Angiotensin type 1a receptors in the paraventricular nucleus of the hypothalamus protect against diet-induced obesity

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Abstract

Obesity is associated with increased levels of angiotensin-II (Ang-II), which activates angiotensin type-1a receptors (AT$_{1a}$) to influence cardiovascular function and energy homeostasis. To test the hypothesis that specific AT$_{1a}$ within the brain control these processes, we utilized the Cre/lox system to delete AT$_{1a}$ from the paraventricular nucleus of the hypothalamus (PVN) of mice. PVN AT$_{1a}$ deletion did not affect body mass or adiposity when mice were maintained on standard chow. However, maintenance on a high-fat diet revealed a gene by environment interaction whereby mice lacking AT$_{1a}$ in the PVN had increased food intake and decreased energy expenditure that augmented body mass and adiposity relative to controls. Despite this increased adiposity, PVN AT$_{1a}$ deletion reduced systolic blood pressure, suggesting that this receptor population mediates the positive correlation between adiposity and blood pressure. Gene expression studies revealed that PVN AT$_{1a}$ deletion decreased hypothalamic expression of corticotrophin-releasing hormone and oxytocin, neuropeptides known to control food intake and sympathetic nervous system activity. Whole cell patch clamp recordings confirmed that PVN AT$_{1a}$ deletion eliminates responsiveness of PVN parvocellular neurons to Ang-II, and suggest that Ang-II responsiveness is increased in obese wild-type mice. Central inflammation is associated with metabolic and cardiovascular disorders and PVN AT$_{1a}$ deletion reduced indices of hypothalamic inflammation. Collectively, these studies demonstrate that PVN AT$_{1a}$ regulate energy balance during environmental challenges that promote metabolic and cardiovascular pathologies. The implication is that the elevated Ang-II that accompanies obesity serves as a negative feedback signal that activates PVN neurons to alleviate weight gain.

Introduction

Body weight is maintained by endocrine and neural signals that act in the hypothalamus to coordinate the amount
of energy consumed with that expended. Recently, the renin-angiotensin-system (RAS) has emerged as a potential mediator of the hypothalamic control of body weight (Grobe et al., 2010; de Kloet et al., 2011b). Obese humans and animals have increased RAS activity resulting in elevated levels of angiotensin-II (Ang-II) (Cooper et al., 1998; Boustany et al., 2004; Rahmouni et al., 2004), which stimulates angiotensin type 1a receptors (AT\textsubscript{1a}) in a variety of tissues including the brain (de Kloet et al., 2010). Transgenically (e.g., via the co-expression of human renin and angiotensinogen in mice) or pharmacologically augmenting central RAS activity promotes decreased body weight by inhibiting food intake and elevating energy expenditure (Porter et al., 2003; Furuhashi et al., 2004; Porter and Potratz, 2004; de Kloet et al., 2009; Grobe et al., 2010; de Kloet et al., 2011b). Thus, while it is clear that augmenting brain RAS activity to perhaps supraphysiological levels promotes negative energy balance, the role that central angiotensinergic circuits play in the physiological regulation of body weight subsequent to metabolic challenges that promote obesity and enhance RAS activity has not been discerned, nor has been the neural mechanism(s) underlying these effects. We hypothesized that exposure to an obesigenic environment increases AT\textsubscript{1a} activation within the brain to decrease food intake and increase energy expenditure, and consequently, to alleviate weight gain.

Neurons within the paraventricular nucleus of the hypothalamus (PVN) are well-suited to mediate the effects of Ang-II on body weight regulation. Parvocellular neurons within the PVN are known to influence food intake and energy expenditure (Woods and D'Alessio, 2008) and these same neurons also robustly express AT\textsubscript{1a} (Lenkei et al., 1997). Consequently, AT\textsubscript{1a} on PVN neurons may represent the mechanism and site of action underlying Ang-II mediation of energy balance. The present studies utilized the Cre/lox system in mice to delete the AT\textsubscript{1a} expressed on PVN neurons. Subsequently, the contribution of this receptor population to the regulation of energy balance was determined during maintenance on standard low-fat chow as well as during the consumption of increased dietary fat, a manipulation that facilitates weight gain and enhances RAS activity (Boustany et al., 2004). These studies reveal that deletion of AT\textsubscript{1a} specifically within the PVN exacerbates diet-induced obesity.

Materials and Methods

Animals

PVN AT\textsubscript{1a} KO mice were generated by crossing AT\textsubscript{1a} flox mice (obtained from Dr. Alan Daugherty, University of Kentucky (Li et al., 2011)) on a C57BL/6 background with Sim1Cre mice (generated by Dr. B. Lowell, Beth Israel Deaconess Medical Center and Harvard Medical School (Balthasar et al., 2005)) on a C57BL/6 × 129 background. Male PVN AT\textsubscript{1a} KO mice (homozygous for AT\textsubscript{1a} flox [AT\textsubscript{1a} flox/flox]) and expressing Sim1Cre) and littermate control mice (AT\textsubscript{1a} flox/flox) were used for all studies and were 10–12 wks old at the time experiments began. In order to generate litters that contained both of these genotypes, the dams were AT\textsubscript{1a} flox/flox and did not express Sim1Cre, while the sires were AT\textsubscript{1a} flox/flox and carried one copy of the Sim1Cre transgene. For the electrophysiological experiment conducted in wild-type C57BL/6 mice, mice were obtained from Harlan Laboratories. Mice were fed either standard low-fat rodent chow (CHOW; Harlan Teklad [LM485], Indianapolis, IN; 3.1 kcal/g; ~5% fat) or a high-fat diet (HFD; Research Diets [D03082706], New Brunswick, NJ; 4.54 kcal/g; ~40% fat). Unless otherwise noted, food and water were provided ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committees at the University of Florida and the University of Cincinnati.

Food, water and NaCl intake

Food intake was assessed manually every 2–3 d for the duration of the 31 d study and is reported as cumulative food intake. Water intake was assessed by weighing the water bottles every 2–3 d during 1 wk of CHOW-feeding or HFD-feeding and is reported as mean daily water intake. Mice maintained on CHOW or HFD were given
access to water and a solution of 500 mM NaCl for 3 d, and mean daily NaCl intake was determined. Mice were maintained on HFD for 4 wks before assessment of water and NaCl intake.

**Body weight and body composition**

Body composition was determined using NMR technology (Echo NMR, Waco, TX) on unanesthetized mice as previously described (Taicher et al., 2003). Body weight was assessed every 2–3 d for the duration of the study.

**Indirect calorimetry**

Energy expenditure during fasting and ad libitum-feeding conditions was assessed via indirect calorimetry using the Columbus instruments CLAMS (AccuScan Instruments, Columbus, OH) system. 12 wk-old mice were fed HFD for 4 wks prior to the initiation of energy expenditure assessment. At the time of the experiment, mice were 16 wks of age. Mice were allowed to acclimate to the metabolic cages for 1 d. On the first day of energy expenditure assessment, mice were given ad libitum access to HFD. Subsequently, mice were fasted for 16 h, during which time energy expenditure was continuously-monitored. VO₂ and VCO₂ were normalized to lean body mass. Energy expenditure was determined using the following equation: 3.815 * VO₂ + 1.232 * VCO₂ and reported in cal/ (kg lean mass)/min (Sarruf et al., 2010). Respiratory quotient (RQ) was determined using the following equation: RQ = VCO₂/VO₂.

**Tissue collection**

After 32 d of HFD or CHOW-feeding, mice were fasted for 2 h in order to ensure minimal variability in energy status. Mice were then euthanized by conscious decapitation for tissue collection at 1000 h (4 h after the onset of the light-phase). Whole brains were removed and flash frozen in dry ice-cooled isopentane. Kidney, adrenal and pituitary were obtained and flash frozen in dry ice-cooled isopentane for gene expression analysis. Plasma was separated from 600 µl of trunk blood collected in 20 µl of EDTA (100 mM).

**Cardiovascular and activity recording via telemetry**

While AT₁a flox/flox mice and PVN AT₁a KO were under isoflurane anesthesia, radiotelemetry transmitters (Data Sciences International, St. Paul, MN) were placed into the distal left carotid artery and positioned subcutaneously near the left rear flank region. Following recovery and acclimation for 2 wks, mice were maintained on CHOW (AT₁a flox/flox, n = 4; PVN AT₁a KO, n = 4) or placed on HFD (AT₁a flox/flox, n = 4; PVN AT₁a KO, n = 4) and cardiovascular and activity measurements were continuously recorded for 8 d or 4 wks, respectively. Data were obtained at 10-s intervals, which were collapsed into 1 h means to generate a 24 h profile for each mouse. These 24 h profiles were then averaged for each mouse to produce a 24 h profile representing the 8 d or 4 wks on CHOW or HFD, respectively. Finally, these data sets were averaged to generate the 24 h profiles representing the cardiovascular and activity measurements obtained from AT₁a flox/flox and PVN AT₁a KO mice during maintenance on CHOW or HFD for 8 d or 4 wks, respectively.

**RNA isolation and cDNA synthesis**

RNeasy columns (Qiagen, Valencia, CA) were used to isolate RNA from the hypothalamus, adrenal, pituitary and renal cortex. DNase treatment (Qiagen, Valencia, CA) was performed to minimize genomic DNA contamination of RNA extracts. For hypothalamic gene expression analysis, hypothalami were dissected from flash-frozen brains in the chamber of a cryostat (at −15 C) on the day of RNA extraction. Brains were placed in a cooled metal brain block and razor blades were used to remove the tissue rostral and caudal to the hypothalamus. This portion of the brain containing the hypothalamus was then placed on the stage of the cryostat and the brain tissue lateral and dorsal to the hypothalamus was removed. The isolated hypothalamus was then submerged in 700 µl of
RLT buffer from the Qiagen RNeasy kit. RNA extraction and DNase treatment procedures were performed according to the manufacturer’s instructions. Subsequently, iScript (Bio-Rad, Hercules, CA) was used to synthesize cDNA from 1 µg total RNA.

**Semi-quantitative real-time PCR**

Gene expression was assessed in the hypothalamus, kidney, adrenal and pituitary using semi-quantitative real-time PCR. For semi-quantitative real-time PCR analysis of agouti-related peptide (AgRP; Mm00475829), pro-opiomelanocortin (POMC; Mm00435874), angiotensin type-1a receptor (AT$_{1a}$; Mm01166161), single-minded 1 (Sim-1; Mm00441390), renin (REN; Mm02342887), arginine vasopressin (AVP; Mm00437761), corticotrophin-releasing hormone (CRH; Mm1293920), oxytocin (OXT; Mm01329577), tumor necrosis factor α (TNF-α; Mm00443260), interleukin-6 (IL-6; Mm00446190), interleukin-1β (IL-1β; Mm00434228) and a cluster of differentiation molecule 11b (CD11b; Mm00434455) diluted (1:5), cDNA samples were run in duplicate using a 7900HT Fast Real-time PCR system, Taqman Gene Expression Master Mix and validated Taqman probes (Applied Biosystems, Foster City, CA). Expression patterns of genes of interest were normalized to constitutively-expressed ribosomal protein L32 (Mm02528467) and relative expression was quantified using the $2^{ΔΔCt}$ method.

**Analysis of plasma measurements**

Plasma glucose was determined using the glucose oxidation method as previously described (de Kloet et al., 2009). Plasma renin activity (PRA) levels were determined using a $^{125}$I RIA kit from Diasorin, and corticosterone levels were assessed using a $^{125}$I RIA kit from MP Biomedicals (Krause et al., 2008; de Kloet et al., 2009). Plasma sodium concentration was determined using a dual-channel flame photometer (Cole-Parmer, Vernon Hills, IL). Plasma proteins and hematocrit were determined using a refractometer and microcapillary reader, respectively.

**Semi-quantitative receptor autoradiography**

Receptor autoradiography was used to assess the efficacy of Cre-mediated deletion of AT$_{1a}$ from the PVN. Autoradiographic measurement of Ang-II binding to central angiotensin receptors was performed as previously described (Krause et al., 2008; Krause et al., 2011).

**In vitro whole-cell recording**

Male AT$_{1a}$flox/flox and PVN AT$_{1a}$KO mice maintained on CHOW and wild-type C57BL/6 mice maintained on CHOW or HFD were anesthetized with isoflurane and decapitated using a small-animal guillotine. Their brains were rapidly removed and immersed in ice cold artificial cerebral spinal fluid (ACSF). Coronal sections through the hypothalamus were cut at 300 µm using a Pelco Series 3000 Vibratome (Pelco, Redding, CA) and immediately submerged in an incubator containing ACSF preheated to 30–35°C. Following a 30 min incubation period, slices were allowed to equilibrate to room temperature for an additional 30 min before use. ACSF for sectioning and incubation contained (in mM): 124 NaCl, 2.5 KCl, 1.2 NaH$_2$PO$_4$, 2.5 MgSO$_4$, 10 D-glucose, 1 CaCl$_2$, and 25.9 NaHCO$_3$, saturated with 95% O$_2$ and 5% CO$_2$. ACSF for recording contained (in mM): 126 NaCl, 3 KCl, 1.2 NaH$_2$PO$_4$, 1.5 MgSO$_4$, 11 D-Glucose, 2.4 CaCl$_2$ and 25.9 NaHCO$_3$. This solution was saturated with 95% O$_2$ and 5% CO$_2$, maintained at 30 °C, and washed over slices at a rate of 2 ml/min. Whole-cell voltage-clamp recordings were performed using micropipettes pulled from borosilicate glass using a Flaming/Brown electrode puller (Sutter P-97, Sutter Instruments, Novato, CA). Electrode tip resistance was between 4–6 MΩ when filled with an internal solution that contained (in mM): 130 K-gluconate, 10 KCl, 10 NaCl, 2 MgCl$_2$, 1 EGTA, 2 Na$_2$ATP, 0.3 NaGTP, 10 HEPES. This solution was pH adjusted to 7.3 using KOH.
and volume adjusted to 285–300 mOsm. For each cell, 1 µM of Ang-II was bath-applied after 6 min of stable recording in current clamp. The initial membrane potential was defined as the mean voltage observed during a continuous 60 s period extracted from the last 3 min of baseline recording. The effect of Ang-II was measured in an identical 60 s window observed between 2–5 min after application. The precise timing of these windows was adjusted as necessary to ensure that measurement of the pre- or post Ang-II mean membrane voltage was not contaminated by action potentials.

Statistics

For food intake and body composition studies in HFD- and CHOW-fed, PVN AT_{1a} KO and AT_{1a} flox/flox mice, statistical significance was ascertained with two-way ANOVA (genotype and diet as the factors). For endpoints assessed exclusively in CHOW-fed or HFD-fed mice, including hypothalamic, adrenal, pituitary and kidney gene expression, plasma measures, water intake, and NaCl intake, an unpaired Student’s t-test was used to determine statistical significance. A repeated-measures ANOVA was used for assessment of body mass, energy expenditure and cardiovascular/activity parameters. In the electrophysiological studies, an unpaired Student’s t-test was used to compare the mean effect of Ang-II on membrane voltage in the PVN AT_{1a} KO mice vs. the littermate AT_{1a} flox/flox controls. Basic principles in probability theory were used to test the hypothesis that a higher percentage of cells were responsive to Ang-II in the HFD animals. Statistical significance was set at p < 0.05 (two-tailed).

Results

AT_{1a} receptor expression in the brain and peripheral tissues

Co-expression of the Sim1Cre and AT_{1a} flox genes led to reduced AT_{1a} binding within the PVN (t_{14} = 7.07; p < 0.05), but not in other Sim1- or AT_{1a}-containing brain nuclei, including the supraoptic nucleus (SON, contains Sim1, but not AT_{1a}), subfornical organ (SFO; contains AT_{1a}, but not Sim1), median preoptic nucleus (MnPO; contains AT_{1a} and scattered Sim1) or periventricular nucleus (PeVN; contains AT_{1a}, but not Sim1), suggesting that within the brain, AT_{1a} deletion was specific to the PVN (Figure 1). Co-expression of the Sim1Cre and the AT_{1a} flox transgenes did not affect AT_{1a} gene expression within the adrenal and pituitary; however, in addition to reduced hypothalamic AT_{1a} gene expression (t_{15} = 4.82; p < 0.05) PVN AT_{1a} KO mice had a ~25 % reduction of AT_{1a} levels within the renal cortex (t_{12} = 2.80; p < 0.05; Figure 2). This reduction in renal AT_{1a} expression is consistent with the scattered Cre expression observed by Balthasar et al. (2005). For detailed discussion of AT_{1a} expression in central and systemic tissues refer to (Lenkei et al., 1997; Allen et al., 1999; Chen et al., 2012).

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Sim1Cre and AT_{1a} flox/flox co-expression does not affect kidney function or hydromineral balance

Due to the slight decrease in kidney AT_{1a}, it was necessary to assess the impact of the genetic manipulation on kidney function. Thus, indices of both kidney function and hydromineral balance were assessed in AT_{1a} flox/flox and PVN AT_{1a} KO mice fed CHOW or HFD for 4 wks. PVN AT_{1a} deletion did not alter hematocrit, plasma
protein, plasma Na⁺, glucose, CORT, or ACTH levels when mice were maintained on CHOW or HFD (Table 1). Furthermore, plasma renin activity (PRA; Table 1) and kidney renin mRNA (Figure 2) expression were not different between the AT₁a flox/flox and PVN AT₁a KO mice maintained on CHOW or HFD. Water (4.52 ± 0.33 ml/d vs. 4.87 ± 0.19 ml/d when maintained on CHOW [n = 8–9/group]; 2.38 ± 0.20 ml/d vs. 2.34 ± 0.11 ml/d when maintained on HFD [n = 5–6/group]) and 500 mM NaCl (0.57 ± 0.06 ml/day vs. 0.58 ± 0.08 ml/day when maintained on CHOW [n = 8–9/group]; 1.05 ± 0.15 ml/day vs. 1.05 ± 0.14 ml/day when maintained on HFD [n = 5–6/group]) intake were also not different between the groups. Collectively, these data suggest that PVN AT₁a KO mice do not have altered kidney function or hydromineral imbalance when maintained on CHOW or HFD.

Table 1
Plasma Measurements

PVN AT₁a deletion enhances susceptibility to diet-induced body and adipose mass gain

At 10–12 wks of age, AT₁a flox/flox and PVN AT₁a KO mice were body mass-matched and sub-divided into CHOW and HFD-fed groups. At the initiation of the study, AT₁a flox/flox and PVN AT₁a KO mice had similar body masses (29.00 ± 0.57 g vs. 29.37 ± 0.63 g). AT₁a flox/flox and PVN AT₁a KO mice maintained on CHOW, gained similar amounts of body mass, adipose mass and lean mass, regardless of genotype (Figure 3). When given a HFD, both AT₁a flox/flox and PVN AT₁a KO mice gained significantly more body mass and adipose mass than CHOW fed mice (Figure 3: main effect of diet; F(1,29) = 5.67; p < 0.05). Starting on Day 9, however, PVN AT₁a KO mice given HFD gained significantly more body mass than AT₁a flox/flox mice given HFD (gene × diet interaction; F(1,29) = 9.52; p < 0.01). This difference in body mass is mostly attributable to an increase in adiposity, since after 28 d of HFD feeding, PVN AT₁a KO mice gained ~3 g more adipose mass than did AT₁a flox/flox mice (gene × diet interaction; F(1,29) = 6.86; p < 0.05). There was no effect of genotype on lean mass although there was a main effect of diet on lean mass (F(1,29) = 14.10; p < 0.001).

Figure 3
PVN AT₁a deletion enhances susceptibility to diet-induced body and adipose mass gain

PVN AT₁a deletion increases energy consumption and reduces energy expenditure in HFD-fed mice

Throughout the study, food intake and energy expenditure were assessed as potential mechanisms for the reduced body mass. When mice were maintained on CHOW, PVN AT₁a deletion had no effect on food intake.

Conversely, PVN AT₁a KO mice exhibited increased consumption of HFD, relative to AT₁a flox/flox mice maintained on HFD (Figure 4; gene × diet interaction; F(1,27) = 8.50; p < 0.001). Energy expenditure during fasting and ad libitum-feeding was assessed in AT₁a flox/flox and PVN AT₁a KO mice maintained on HFD for 4 wks (Figure 4). As depicted in Figure 4, PVN AT₁a deletion resulted in a significant reduction in energy expenditure regardless of whether the mice were fasting or ad libitum-feeding (main effect of genotype during fasting [F(1,14) = 13.66; p < 0.01] and ad libitum-feeding [F(1,14) = 13.66; p < 0.01]). Additionally, there was a significant effect of genotype on respiratory quotient (RQ) during ad libitum-feeding (F(1,14) = 4.81; p < 0.05). In general, the PVN AT₁a KO mice had an elevated RQ relative to AT₁a flox/flox mice, suggestive of a reduced utilization of fat (enhanced utilization of carbohydrates) in the PVN AT₁a KO mice. Conversely, during the fast, there was no significant effect of genotype on RQ, although it did significantly change over time (F(26,364) =
15.56; p < 0.001) from an initial level of ~0.79 to a fasting level of ~0.71. These data indicate that both genotypes shifted to a greater utilization of fat during the fast.

**Figure 4**

**PVN AT<sub>1α</sub> deletion increases energy consumption and reduces energy expenditure in HFD-fed mice**

**PVN AT<sub>1α</sub> deletion reduces blood pressure and activity during HFD-feeding**

Cardiovascular parameters were assessed in AT<sub>1α</sub> flox/flox and PVN AT<sub>1α</sub> KO mice maintained on CHOW or HFD for 8 d or 4 wks, respectively (Figure 5). As expected, in the CHOW condition there was an effect of time on systolic blood pressure (F(23,138) = 31.9; p < 0.001), heart rate (F(23,138) = 35.16; p < 0.001), and activity (F(23,138) = 10.21; p < 0.001) with larger values occurring during the dark phase. There was no effect of genotype with AT<sub>1α</sub> flox/flox and PVN AT<sub>1α</sub> KO mice having similar systolic blood pressure, heart rate and activity when given CHOW (Figure 5 A–C). However, maintenance on HFD revealed a genotype by time interaction, with PVN AT<sub>1α</sub> KO mice having reduced systolic blood pressure (F(23,138) = 1.92; p < 0.05) and activity (F(23,138) = 2.41; p < 0.001) during the dark phase relative to AT<sub>1α</sub> flox/flox mice (Figure 5D and 5F). Heart rate, on the other hand, was not affected by genotype, although there was a main effect of time (F(23,138) = 38.75; p < 0.001; Figure 5E).

**Figure 5**

**PVN AT<sub>1α</sub> deletion reduces blood pressure and activity during HFD-feeding**

**PVN AT<sub>1α</sub> deletion modulates hypothalamic gene expression**

In order to explore some potential mechanisms downstream of AT<sub>1α</sub> for Ang-II’s energy balance regulatory functions, hypothalamic gene expression was assessed in AT<sub>1α</sub> flox/flox and PVN AT<sub>1α</sub> KO mice maintained on HFD (Figure 6). Mice lacking the AT<sub>1α</sub> receptor specifically in the PVN have reduced expression of corticotrophin-releasing hormone (CRH; t<sub>15</sub> = 2.30; p < 0.05) and oxytocin (OXT; t<sub>15</sub> = 2.19; p < 0.05) mRNAs; however AVP mRNA expression was not different between the genotypes. Additionally, the hypothalamic arcuate nucleus (ARC)-specific genes, AgRP and POMC were not altered in mice lacking AT<sub>1α</sub> in the PVN. During HFD-feeding, PVN AT<sub>1α</sub> deletion also causes a significant reduction in gene expression for TNFα (t<sub>15</sub> = 2.81; p < 0.05), a pro-inflammatory cytokine, and CD11b (t<sub>15</sub> = 2.49; p < 0.05), a marker for active microglia.

**Figure 6**

**PVN AT<sub>1α</sub> deletion modulates hypothalamic gene expression**

**PVN AT<sub>1α</sub> deletion eliminates the response of parvocellular neurons in the PVN to bath-applied Ang-II**

We used whole cell recording techniques (see methods) to examine the effects of bath-applied Ang-II on PVN parvocellular neurons in both AT<sub>1α</sub>flox/flox and PVN AT<sub>1α</sub> KO mice. In both groups parvocellular neurons had high input resistance (AT<sub>1α</sub>flox/flox: 842 ± 158 MΩ, PVN AT<sub>1α</sub> KO: 814 ± 104 MΩ), and low capacitance (AT<sub>1α</sub>flox/flox: 7.7 ± 1.3 pF, PVN AT<sub>1α</sub> KO: 11 ± 1.8 pF) as assessed with brief (25 msec) voltage steps from −70 mV to −80 mV in voltage clamp mode. Similarly, in both groups some parvocellular neurons exhibited
intrinsic electrophysiological properties suggestive of preautonomic neurons, while other neurons were likely neurosecretory. For more information on how intrinsic properties were used to select cells please see Fig. 7A–C (and also, Hoffman et al., 1991; Tasker and Dudek, 1991; Stern, 2001; Luther et al., 2002). We focused on parvocellular populations, due to their known role in regulation of body weight. In order to test the effects of bath application of Ang-II, the membrane potential was adjusted in current clamp mode to near $-60 \text{ mV}$ ($\text{AT}_{1a} \text{flox/flox } : -63 \pm 2.4 \text{ mV, PVN AT}_{1a} \text{KO } : -61 \pm 1.7 \text{ mV}$) using mild holding currents ($\text{AT}_{1a} \text{flox/flox } : -31 \pm 11 \text{ pA, PVN AT}_{1a} \text{KO } : -0.5 \text{ pA} \pm 8.5 \text{ pA, t}_{18} = 2.3; p = 0.04$). Consistent with prior reports (Li et al., 2003; Cato and Toney, 2005; Latchford and Ferguson, 2005) bath application of 1 $\mu$M Ang-II in the control group produced a clear depolarization in 6 of 10 cells tested (overall mean depolarization: $8.4 \pm 2.7 \text{ mV, t}_{9} = 3.10; p = 0.01$, responders only: $14 \pm 2.7 \text{ mV, t}_{5} = 5.12; p = 0.004$). In sharp contrast, this effect was eliminated in PVN $\text{AT}_{1a}$ KO mice (mean depolarization: $1.6 \pm 0.6 \text{ mV, t}_{18} = 3.64; p = 0.002$ vs. all controls, $t_{14} = 5.6; p < 0.0001$ vs. control responders). These results indicate that PVN-specific $\text{AT}_{1a}$ deletion effectively removes the physiological response of PVN parvocellular neurons to acute application of Ang-II.

**Figure 7**
PVN $\text{AT}_{1a}$ deletion eliminates the response of parvocellular neurons in the PVN to bath-applied Ang-II

**HFD-induced obesity increases the percentage of PVN parvocellular neurons that are responsive to Ang-II**

In a final series of experiments, we used electrophysiological techniques to examine the effects of bath-applied Ang-II on PVN parvocellular neurons in wild-type C57BL/6 mice maintained on CHOW or HFD (see methods). Parvocellular neurons were selected as in prior experiments. Input resistance and whole-cell capacitance were not affected by diet, and were similar to those observed in $\text{AT}_{1a}$ flox/flox and PVN $\text{AT}_{1a}$ KO mice ($R_m$ CHOW: $742 \pm 80.4 \text{ M}\Omega$, HFD: $804 \pm 79.7 \text{ M}\Omega$; $C_m$ CHOW: $9.2 \pm 1.0 \text{ pF}$, HFD: $10.8 \pm 1.24 \text{ pF}$). Overall, our results indicate that maintenance on the HFD led to a significant increase in body weight (CHOW: $24.3 \pm 0.38 \text{ g}$, HFD: $30.1 \pm 1.21 \text{ g, t}_{23}=4.1; p < 0.001$), but across all cells tested, did not produce a statistically significant increase in the mean depolarization produced by Ang-II (CHOW: $2.4 \pm 1.3 \text{ mV}$, HFD: $3.6 \pm 1.0 \text{ mV}$). However, this is not an optimal analysis because some parvocellular neurons lacked $\text{AT}_{1a}$ and some mice were resistant to diet-induced obesity. Further, it is possible that the primary effect of the HFD is on the percentage of cells responsive to Ang-II rather than the extent to which individual cells are depolarized. To test this hypothesis, we defined a responsive cell as one in which bath application of Ang-II depolarized the membrane potential by an amount that was $\geq$ to two times the SD observed across all cells tested in the CHOW group. This test was applied to all cells tested in both CHOW and HFD groups. Similarly, we then defined a HFD (obese) animal as one whose body weight was $\geq$ to the 95% confidence interval for mean body weight observed in the CHOW group (e.g., $\geq$ CHOW mean weight + [2*SD of the mean]), while a HFD (lean) animal was one maintained on HFD whose body weight did not meet that standard. With these definitions in place, it became clear that the probability of observing a response to Ang-II was indeed significantly increased in the HFD (obese) population (from 4/11 [36.4%] in CHOW to 8/9 [88.9%], $p = 0.002$), but was unaltered in the HFD (lean) population (2/5 [40%], see Figure 8).

**Figure 8**
HFD-induced obesity increases the percentage of PVN parvocellular neurons that are responsive to Ang-II

**Discussion**

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The present data highlight an environment and gene interaction whereby increased fat intake augments PVN AT\textsubscript{1a} activation to alleviate weight gain. PVN AT\textsubscript{1a} KO mice had an enhanced susceptibility to HFD-induced body and adipose mass gain. The increased body weight and adiposity of PVN AT\textsubscript{1a} KO mice fed HFD was the result of increased food intake and decreased energy expenditure. Measures of hypothalamic gene expression in conjunction with electrophysiological studies suggest that PVN AT\textsubscript{1a} mediate hypothalamic inflammation, which in turn may influence the activation of PVN neurons that express CRH or OXT to regulate energy intake and expenditure. Taken together, these studies underscore an important role for PVN AT\textsubscript{1a} in the regulation of energy balance during HFD-induced obesity, a condition that increases RAS activity (Boustany et al., 2004; Rahmouni et al., 2004).

In the brain, mice homozygous for the AT\textsubscript{1a} flox gene that express Cre-recombinase under control of the Sim1 promoter exhibit PVN-selective deletion of AT\textsubscript{1a}. In peripheral tissues, AT\textsubscript{1a} expression in adrenal and pituitary was not different between AT\textsubscript{1a} flox/flox and PVN AT\textsubscript{1a} KO mice; however, PVN AT\textsubscript{1a} KO mice had a slight reduction of renal cortex AT\textsubscript{1a} expression. Despite this reduction, there were no differences in indices of renal function. Moreover, PRA and kidney renin gene expression were not different between the groups, suggesting that AT\textsubscript{1a} deletion from Sim1-containing cells does not affect systemic RAS activity.

Our study reveals that deletion of AT\textsubscript{1a} from the PVN exacerabes diet-induced body and adipose mass gain, findings that are consistent with reports suggesting a role for Ang-II in the regulation of energy balance (Furuhashi et al., 2004; Porter and Potratz, 2004; Grobe et al., 2010; de Kloet et al., 2011b; Yamamoto et al., 2011). Elevations in central Ang-II either via exogenous administration or transgenically via the co-expression of human renin and angiotensinogen reduces body weight and adiposity (Porter et al., 2003; Furuhashi et al., 2004; Grobe et al., 2010; de Kloet et al., 2011b), supporting the hypothesis that high, perhaps supraphysiological, levels of Ang-II are sufficient to reduce adiposity. These previous studies, however, did not address the necessity of Ang-II in energy balance regulation during physiological conditions that elevate RAS activity. To this end, the necessity of the RAS in the regulation of energy balance has been investigated by targeting RAS components throughout the entire body. These non-selective manipulations cannot reveal the central mechanism(s) of the influence of Ang-II on metabolic regulation and have produced phenotypes that often contrast the obesity produced by selective deletion of PVN AT\textsubscript{1a}. Rodents given pharmacological RAS inhibitors (Weisinger et al., 2008; de Kloet et al., 2009) and mice lacking angiotensinogen (Massiera et al., 2001), angiotensin-converting enzyme (Jayasooriya et al., 2008), renin (Takahashi et al., 2007) or angiotensin receptors (Kouyama et al., 2005; Yvan-Charvet et al., 2005) are lean, consistent with a role of the systemic RAS in promoting energy storage. The divergence between the central and peripheral effects of the RAS suggests the presence of a negative feedback pathway that is activated when adipose angiotensinogen levels are high, allowing adipose-derived Ang-II to access the brain and provide a brake on peripheral Ang-II action. The present data indicate that PVN AT\textsubscript{1a} may coordinate this negative feedback.

The increased adiposity of PVN AT\textsubscript{1a} KO mice was the result of increased food intake and decreased energy expenditure. Although CHOW intake was not altered by PVN AT\textsubscript{1a} deletion, these mice consumed more HFD, suggesting that PVN AT\textsubscript{1a} regulate of food intake during HFD-feeding (an environmental manipulation that increases RAS activity (Boustany et al., 2004)). Moreover, when fed HFD, these mice exhibited reduced energy expenditure, activity and blood pressure. These results are consistent with previous findings that central administration of Ang-II produces anorexia and elevates energy expenditure (Porter and Potratz, 2004; Grobe et al., 2010; de Kloet et al., 2011b), while transgenically reducing brain RAS activity via the brain expression of antisense oligonucleotides targeting angiotensinogen, elevates food intake (Kasper et al., 2005). Mice with whole-body deficits in RAS components can be lean or obese; however, in both cases they are hyperphagic (Kouyama et al., 2005; Yamamoto et al., 2011), consistent with a central role of the RAS in reducing food intake.
The reduced energy expenditure observed in our studies was associated with elevated RQ, representing a shift in caloric utilization from fat to carbohydrates. This shift likely contributes to the increased adiposity of PVN AT1a KO mice maintained on HFD and indicates that the PVN AT1a may regulate fat deposition. White and brown adipose tissue depots are innervated by the sympathetic nervous system and parvocellular AT1a-expressing PVN neurons regulate sympathetic outflow. Administration of Ang-II increases the expression of indices of sympathetic activation of adipose tissue, which may increase lipolysis (Porter and Potratz, 2004; de Kloet et al., 2011b). The present data, in conjunction with these previous studies, suggest that Ang-II regulates energy expenditure via sympathetic activation of adipose tissue.

It can be inferred from these studies that Ang-II regulates energy expenditure during HFD-feeding by controlling blood pressure and activity. Despite increased adiposity of HFD-fed PVN AT1a KO mice, blood pressure and activity were reduced relative to HFD-fed AT1a/flox/flox controls. These data suggest a key role for PVN AT1a in blood pressure regulation subsequent to the increased RAS activity that occurs during HFD-induced obesity, and are consistent with studies implicating PVN AT1a in sympathetic nervous system regulation (Li et al., 1996; Zhu et al., 2002; Li et al., 2003; Li and Pan, 2005).

Although we cannot rule out a role for reduced kidney AT1a, the hypothalamic gene expression analysis and electrophysiological studies point to a central mechanism. PVN AT1a deletion reduced CRH and OXT gene expression within the hypothalamus and abolished the Ang-II-induced depolarization of PVN neurons exhibiting neurosecretory or preautonomic electrophysiological properties. Interestingly, the probability that neurosecretory and preautonomic neurons respond to Ang-II was increased in wild-type mice rendered obese on HFD. Within the PVN, AT1a influence the activity of neurosecretory (Sumitomo et al., 1991; Aguilera et al., 1995) and preautonomic (Cato and Toney, 2005) neurons that are known to express CRH and OXT (Cechetto and Saper, 1988). Elevated Ang-II increases CRH expression (Sumitomo et al., 1991) and activation of CRH receptors induces anorexia, elevates energy expenditure, and enhances sympathetic outflow (Richard et al., 2002; Yamamoto et al., 2011), while antagonism of CRH receptors has the opposite effect (Krahn et al., 1986; Hotta et al., 1999). Similarly, Ang-II influences the activation of OXT neurons and increased OXT produces anorexia and elevates energy expenditure (Olson et al., 1991). These prior studies, in conjunction with our results, suggest that Ang-II influences energy balance during HFD-feeding by up-regulating the activation of AT1a on PVN neurons that express CRH or OXT.

Ang-II is a proinflammatory factor and inflammation is an important regulator of energy homeostasis (Kim et al., 2008). For example, TNFα promotes negative energy balance (de Kloet et al., 2011a) and is reduced in PVN AT1a KO mice consistent with a role for inflammation in mediating some of these effects. Additionally, inflammation and microglial activation within the PVN are associated with elevated blood pressure and augmented sympathetic activity (Li et al., 2008; Shi et al., 2010). Given that obesity up-regulates the RAS, it is possible that a reduction in Ang-II-induced inflammation enhances the susceptibility to HFD-induced obesity in PVN AT1a KO mice. Conversely, increased inflammation and microglial activation may contribute to the enhanced responsiveness of PVN neurons to Ang-II.

The present data reveal a key role of the PVN in the angiotensinergic regulation of energy balance; however, there are other brain nuclei that express AT1a and it is possible that one or more of these play a role in Ang-II’s metabolic regulatory action. Yoshida et al. (2012) found that Ang-II produces anorexia by reducing expression of the orexigenic factors, Neuropeptide Y and orexin, suggesting that the ARC and the lateral hypothalamus may be involved. However, the lack of an effect of genotype on AgRP and POMC levels in the present study suggests that PVN AT1a deletion does not affect the ARC-specific energy balance pathway. The SFO is another potentially important brain nucleus in this regard, as it is critical for communicating the levels of systemic RAS...
activity to the CNS (Miselis, 1981; Lind et al., 1984; Swanson and Lind, 1986) and has direct projections to the PVN (Krause et al., 2011). The SFO is a potential contributor to the regulation of energy balance (Smith et al., 2009) and a site where adiposity signals like leptin and Ang-II may interact (Hilzendeger et al., 2012). Consequently, the SFO may coordinate systemic and central RAS activity to regulate energy balance.

The RAS has emerged as a key contributor to the regulation of metabolic homeostasis; however, targeting this system as a therapeutic for both pathologies is complicated by the fact that the RAS is present in and has diverse effects on many metabolic tissues. Understanding the distinctive roles of the RAS within various tissues may shed light on how the system can be targeted to treat not only hypertension, but also obesity.

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