 ($n = 6$ and 8 for DNMT1, and $n = 9$ and 11 for DNMT3a, respectively) and +174 to +366 relative to the TSS ($n = 4$ and 4 in DNMT1, and $n = 10$ and 8 in DNMT3a, respectively), in the PVN of control and Dex-treated offspring. Filled circles, control; open circles, Dex-treated offspring. Throughout, data represent the means \pm SEM. * $P < 0.05$ versus control offspring (t test).

Salt-sensitive hypertension in offspring of pregnant hypothalamus-specific Dnmt3a-deficient mice. To confirm the involvement of DNMT3a in aberrant DNA methylation of *Agtr1a* and the resultant salt-sensitive hypertension, we administered the HS diet to Sim1-Cre *Dnmt3a*-KO mice. As previously reported (23), BW at week 12 was higher in the KO mice than in control flox mice (Figure 5A). *Dnmt3a*-deficient mice showed increased expression of *Agtr1a* mRNA in the PVN (Figure 5B), in the absence of dexamethasone treatment during pregnancy (Supplemental Figure 1), together with an increase in BP (Figure 5C). HS diet further increased BP in *Dnmt3a*-deficient mice but not in the flox mice (Figure 5C). Thus, chronic deletion of *Dnmt3a* from the hypothalamic neuron develops salt-sensitive hypertension by upregulation of *Agtr1a* in the PVN, even without prenatal exposure to excess glucocorticoid.

The PVN is composed of neurons, glia, and astrocytes. To determine where *Agtr1a* is expressed, we performed in situ hybridization of the PVN in *Dnmt3a*-deficient mice (Figure 5D) and dexamethasone-treated offspring (Supplemental Figure 8). We observed no staining of *Agtr1a* mRNA in cells positive for markers of glia and astrocytes (Figure 5E), confirming that *Agtr1a* is expressed mainly in neurons, as previously reported (29).

The absence of salt-sensitive hypertension in offspring of pregnant Agtr1a-deficient mice treated with dexamethasone. Expression of *Agtr1a* mRNA in the PVN was significantly higher in offspring of C57BL6/J WT mice treated with dexamethasone during pregnancy (Figure 6A), and this increase was accompanied by salt-induced BP elevation and reduced expression of *Dnmt3a* mRNA in the PVN (Figure 6B), consistent

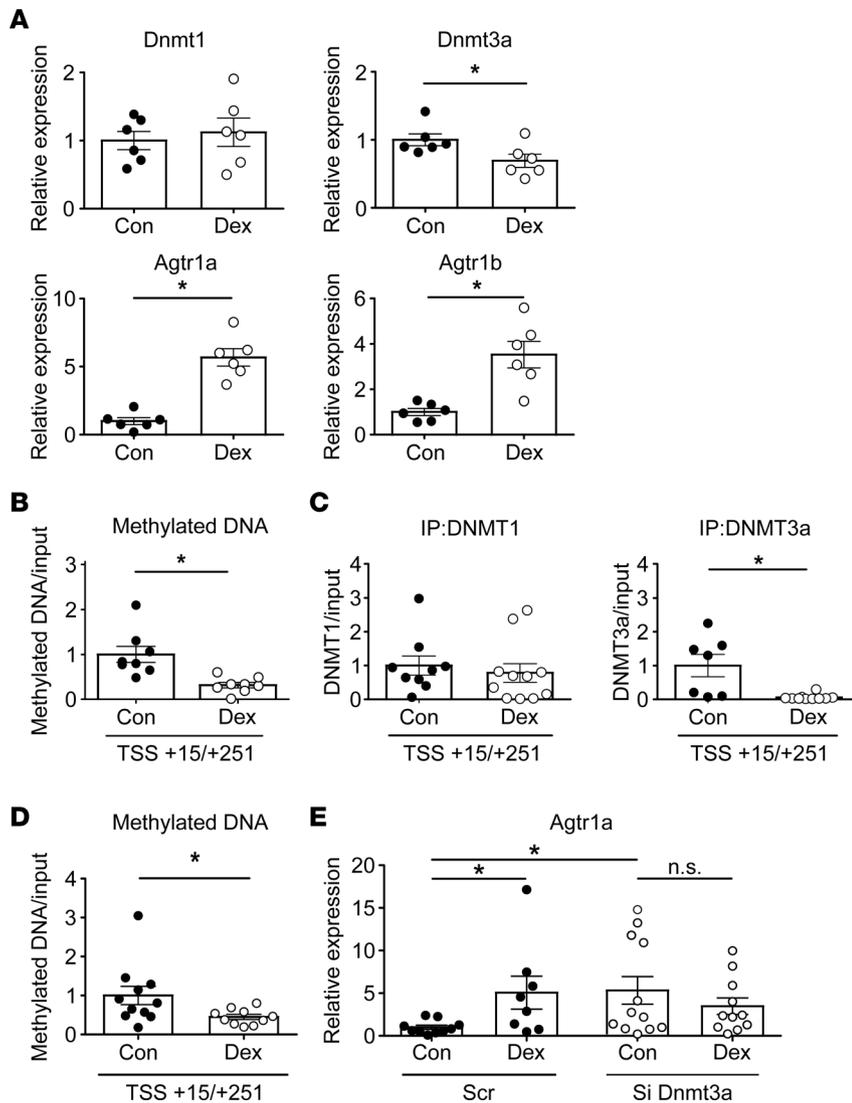


Figure 4. Effect of dexamethasone (Dex) on *Agtr1a* mRNA and DNA methylation of the *Agtr1a* promoter in the immortal mouse fetal PVN neuronal cell line, N39. (A) Real-time PCR of *Dnmt1*, *Dnmt3a*, *Agtr1a*, and *Agtr1b* mRNA following treatment for 6 days with vehicle ($n = 6$) or Dex ($n = 6$). **(B)** Quantitation of DNA methylation (by MeDIP) at the *Agtr1a* gene +15/+251 from the transcription start site in vehicle-treated or Dex-treated cells (each $n = 8$). **(C)** ChIP assays of DNMT1 (left) and DNMT3a (right) binding to the site +15/+251 relative to the TSS in vehicle-treated or Dex-treated cells ($n = 9$ and 10 in DNMT1, and $n = 7$ and 10 in DNMT3a, respectively). **(D)** siRNA against DNMT3a ($n = 10$) significantly decreased DNA methylation of *Agtr1a* relative to scrambled siRNA ($n = 11$). **(E)** Effect of Dex treatment on *Agtr1a* mRNA expression in cells transfected with siDnmt3a (control and Dex, $n = 12$ and 11, respectively) or scrambled RNA (control and Dex, $n = 10$ and 8, respectively). **(A–C)** Filled circles, vehicle-treated cells; open circles, Dex-treated cells. **(D–E)** Filled circle, cells treated with scrambled RNA; open circles, cells treated with siRNA against *Dnmt3a*. Data represent means \pm SEM. * $P < 0.05$ (A–D) *t* test; (E) Kruskal-Wallis test with post hoc Dunn's multiple comparison.

with the results obtained in dexamethasone-treated rat offspring (Figure 3A and Figure 1E, respectively). By contrast, *Agtr1a* deficiency abolished salt-induced elevation of BP in the dexamethasone-exposed offspring (Figure 6B), concomitant with reduced expression of *Dnmt3a* and *Dnmt1* in the PVN (Figure 6C). The sustained downregulation of *Dnmt3a* mRNA without salt-induced elevation of BP implies that aberrant DNA methylation is persistent, rather than secondary to the BP rise. Given that BP elevation can be induced by optogenetic stimulation of PVN AT1a neurons (17), these findings indicate that epigenetic modulation of *Agtr1a* plays a critical role in salt-sensitive hypertension in offspring exposed prenatally to excessive glucocorticoid (Figure 6D).

Discussion

We present evidence demonstrating that an epigenetic mechanism underlies hypothalamic angiotensin signaling and leads to the development of salt-sensitive hypertension induced by the prenatal programming resulting from parental exposure to excess glucocorticoid. Specifically, we show that DNA demethylation caused by decreased binding of DNMT3a to the *Agtr1a* gene induces upregulation of *Agtr1a* mRNA in the PVN and that the increased angiotensin signaling in the hypothalamus, in turn, leads to salt-induced BP elevation in offspring of pregnant mothers receiving a LP or treated with dexamethasone. Given the importance of central angiotensin signaling in prenatal programmed hypertension (13, 15), *Agtr1a* deficiency attenuated salt-induced BP elevation induced by prenatal dexamethasone treatment, and dexamethasone treatment — in vitro — increased expression of *Agtr1a* mRNA in cultured PVN cells. Chronic deletion of *Dnmt3a* from the hypothalamus induces upregulation of *Agtr1a* mRNA in the PVN and subsequently results

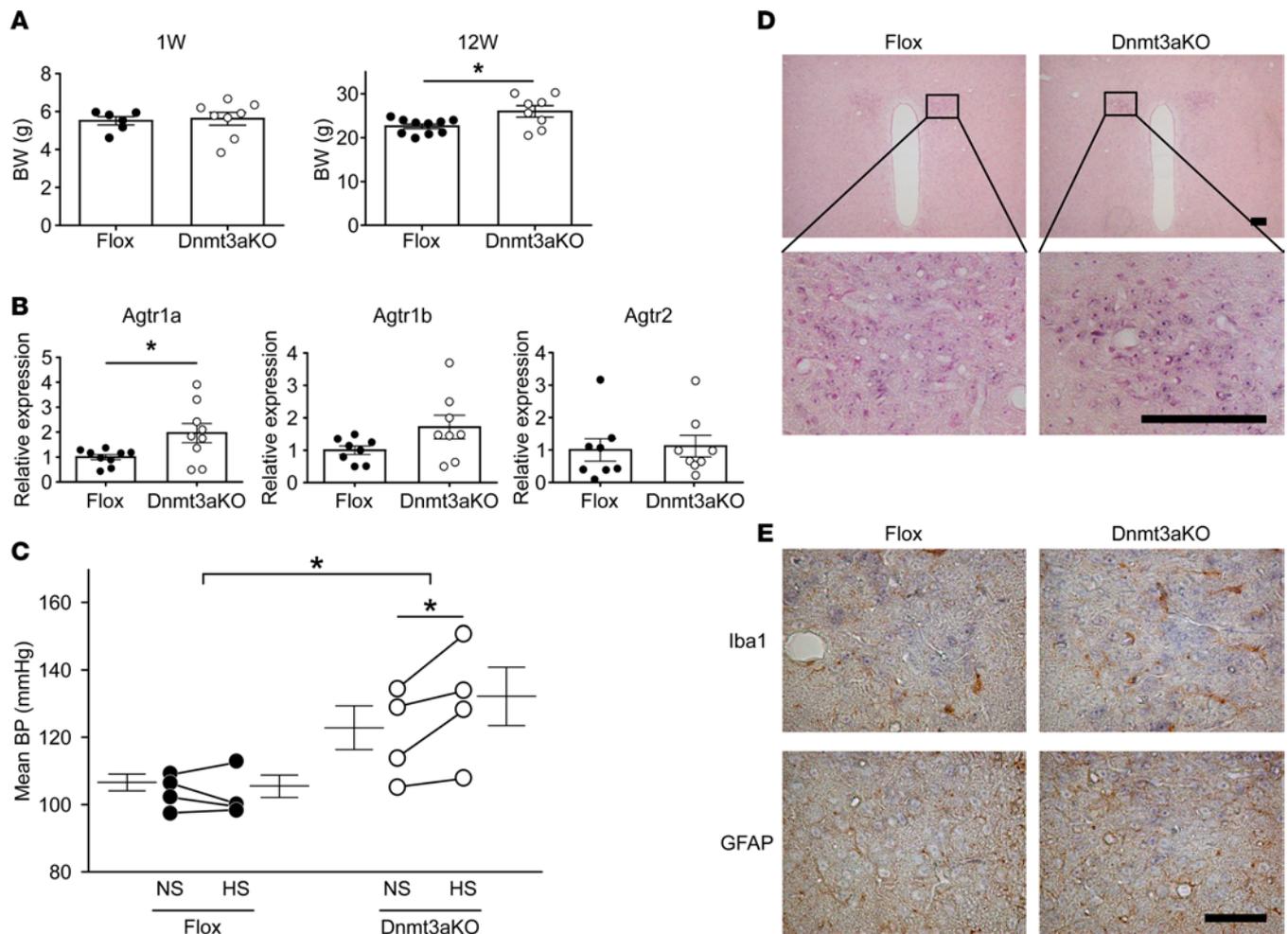


Figure 5. Salt-sensitive hypertension in offspring of hypothalamic neuron-specific Dnmt3a-KO mice without dexamethasone (Dex) treatment during pregnancy. (A) BW at weeks 1 and 12 in the flox mice ($n = 6$ and 10 , respectively) and hypothalamic neuron-specific Dnmt3a-KO mice ($n = 8$ and 8 , respectively). Filled circle, Dex-untreated flox mice; open circle, Dex-untreated Dnmt3a-KO mice. (B) Real-time PCR of *Agtr1a* ($n = 9$), *Agtr1b* ($n = 8$), and *Agtr2* ($n = 8$) mRNA in PVN of flox and Dnmt3a-KO mice. Filled circle, Dex-untreated flox mice; open circle, Dex-untreated Dnmt3a-KO mice. (C) Mean BP by radiotelemetry before and after 1 week of HS in Dex-untreated flox mice ($n = 4$, left) and Dex-untreated Dnmt3a-KO mice ($n = 4$, right). Filled circle, Dex-untreated flox mice; open circles, Dex-untreated Dnmt3a-KO mice. In A and B, $*P < 0.05$ versus Dex-untreated flox mice (t test); in C, $*P < 0.05$ versus HS-treated flox mice or NS-treated Dnmt3a-KO mice (2-way repeated ANOVA, Bonferroni post hoc test). (D) In situ hybridization of *Agtr1a* mRNA in the PVN of Sim1-Cre Dnmt3a-KO (right) and flox mice (left) (upper panels, low power; lower panels, high power). Hybridization using an antisense probe indicates expression of *Agtr1a* mRNA (purple, lower panel). Hybridization using the sense probe yielded no detectable signals in the PVN (data not shown). Scale bar: $100 \mu\text{m}$. (E) Upper panels show staining for Iba1, a marker of glia cells (brown), and lower panels show GFAP, a marker of astrocytes (brown). Agtr1a (blue) colocalized with neither Iba1 nor GFAP, suggesting that it is expressed mainly in neuronal cells. The expression pattern was not affected by Sim1-Cre Dnmt3a-KO. Scale bars: $50 \mu\text{m}$.

in the development of salt-sensitive hypertension in offspring, even without dexamethasone treatment or a LP. Taken together, our results suggest that aberrant DNA methylation by a glucocorticoid-induced decrease in DNMT3a expression and activity, and concurrent upregulation of *Agtr1a* mRNA in the hypothalamus, plays a critical role in the development of prenatal programmed salt-sensitive hypertension.

In agreement with the proposed link between obesity and hypertension, offspring of pregnant mothers receiving a LP or dexamethasone treatment exhibited a low weight at birth but gradually gained weight after birth and became obese at week 12, the time at which they acquired salt-sensitive hypertension. Obesity is associated with salt-sensitive hypertension (30–32). Specifically, high-fat diet-induced upregulation of *Agtr1a* expression in the hypothalamus is mediated by hyperleptinemia (33), and the elevated sensitization of central Ang II results, in turn, in sympathetic overactivity (12, 13, 16, 33–35). However, neither expression of *Lep* mRNA in adipose tissue at week 1 nor serum leptin concentration at week 12 was elevated in LP-treated offspring, implying that leptin makes little if any contribution to prenatal programmed hypertension. Injection of *Agtr1a* antisense into the PVN decreases salt-sensitive hypertension in transgenic rats overexpressing

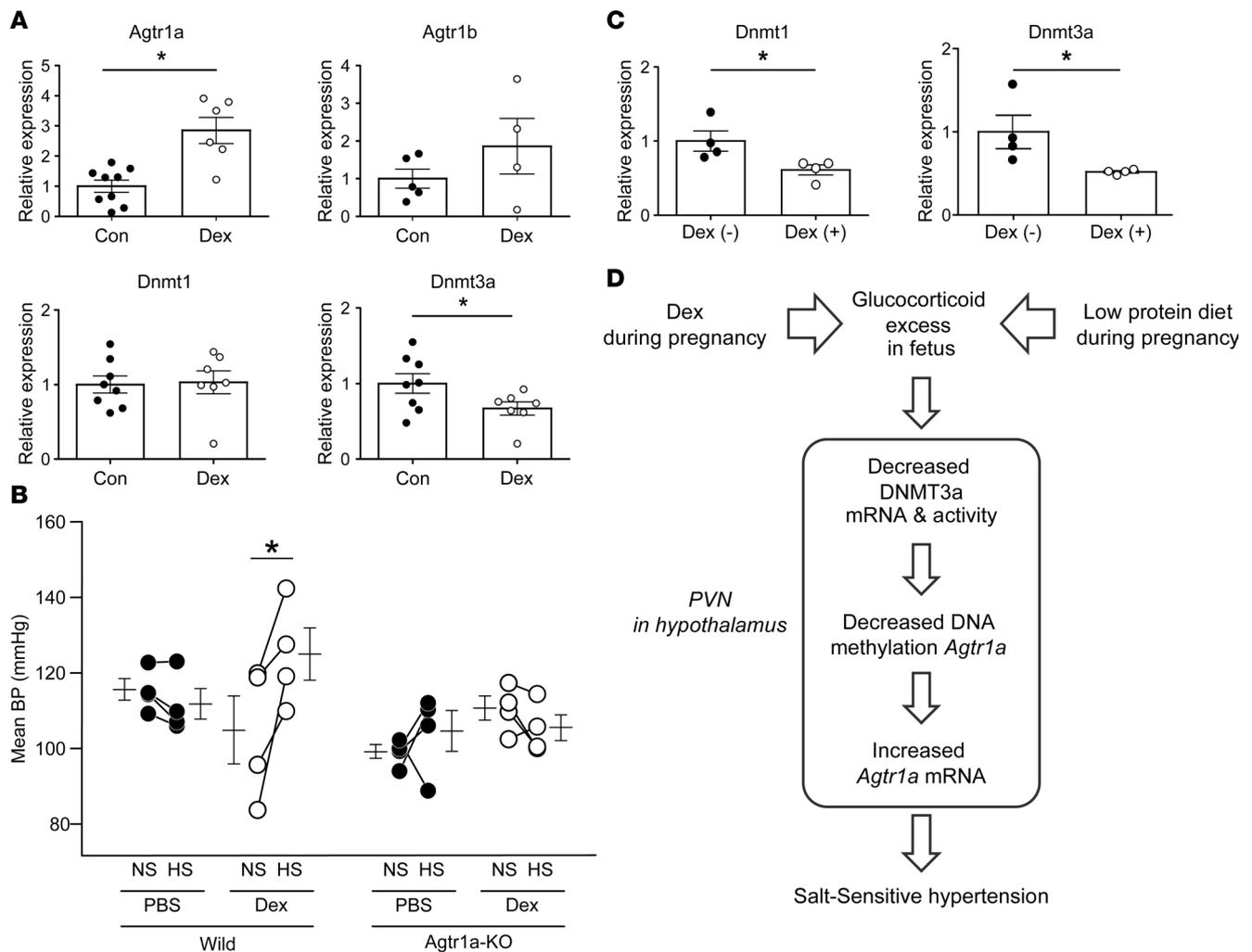


Figure 6. Offspring of pregnant C57BL6/J WT mice and pregnant Agtr1a-KO mice treated with dexamethasone (Dex). (A) Real-time PCR of *Agtr1a*, *Agtr1b*, *Dnmt1*, and *Dnmt3a* mRNA in the paraventricular nucleus (PVN) of offspring of Dex-untreated ($n = 6-9$) and Dex-treated pregnant C57BL6/J mice ($n = 4-7$). Filled circles, Dex-untreated WT mice (Con); open circles, Dex-treated WT mice (Dex). (B) Mean BP by radiotelemetry before and after 1 week of HS treatment in offspring of pregnant WT mice (left) and pregnant Agtr1a-KO mice (right) treated with or without Dex. Filled circles, Dex-untreated WT mice and Agtr1a-KO mice; open circles, Dex-treated WT mice and Agtr1a-KO mice (WT, $n = 4$; Agtr1a-KO, $n = 4$). (C) Real-time PCR of *Dnmt1* and *Dnmt3a* mRNA in Dex-untreated Agtr1a-KO mice ($n = 4$) and Dex-treated Agtr1a-KO mice ($n = 4$). Filled circles, Dex-untreated Agtr1a-KO mice; open circles, Dex-treated Agtr1a-KO mice. Throughout, data represent means \pm SEM. In A and C, * $P < 0.05$ versus Dex-untreated WT mice or Dex-untreated Agtr1a-KO mice (t test); in B, * $P < 0.05$ versus NS-treated offspring of pregnant WT mice treated with Dex (2-way repeated ANOVA, Bonferroni post hoc text). (D) Model of prenatal programmed hypertension. A low-protein diet, as well as treatment of Dex, during pregnancy induces exposure to excessive glucocorticoid in the fetus, through the decreased 11 β -HSD2 activity. Excessive glucocorticoid decreases *Dnmt3a* mRNA and activity, concomitant with DNA demethylation and *Agtr1a* upregulation in the PVN of hypothalamus of the offspring; the increased angiotensin signaling in the hypothalamus, in turn, develops salt-sensitive hypertension.

RAS components in brain tissue (16), and PVN-specific deletion of *Agtr1a* inhibits obesity-induced hypertension through inhibition of sympathetic overactivity, concomitant with augmentation of diet-induced BW gain (36). Together with these previous findings, our results demonstrate that upregulation of hypothalamic *Agtr1a* expression not only leads to salt-sensitive hypertension, but also serves as negative feedback signal that activates neurons, thereby alleviating weight gain and obesity in offspring exposed prenatally to excessive glucocorticoid. Several investigators have demonstrated that activation of proinflammatory protein NF- κ B in pro-opiomelanocortin neurons of the hypothalamus underlies obesity-related hypertension (33, 35, 37, 38). Obesity-induced inflammation in the hypothalamus increases *Agtr1a* expression in the PVN and increases BP responsiveness to icv infusion of Ang II. By contrast, icv infusion of the TNF- α synthesis inhibitor decreases *Agtr1a* expression, thereby inhibiting the increase in angiotensin responsiveness (34). Of note, there is accumulating evidence showing that, in contrast to the antiinflammatory action of low physiological doses of glucocorticoids, excessive glucocorticoid has proinflammatory effects in the brain (39). Thus, neonatal programmed hypertension

induced by exposure to excessive glucocorticoid might be attributable to obesity-induced inflammation in the hypothalamus and the subsequent overactivity of central angiotensin signaling.

Based upon the importance of central angiotensin signaling in salt-sensitive hypertension, the programming of hypothalamic *Agtr1a* gene expression in offspring may lead to salt-sensitive hypertension possibly through sympathetic overactivity. Wang et al. showed upregulation of *Agtr1a* mRNA in the hypothalamic nuclei of salt-sensitive hypertensive rats compared with salt-resistant normotensive rats and also reported that icv administration of captopril caused a clear decrease in BP (27). Moreover, either injection of *Agtr1a* antisense to the PVN or renal denervation attenuates salt-induced elevation of BP in a rat model of salt-sensitive hypertension with an abnormal hypothalamic-renal sympathetic nervous system (8–10, 16), suggesting that central angiotensin signaling plays a key role in increased sympathetic activity during the development of salt-sensitive hypertension (40). Regarding the mechanism of AT1a activation–induced sympathetic overactivity, Stern et al. demonstrated that AT1a in the astrocytes of the PVN plays a key role in the activation of the sympathetic nervous system (41). Specifically, they showed that hypothalamic AT1a activation excites PVN neurons indirectly via astrocyte AT1a activation–induced inhibition of glutamate reuptake in astrocytes, leading to enhanced glutamate receptor excitation of presympathetic PVN neurons, and results in elevated downstream activity in sympathetic fibers and BP. Thus, it is possible that AT1a activation in astrocytes plays a critical role in the excitation of PVN neurons in prenatal programmed hypertension. However, in situ hybridization revealed no staining of *Agtr1a* mRNA in cells positive for markers of glia and astrocytes, as previously reported (27), and the *Agtr1a* expression pattern was not affected by Sim1-Cre Dnmt3a–KO or dexamethasone treatment, suggesting that *Agtr1a* is expressed mainly in neurons. Moreover, chronic deletion of DNMT3a from hypothalamic neurons increased mRNA expression of PVN *Agtr1a*, and the mice developed salt-sensitive hypertension even without receiving a LP or dexamethasone treatment during pregnancy. These results suggest that DNA demethylation of *Agtr1a* by DNMT3a downregulation in hypothalamic neurons plays a critical role in the upregulation of PVN *Agtr1a* expression by prenatal glucocorticoid excess, leading to prenatal programmed salt-sensitive hypertension, possibly via sympathetic overactivity.

The epigenome, which includes the covalent modifications of DNA and its transcriptional machinery, is the key determinant of gene expression, second in importance only to transcription factor binding (21), leading to persistent phenotypic changes in individual organs (41, 42). Neonatal glucocorticoid exposure makes the initial contribution to the elevated expression of *Agtr1a*, and epigenetic modulation might contribute to the persistent *Agtr1a* upregulation in the hypothalamus of offspring (15). Certainly, transient exposure of PVN cells to dexamethasone in vitro causes epigenetic changes by DNMT3a and altered expression of *Agtr1a*, and both epigenetic and gene expression changes persist despite a background of normal glucocorticoid exposure during adulthood. Indeed, a maternal LP leads to hypomethylation of the CpG islands in the promoters of RAS genes, and upregulation of these genes in the whole brain, in offspring (24, 43). Of note, the whole brain, and even the hypothalamus, is composed of various nuclei that have individual functions. Among the hypothalamic nuclei, the PVN has emerged as one of the most important autonomic control centers in the brain and contains neurons that play essential roles in energy balance and circulatory regulation. We observed aberrant DNA methylation and concurrent upregulation of *Agtr1a* mRNA expression in the PVN of dexamethasone-treated offspring. Upregulation of *Agtr1a* mRNA occurred concomitantly with the reduction in the mRNA expression of *Dnmt3a* associated with reduced activity and binding of DNMT3a, but not DNMT1, to the *Agtr1a* locus. Thus, *Agtr1a* is a target gene of DNMT3a, but not rather than DNMT1. Given the close link between DNMT3a and *Agtr1a* mRNA expression in prenatal programmed hypertension, the mechanism underlying *Dnmt3a* transcription during salt-induced BP elevation needs to be clarified. In contrast with PVN *Agtr1a* mRNA, which is expressed mainly in neural cells, the ubiquitous expression of *Dnmt3a* in the PVN, which consists of multiple cell types including both neurons and glia, makes it difficult to assess the full extent of DNMT3a alterations. Nevertheless, chronic deletion of *Dnmt3a* from hypothalamic neurons could induce DNA demethylation and concurrently increased expressions of *Agtr1a* mRNA in the PVN, leading to salt-sensitive hypertension. Together, these findings strongly suggest that hypothalamic neuron-specific regulation of DNMT3a activity, and the subsequent DNA methylation changes of *Agtr1a*, represent a potential mechanism underlying prenatal programmed salt-sensitive hypertension. From the standpoint of mechanism, however, further study is needed to determine whether sensitization of BP responses to a HS diet after LP pregnancy can actually be blocked by replacement of DNMT3a and blockade of *Agtr1a* within the PVN or, alternatively, by an antagonist-mediated reduction in glucocorticoid signaling.

In conclusion, the RAS in the CNS, which has emerged as a key contributor to the regulation of BP (44), plays an important role in prenatal programmed hypertension. Aberrant DNA methylation resulting from glucocorticoid excess during pregnancy underlies persistent alterations of *Agtr1a* mRNA expression in the hypothalamus, likely leading to elevated BP salt-sensitivity. A greater understanding of epigenetic modulations, including DNA methylation of the RAS component genes, may shed light on how the system could be targeted to prevent and treat prenatal programmed hypertension.

Methods

Animals and experimental design. For the dexamethasone exposure model, pregnant female Sprague-Dawley (SD) rats, C57BL6/J mice, or *Agtr1a*-KO mice were administered dexamethasone (200 µg/kg/d for rats and 100 µg/kg/d for mice) i.p. from F15–21. For the LP model, pregnant female rats were fed a diet containing 9% protein from conception until birth. After delivery, the food was reverted to normal chow (23% protein) and provided ad libitum. Conventional *Agtr1a*-KO mice were purchased from RIKEN (B6; B6CB-Agtr1a<tm1Afu> [RBRC no. RBRC04696]). Sim1-Cre/*Dnmt3a*-KO mice, which harbor hypothalamic neuron-specific *Dnmt3a* deletion, were obtained from Daisuke Kohno (Gunma University). All mice were backcrossed on the C57BL6/J background for 6 or more generations. To generate the Sim1-Cre-specific *Dnmt3a* deletion mice, female mice homozygous for floxed *Dnmt3a* allele1 (45) were crossed with male mice homozygous for the Sim-Cre transgene (46). For HS loading, food containing 8% NaCl was administered for 1 week. Mice and rats were kept at room temperature (22°C–24°C) with a 12-hour/12-hour light/dark cycle, and given access to water ad libitum. We randomly chose the animals for BP measurement, and all the other offspring were used for gene analysis.

BP measurement. Systolic BP and mean arterial pressures (MAP) of conscious rats and mice were monitored using a radiotelemetry system. The animals were anesthetized with pentobarbital sodium, and the tip of the catheter was inserted in the carotid artery of the mouse (PA-C10, Data Sciences International [DSI]) or the abdominal aorta of the rat (PA-C40, DSI), with the telemetry body positioned in a s.c. pocket on the right flank. Baseline measurements were recorded for 2 consecutive days (days 0–2) in rats or mice fed an NS diet. At day 5, posttreatment measurements were recorded for the final 3 consecutive days of the HS diet (days 5–7).

Microarray assay and gene ontology analysis. We extracted RNA from the PVN of control and dexamethasone-treated offspring at 12 weeks of age (each $n = 3$). An Agilent Gene Chip Whole Rat Genome Microarray Ver3.0 was hybridized for each sample. The raw signal intensities and flags for each probe were calculated from the hybridization intensities and spot information according to procedures recommended by Agilent Technologies using the Flag criteria in the GeneSpring Software. In addition, the raw signal intensities of all samples were normalized using the quantile algorithm in Bioconductor. We selected 912 genes that were upregulated by ≥ 2 -fold relative to the control group and subjected them to pathway analysis using the DAVID 6.8 bioinformatics resource. The data files have been uploaded to the National Center for Biotechnology Information Gene Expression Omnibus (GSE119235; to review GSE119235, please go to <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE119235> and enter token gbgiiegktxslbel into the box).

DNMT3a activity assay. To evaluate DNMT3a activity in the rat PVN, we lysed cells in hypotonic buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl) and homogenized by repeatedly passed through a 26-gauge needle syringe. After centrifuge, we extracted nuclear protein with elution buffer (20 mM HEPES, 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, 25% [v/v] glycerol). Nuclear protein was immunoprecipitated overnight at 4°C with anti-DNMT3a antibody (ab2850, Abcam). After the pulldown, DNMT3a activity was measured using the DNMT Universal Chemiluminescent Assay Kit (BPS Bioscience).

Cell culture. The immortalized embryonic mouse hypothalamic neuron cell line N39 was purchased from Cosmo Bio. Cells were maintained in high-glucose DMEM (Invitrogen) containing 10% FBS (Nichirei Bioscience) supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Wako). Mycoplasma contamination was confirmed by using PCR (EZ-PCR mycoplasma Test kit, Cosmo Bio). N39 cells were seeded at 1×10^6 cells per well in 10-cm dishes and maintained in a 37°C humidified 5% CO₂ incubator. The next day, 10–100 nM dexamethasone (Wako) was applied, and the cells were incubated for an additional 1–6 days.

Transfection of siRNA. We transfected 1 nM siRNA into N93 cells using Lipofectamine RNAiMAX (Thermo Fisher Scientific). The sequence of DNMT3a siRNA was UUUCAGUGCACCACAGGAUGUCCUC.

Scrambled RNA was purchased from Invitrogen. For measurement of gene expression, 1×10^6 cells were collected, whereas for MeDIP, 2×10^7 cells were collected. Total RNA or DNA was extracted 6 days after transfection. To determine the efficacy of knockdown, total RNA was extracted as noted above, and the level of the target mRNA was measured (Supplemental Figure 7).

ChIP assay. Brain tissues of the PVN were placed in 0.5 ml of PBS and homogenized by 20 passages through a 25-gauge needle on a syringe. After cell separation, ChIP was performed using the ChIP-IT Express kit (Active Motif). In brief, cells were fixed with 1% formaldehyde for 10 minutes at room temperature; then, crosslinking was stopped by the addition of 125 mM glycine. The cells were lysed with lysis buffer, and chromatin was sheared using a sonicator (Bioruptor UCD-250, CosmoBio) for 10 pulses of 30 seconds each. The efficacy of shearing was confirmed by agarose gel electrophoresis (200–1,000 bp). ChIP was carried out by mixing 20 μ g of chromatin, protein G magnetic beads, 2 μ g of the indicated antibody (mouse monoclonal anti-DNMT1, 39204, Active Motif; rabbit polyclonal anti-DNMT3a antibody, ab2850, Abcam), and ChIP buffer 1 and incubating overnight at 4°C. Subsequently, the beads were washed 3 times with buffers included in the kit, and crosslinks were reversed by incubation with elution buffer AM2 for 15 minutes at 95°C. The eluted DNA was subjected to qPCR using primers hybridizing to the indicated regions near the TSS of *Agtr1a* (Supplemental Table 2). For input DNA, *Agtr1a* DNA was used except in experiments comparing rats on a LP versus a NP (Figure 2D), in which *Agtr1a* PCR was not unsuccessful for unknown reasons. *Actb* DNA was used instead. The *Actb* primers were: F, 5'-TCCAATCCAGAC-CCCGTGTG-3'; R, 5'-GCCTGTATTCTGGGGTAC-3'.

MeDIP. MeDIP was performed using Methyl Collect Ultra Tissue Methylated DNA Immunoprecipitation Kit (Active Motif). Genomic DNA was extracted from a mouse embryonic hypothalamic cell line (N39), enzymatically digested into fragments ranging in size from 200–1,000 bp and divided into IP and input portions. IP DNA was incubated with His-tagged-MBD2b/MBD3L1 protein complex in the kit to bind methylated DNA; normal mouse IgG was used as a negative control (sc-2025, Santa Cruz Biotechnology). Methylated DNA was subjected to qPCR using the primers described above. Relative enrichment of target sequences after MeDIP was expressed as ratios of the signals in IP DNA versus input DNA. Details of the primers used are provided in Supplemental Table 2.

Bisulfite sequencing. Sodium bisulfite treatment of genomic DNA was performed with the CpGenome Turbo Bisulfite Modification Kit (MilliporeSigma). Sequential PCR amplification of the genes of interest was performed using specific primers. The amplicons were ligated into vector pMD20T (Takara Bio) and sequenced. Six or 7 bacterial colonies were picked per amplicon. A web-based quantification tool for methylation analysis was used for bisulfite sequencing analysis of CpG methylation (<http://quma.cdb.riken.jp/>)

qPCR analysis. Total RNA was extracted from cells and PVN (QIAGEN) or ISOGEN lysis buffer (Wako). For qPCR, TaqMan Gene Expression Assays were utilized with a 7300 RT-PCR system (Thermo Fisher Scientific). Denaturation was carried out at 95°C for 15 seconds, and annealing and extension were performed at 60°C for 1 minute for 40 cycles. To compare gene expression levels, the comparative Ct method was used. *Actb* (encoding β -actin) was used as an endogenous control.

In situ hybridization of *Agtr1a*. To evaluate *Agtr1a* expression in the PVN, tissue was fixed with paraformaldehyde/phosphate buffer at 4°C for 24 hours. Paraffin-embedded sections were cut to a thickness of 4 μ m. A 180 bp DNA fragment corresponding to nucleotide positions 1,651–1,830 of mouse *Agtr1a* (GeneBank accession number NM_177322.3) was subcloned into vector pGEMT-Easy (Promega) and used for generation of sense or antisense RNA probes. RNA probes were synthesized using the DIG RNA labeling kit (Roche Diagnostics). Mouse tissues were fixed with G-Fix (GenoStaff) and sectioned at a thickness of 8 μ m. In situ hybridization was performed with the ISH Reagent Kit (GenoStaff). Tissue sections were fixed with G-Nox and rehydrated through an ethanol series and PBS. The sections were fixed with 10% Formalin in PBS for 30 minutes at 37°C, washed in distilled water, and placed in a Coplin jar containing 1 \times G-Wash (GenoStaff), equivalent to 1 \times SSC. Hybridization was performed for 16 hours at 60°C with the indicated probes at a concentration of 250 ng/ml in G-Hybo (GenoStaff). After hybridization, the sections were washed in 1 \times G-Wash for 10 minutes at 60°C. Brain sections were visualized by NBP/BCIP (MilliporeSigma). The sections were counterstained with Kernechtrot (Muto Pure Chemicals).

To determine whether *Agtr1a* is expressed in glia or astrocytes, we stained cells with anti-Iba1 (019-19741, Wako) or anti-GFAP rabbit polyclonal antibody (Z0334, DAKO) overnight at 4°C. After 2 washes with TBS Tween 20 and 1 wash with TBS, the secondary antibody (anti-rabbit Ig Biotin

[E0432, DAKO]) was applied for 30 minutes at room temperature. The samples were washed with TBS and developed with HRP-streptavidin (426062, Nichirei).

Serum leptin concentration. Serum leptin was measured by a commercial laboratory (SRL Inc.) using the multispecies Leptin-RIA kit (MilliporeSigma). Before sampling of blood, rats were starved for at least 6 hours.

Statistics. Some PCR data were omitted when the values were 3 SD outside of the mean or RNA/DNA purity was poor. All data are presented as means \pm SEM. For gene expression analysis, statistical differences were determined by 2-tailed *t* test or by 1-way ANOVA with a post hoc test (Dunnett or Student–Newman–Keuls) unless otherwise noted. Differences with *P* values less than 0.05 were considered statistically significant. BP differences were analyzed by 2-way repeated ANOVA.

Study approval. Animal experiments protocols were approved by the Experimental Animal Research Committee at the University of Tokyo (no. P15008).

Author contributions

KMF, TF, and MN conceived and designed the project. KMF and MN performed the experiments. KMF and MN analyzed the data. WK, NA, KU, DH, RL, DK, SO, TM, and TS helped with experimental procedures, analyzed data, and discussed the results. DK provided Dnmt3a-KO mice. TF, KMF, MN, and TM wrote the manuscript.

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Address correspondence to: Toshiro Fujita, Division of Clinical Epigenetics, Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguroku, Tokyo 153-8904, Japan. Phone: 81.3.5452.5070; Email: Toshiro.FUJITA@rcast.u-tokyo.ac.jp. Or to: Mitsuhiro Nishimoto, Division of Clinical Epigenetics, Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguroku, Tokyo 153-8904, Japan. Phone: 81.3.5452.5070; Email: nishimoto-tky@umin.ac.jp.

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