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Control of Energy Balance by Hypothalamic Gene Circuitry Involving Two Nuclear Receptors, Neuron-Derived Orphan Receptor 1 and Glucocorticoid Receptor

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Nuclear receptors (NRs) regulate diverse physiological processes, including the central nervous system control of energy balance. However, the molecular mechanisms for the central actions of NRs in energy balance remain relatively poorly defined. Here we report a hypothalamic gene network involving two NRs, neuron-derived orphan receptor 1 (NOR1) and glucocorticoid receptor (GR), which directs the regulated expression of orexigenic neuropeptides agouti-related peptide (AgRP) and neuropeptide Y (NPY) in response to peripheral signals. Our results suggest that the anorexigenic signal leptin induces NOR1 expression likely via the transcription factor cyclic AMP response element-binding protein (CREB), while the orexigenic signal glucocorticoid mobilizes GR to inhibit NOR1 expression by antagonizing the action of CREB. Also, NOR1 suppresses glucocorticoid-dependent expression of AgRP and NPY. Consistently, relative to wild-type mice, NOR1-null mice showed significantly higher levels of AgRP and NPY and were less responsive to leptin in decreasing the expression of AgRP and NPY. These results identify mutual antagonism between NOR1 and GR to be a key rheostat for peripheral metabolic signals to centrally control energy balance.

The first group of neurons that encounters peripheral metabolic signals, such as leptin, insulin, and ghrelin, thereby transducing their action to control energy balance to the rest of the central nervous system (CNS), is clustered in the arcuate nucleus region of the hypothalamus (ARC) (1, 2). Two types of metabolic neurons in the ARC have been particularly well characterized, i.e., neurons that express the orexigenic neuropeptides agouti-related peptide (AgRP) and neuropeptide Y (NPY), herein named AgRP neurons, and neurons expressing the anorexigenic neuropeptides α-melanocyte-stimulating hormone (αMSH), a proteolytic product of pro-opiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART), herein named POMC neurons. The anorexigenic signals insulin and leptin, which are critical adiposity signals that circulate in proportion to the body fat mass, stimulate POMC neurons and inactivate AgRP neurons to inhibit food intake and increase energy expenditure (1, 2). In contrast, the orexigenic signal ghrelin, a circulating peptide secreted from the stomach, activates AgRP neurons, thereby stimulating food intake. Thus, interplays among leptin, insulin, ghrelin, and AgRP and POMC neurons play important roles in maintaining normal energy balance, and deregulation of these communications leads to obesity and type II diabetes (1, 2).

Nuclear receptors (NRs) have been extensively studied as critical regulators of a diverse array of physiological processes in the human body, including the CNS control of energy balance (3). In particular, recent progress has uncovered the roles of NRs in energy balance that involve the arcuate POMC and AgRP neurons. For example, it has been suggested that estrogen exerts its anorexigenic function through AgRP neurons and that, interestingly, this occurs through estrogen receptor α (ERα), expressed in different neurons (4). Consistent with these results, it has also been found that ERα is localized in POMC neurons and binds to the enhancer region that drives the expression of POMC (5). Interestingly, the hypothalamic mTOR pathway has been shown to mediate the T3-induced hyperphagia in hyperthyroidism by triggering the increased expression of AgRP and NPY and the decreased expression of αMSH, likely through the arcuate thyroid hormone receptor α (6). In addition, we have found that the well-defined peripheral orexigenic signal glucocorticoid (Gc) directly upregulates the expression of AgRP in AgRP neurons by triggering the Gc receptor (GR) to functionally bind to a novel Gc response element (GRE) located in the promoter region of *AgRP* that we named the AgRP-GRE (7). Overall, increasing our knowledge of gene regulatory networks of NRs in the CNS control of energy balance remains an interesting challenge. To address this issue, we have focused first on NRs that are expressed in the arcuate POMC and AgRP neurons.

In this report, we present a novel gene network consisting of GR and another NR, neuron-derived orphan receptor 1 (NOR1), in AgRP neurons which directs a regulated expression of orexigenic neuropeptides AgRP and NPY in response to peripheral signals. In this network, GR inhibits NOR1 expression likely via antagonizing the action of cyclic AMP (cAMP) response element-binding protein (CREB), a positive regulator of NOR1 expression, while NOR1 suppresses AgRP/NPY expression. To suppress AgRP expression, NOR1 appears to directly antagonize the positive action of GR on the AgRP-GRE. Consistent with these results, relative to wild-type mice, NOR1-null mice express higher levels of AgRP/NPY and are less responsive to leptin in decreasing the ex-

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pression of AgRP/NPY. Our results identify mutual antagonism between NOR1 and GR to be a key rheostat for peripheral metabolic signals to centrally control energy balance and suggest that defects in this circuitry may at least in part contribute to the development of leptin resistance.

MATERIALS AND METHODS

Animals. All procedures and experiments were carried out with the approval of the Institutional Animal Care and Use Committee of the Oregon Health & Science University. All mice were maintained on a normal 12-h light, 12-h dark cycle with *ad libitum* access to normal chow and water, unless otherwise indicated. NOR1-null mice were gifts of Orla Connelly. Adrenalectomized (ADX) and *Ob/Ob* mice were obtained from Charles River Laboratories (Wilmington, MA) and Jackson Laboratories (Bar Harbor, ME), respectively.

qRT-PCR analysis. Total RNA was extracted from the samples using the TRIzol reagent (Invitrogen) and reverse transcribed by random hexamer primers using a SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. Quantitative reverse transcription-PCR (qRT-PCR) primers for NOR1 were CGC CGA AAC CGA TGT CA (forward) and TGT ACG CAC AAC TTC CTT AAC CA (reverse), those for AgRP were CTT TGG CGG AGG TGC TAG AT (forward) and TGC GAC TAC AGA GGT TCG TG (reverse), and those for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were ACC ACA GTC CAT GCC ATC AC (forward) and TCC ACC ACC CTG TTG CTG TA (reverse). For knockdown experiments, we used a previously described control construct and a construct with small interfering RNA (siRNA) against NOR1 (si-NOR1) (gifts of George Muscat) (8) and lentiviral particles (as a control) and CREB short hairpin RNA from Santa Cruz.

ISH. Mouse brains were fixed by cardiac perfusion of 4% paraformaldehyde while the mice were under deep anesthesia, postfixed overnight in 4% paraformaldehyde, cryoprotected in 30% sucrose, embedded in OCT specimen matrix compound (Tissue-Tek), and frozen in dry ice. The antisense riboprobes were transcribed from AgRP (NM_007427, bases 421 to 659), NPY (NM_023456, bases 1 to 561), and Nur77 (NM_010444, bases 1051 to 1934). The riboprobes for αMSH, Nurr1, and NOR1 were as described previously (9, 10). *In situ* hybridization (ISH) was performed essentially as described previously (11, 12).

Double-fluorescent ISH was performed as described previously (12) with digoxigenin- and fluorescein-labeled riboprobes, detected first with a TSA-Plus fluorescein system (fluorescein; PerkinElmer) and then with a TSA-Plus cyanine 5 system (digoxigenin; PerkinElmer).

ChIP assays. For mouse chromatin immunoprecipitation (ChIP) experiments, the whole hypothalamus was dissected and fixed in 1% formaldehyde solution. We also carried out ChIPs with P19 cells transiently transfected with either vector alone or a NOR1 expression vector. The antibodies used were anti-CREB antibody (Cell Signaling Technology), anti-GR antibody (Santa Cruz Biotechnology, Inc.), our homemade anti-NOR1 antibody, and normal rabbit IgG (Santa Cruz Biotechnology, Inc.). The region between nucleotides −127 and +29 of the mouse NOR1 promoter was amplified using the following primers: ACC CTC GCA CAC GCG GAA C (forward) and TTC GCT CGC TCT CTC GGC AC (reverse). The primers for the AgRP-GRE were as reported previously (7).

Luciferase assays. Transient transfections were performed using the Superfect transfection reagent (Qiagen) according to the manufacturer's protocol. Cells were transfected with a luciferase reporter construct containing the NOR1 promoter (nucleotides −1700 to −1; provided by Dennis Bruemmer) (13) or two copies of the AgRP-GRE (nucleotides −691 to −665) (7) along with a control. An actin β-galactosidase plasmid was cotransfected as a control for transfection efficiency, and empty vectors were used to equalize the total amount of DNA. The luciferase assays were done as described previously (7).

Immunohistochemistry. A series of 14-μm brain tissue sections was prepared with a cryostat and incubated with our homemade antibodies against Bsx (rabbit) and NOR1 (guinea pig) at 4°C overnight. The sections were washed on the next day and incubated for 1 h at room temperature with fluorescein-conjugated secondary antibodies (AlexaFluor 488–anti-rabbit and AlexaFluor 594–anti-guinea pig antibodies).

Statistical analysis. All values are presented as means ± standard errors of the means. Differences between groups were analyzed by two-tailed Student's *t* test or one-way analysis of variance (ANOVA), followed by the Bonferroni *post hoc* test, using Microsoft Excel and Prism 5 software.

RESULTS

NOR1 suppresses the expression of AgRP and NPY in the ARC. To begin studying the gene regulatory networks of NRs in the CNS control of energy balance, we searched for NRs that are expressed in the ARC. Our ISH results revealed that NOR1 is widely expressed throughout the ARC (Fig. 1A). Interestingly, NOR1-positive cells in the ARC also included AgRP neurons, because all NPY/AgRP-positive cells (i.e., AgRP neurons) appeared to express NOR1 in our double-immunofluorescence ISH assays (Fig. 1B and data not shown). These results prompted us to examine whether NOR1 controls the expression of AgRP and NPY in AgRP neurons using ISH. Relative to wild-type mice, NOR1-null mice displayed significantly higher levels of AgRP and NPY under both fed and fasted conditions (Fig. 1C and D; data not shown). In contrast, αMSH levels were similar between wild-type and NOR1-null mice under the fed condition (Fig. 1C and D). These results suggested an unanticipated role for NOR1 in suppressing the expression of AgRP/NPY in AgRP neurons.

On the basis of our results for an increased amount of the orexigenic neuropeptides AgRP/NPY in NOR1-null mice, these animals would be expected to consume more food and spend less energy. However, NOR1-null mice are known to have inner ear defects and partial bidirectional circling behavior (14). We found that this increased activity continues even under fasted conditions (data not shown), at least in part contributing to the insulin sensitivity and glucose tolerance of these animals (data not shown). Nonetheless, we observed that the overall food consumption of NOR1-null mice was higher than that of wild-type mice of both genders (Fig. 2A and E). Moreover, likely with the increased activity, NOR1-null mice lost more body weight than wild-type mice upon fasting (Fig. 2B, C, F, and G) but clearly consumed more food during refeeding (Fig. 2D and H; see the results for 72 h). Of note, likely in relation to the increased activity observed, by the end of the fasting period, NOR1-null mice displayed signs of exhaustion, which explains our initial difficulty in observing differences in food intake between wild-type and NOR1-null mice (Fig. 2D and H; see results for 24 h). Importantly, the higher overall food consumption and the increased food intake of NOR1-null mice during refeeding are consistent with their possession of higher levels of AgRP/NPY.

NOR1 impinges on AgRP-GRE. We have recently found that the fasting-elevated Gc levels trigger GR to bind to the AgRP-GRE, thereby inducing the expression of AgRP (7). Of note, NOR1 is known to antagonize GR transactivation, likely via protein-protein interactions with GR (15). Therefore, we hypothesized that NOR1 directly impinges on GR transactivation of the AgRP promoter. In P19 embryonic carcinoma cells, the levels of AgRP mRNA in the presence of dexamethasone (Dex; a synthetic glucocorticoid) were decreased by ectopic expression of NOR1 (Fig. 3A). In contrast, AgRP expression was increased in the presence of si-NOR1 (8) (Fig. 3B). In addition, the Dex-dependent transactivation of a luciferase reporter driven by two copies of the AgRP-

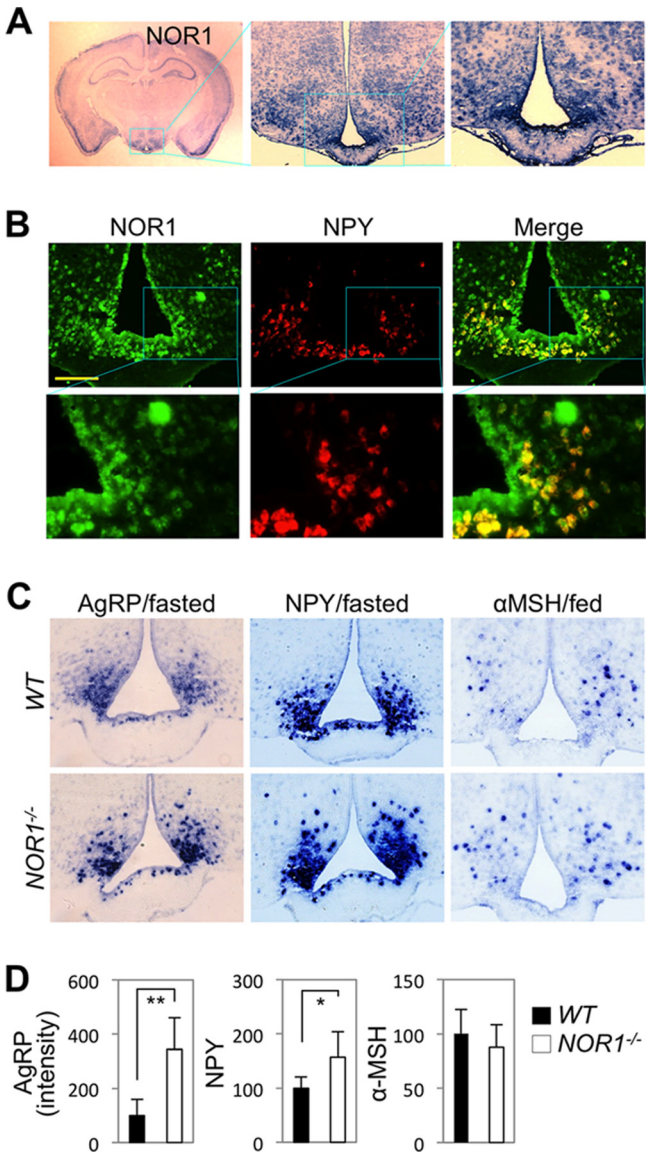

FIG 1 NOR1 as a regulator of AgRP/NPY in AgRP neurons. (A) ISH for NOR1 expression in wild-type male mice under fed conditions. (B) Double-immunofluorescence ISH with the ARC samples of fed wild-type male mice reveals colocalization of NOR1 with NPY. Similar colocalization results were also obtained with AgRP (data not shown). Bar, 100 μm. (C) Representative images of ISH for AgRP, NPY, and αMSH expression in wild-type (WT) and NOR1-null female mice with or without 24 h of fasting (3 mice per each group). (D) Quantification of the results in panel C using ImageJ software. *, $P < 0.05$; **, $P < 0.001$.

GRE was significantly impaired by NOR1 in HEK293 cells (Fig. 3C). Moreover, our ChIP analysis with mouse hypothalamus lysates revealed that the AgRP-GRE is occupied by NOR1 (Fig. 3D). Interestingly, the amount of NOR1 on the AgRP-GRE under fasting conditions was similar to that under fed conditions (Fig. 3D), despite the fact that NOR1 levels were significantly lower under fasting conditions (Fig. 4). One explanation for this observation is the possible recruitment of NOR1 to the AgRP-GRE via GR. Therefore, under fed conditions, in which the level of activated, nuclear GR is minimum due to lower levels of cortisol, only a small fraction of NOR1 was found on the AgRP-GRE (although the overall NOR1 levels were higher). In contrast, under fasting conditions, in which the level of activated, nuclear GR was maximum due to higher levels of cortisol, most NOR1 could be mobilized to the AgRP-GRE. Consistent with this idea, in P19 cells, recruitment of ectopically expressed NOR1 was found on the AgRP-GRE only when the cells were treated with Dex (Fig. 3E). Notably, the level of endogenous NOR1 was quite low in P19 cells (data not shown), and the recruitment of endogenous NOR1 to the AgRP-GRE was not readily observed (Fig. 3E). More importantly, the amount of GR bound to the AgRP-GRE was significantly decreased by ectopic expression of NOR1 (Fig. 3E; compare the results between Dex plus vector and Dex plus NOR1). These results, together with the reported interactions between GR and NOR1 (15), suggest that, in AgRP neurons, NOR1 may suppress the expression of AgRP at least in part by forming a complex with activated, nuclear GR on the AgRP-GRE and somehow weakening the DNA binding activity of GR.

Regulation of NOR1 expression by Gc. Our finding that the expression of AgRP and NPY is suppressed by NOR1 (Fig. 1), coupled with the fact that AgRP/NPY levels are strongly induced during fasting, raised an interesting possibility that NOR1 levels are actively suppressed during fasting. In support of this idea, NOR1 levels were sharply reduced upon fasting in our ISH, qRT-PCR, and immunostaining experiments (Fig. 4A to C). As expected, AgRP expression was induced by fasting, while αMSH levels were suppressed by fasting (Fig. 4A). To test whether the fasting-elevated Gc levels underlie at least in part the reduced expression of NOR1 during fasting, we treated adrenalectomized (ADX) mice (which have no endogenous Gc) with either vehicle or Dex. In ISH and RT-PCR experiments, while the levels of both AgRP/NPY and SGK1, another target of GR (16), were increased by Dex, NOR1 expression was dramatically reduced by Dex (Fig. 4D and E). Notably, our coimmunostaining results revealed that the fasting-induced decrease in NOR1 levels in the ARC clearly involves AgRP neurons, which are marked by Bsx (7) (Fig. 4C; see the decrease in NOR1-Bsx double-positive cells under fasting conditions). Taken together, our results suggested that fasting suppresses the expression of NOR1 in AgRP neurons at least through Gc, whose levels are increased by fasting.

Regulation of NOR1 expression by leptin. Our findings that NOR1 reduces the expression of the orexigenic neuropeptides AgRP/NPY and that NOR1 is negatively regulated by the orexigenic signal Gc led us to investigate whether NOR1 expression is also upregulated by the anorexigenic signals leptin and insulin, thereby contributing to their anorexigenic action to suppress the expression of the orexigenic neuropeptides AgRP/NPY (1, 2). To test this idea, we investigated whether NOR1 expression is induced by leptin using leptin-null *Ob/Ob* mice. Consistent with the idea that leptin is a positive regulator of NOR1 expression, under fed conditions, *Ob/Ob* mice showed higher levels of AgRP, lower levels of αMSH, and a dramatically decreased amount of NOR1 relative to the levels for wild-type mice (Fig. 5A). We also found that, under fasting conditions, leptin reduced the expression of AgRP and induced the expression of αMSH and NOR1 in wild-type mice (Fig. 5B). In further support of our idea, NOR1 levels were greatly induced by leptin in fed *Ob/Ob* mice, while feeding-induced levels of AgRP were much lower in leptin-treated *Ob/Ob* mice than vehicle-treated *Ob/Ob* mice (Fig. 5C).

Our results demonstrate that the expression of NOR1 in the ARC is regulated by both orexigenic (e.g., Gc) and anorexigenic (e.g., lep-

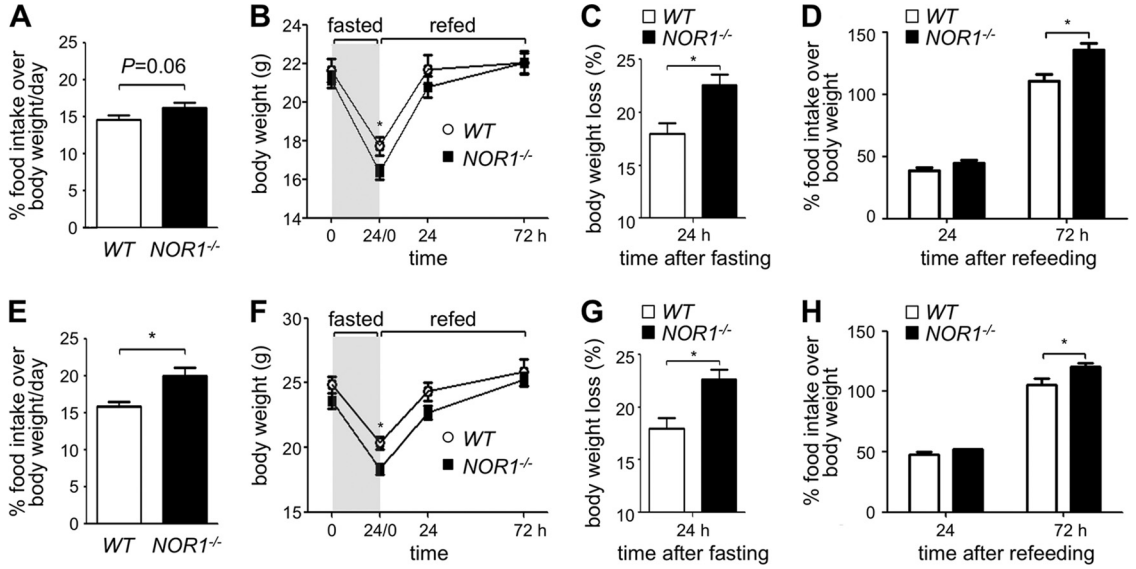

FIG 2 Increased food intake with NOR1-null mice. For male (A to D) and female (E to H) wild-type and NOR1-null mice (5 to 8 mice per each group), we measured daily food intake for 6 days and calculated the percent food intake over body weight per day (A and E), finding that NOR1-null mice consume more food. (B and F) We also measured body weights during fasting (0 and 24 h) and refeeding (24 and 72 h). (C and G) We measured the loss in body weight upon 24 h of fasting relative to the beginning body weight. (D and H) Accumulated food intake (percent) at 24- and 72-h refeeding points. *, $P < 0.05$.

tin) cues and raise an interesting possibility that NOR1 serves as their major effector in regulating the expression of AgRP/NPY.

Interplays of leptin, CREB, and Gc in NOR1 expression. Leptin has been shown to utilize the transcription factor cyclic AMP response element-binding protein (CREB) in enhancing the expression of cyclin D1 (17) and an intestinal epithelial apical membrane transporter, PepT1 (18). In particular, it has been demonstrated that the hypothalamic CREB mRNA level of *Ob/Ob* mice is lower than that of lean control mice and that leptin increases the hypothalamic CREB mRNA level in *Ob/Ob* mice (19). In addition, CREB has been reported to upregulate NOR1 expression via three copies of the cAMP response element (CRE) in the NOR1 promoter (13, 20, 21). Furthermore, mutual cross interference between GR and CREB has been observed (22). Taken together, these results led us to hypothesize that, in the ARC, leptin mobilizes CREB to upregulate the expression of NOR1, while Gc downregulates the expression of NOR1 via antagonizing the action of CREB.

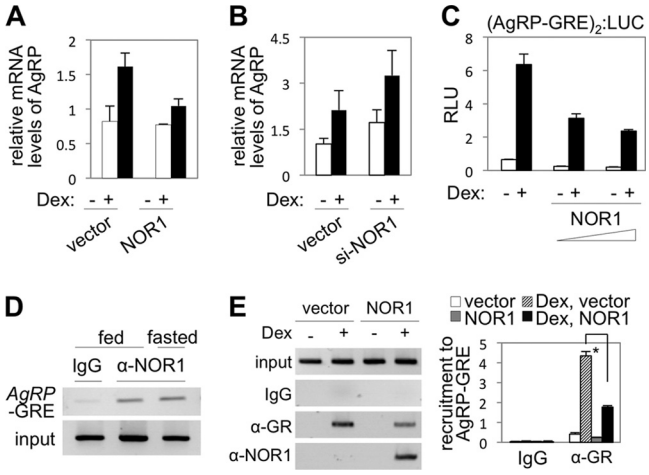

FIG 3 NOR1 interferes with Gc induction of AgRP. (A and B) Quantification of AgRP levels using qRT-PCR in P19 cells treated with either vehicle or 10 nM Dex for 4 h under the indicated conditions. (C) Luciferase (LUC) reporter assays in HEK293 cells for a reporter driven by two copies of the AgRP-GRE in the absence or presence of increasing amounts of the NOR1 expression vector. RLU, relative light units. Representative results from two to four independent experiments are shown (A to C). (D) ChIP with hypothalamus lysates of wild-type mice either fed or fasted overnight. (E) ChIP with P19 cells treated with either vehicle or 50 nM Dex for 4 h under the indicated conditions. P19 cells express endogenous GR but very little NOR1, and thus, we also expressed exogenous NOR1 before carrying out the ChIP experiments. The difference in GR recruitment between cells transiently transfected with either vector alone or the NOR1 expression vector was quantified. *, $P < 0.05$.

In support of our hypothesis, chromatin immunoprecipitation (ChIP) analysis using the ARC lysates of fed *Ob/Ob* mice treated with either vehicle or leptin revealed that recruitment of CREB to the CRE region of the NOR1 promoter is significantly increased in the presence of leptin (Fig. 6A). In wild-type mice, the amount of CREB that bound to the NOR1-CRE region under fed conditions was significantly higher than that under fasted conditions (Fig. 6B), consistent with the higher levels of NOR1 under fed conditions. In contrast, significantly more GR was observed in the NOR1-CRE region under fasted conditions than fed conditions (Fig. 6B), consistent with the inhibitory action of fasting-elevated Gc on NOR1 expression. Interestingly, in fed *Ob/Ob* mice, the transcriptionally active form of CREB, phosphorylated CREB (p-CREB), was significantly increased upon leptin treatment, while the overall level of CREB remained unchanged (Fig. 6C and D). Although no anti-p-CREB antibody suitable for ChIP is available, our results, together with the report that leptin induces a marked increase in p-CREB (23), suggest that leptin may trigger an increased occupancy of p-CREB with the NOR1-CRE region, leading to the leptin-induced expression of NOR1 (Fig. 5B and C), and that Gc-activated GR may at least in part interfere with the binding of p-CREB to the NOR1-CRE region. In further support of this idea, in N42 immortalized hypothalamic neurons (Cellutions Biosystems), CREB was enriched on the NOR1-CRE region

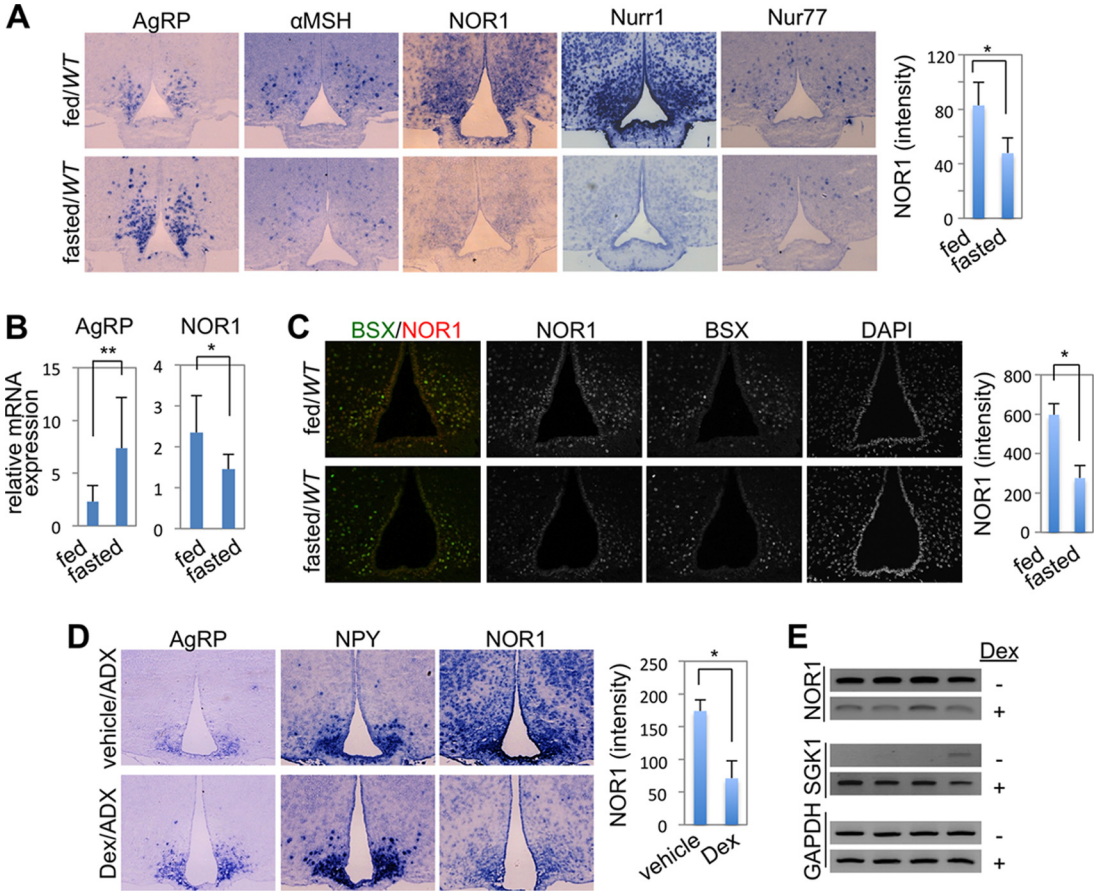

FIG 4 Gc-mediated repression of NOR1 expression. (A) Representative ISH images for AgRP, αMSH, NOR1, Nurr1, and Nur77 expression in wild-type male mice either fed or fasted for 24 h (3 mice per each group) as well as the quantification of the NOR1 results using ImageJ software. (B) Quantification of AgRP/NOR1 levels by qRT-PCR on the hypothalamic samples of wild-type male mice either fed or fasted for 24 h (5 mice per each group). (C) Representative coimmunostaining images of the ARC region of wild-type male mice either fed or fasted for 24 h (3 mice per each group) as well as the quantification of the NOR1 results using ImageJ. (D) Representative ISH images for AgRP, NPY, and NOR1 in fed ADX male mice sacrificed 6 h after intraperitoneal injection of either vehicle or Dex (5 mg/kg of body weight) (3 mice per each group) as well as the quantification of the NOR1 results using ImageJ. (E) RT-PCR analysis of the hypothalamic samples of fed ADX male mice sacrificed 6 h after intraperitoneal injection of either vehicle or Dex (5 mg/kg) (4 mice per each group). *, $P < 0.05$.

upon treatment with forskolin, an activator of protein kinase A and CREB, but not in the presence of both forskolin and Dex (data not shown). Consistent with these results, in N42 cells, NOR1 mRNA levels were induced by leptin, and this induction was abolished by Dex (Fig. 6E, left). Moreover, leptin induction of NOR1 expression in N42 cells was impaired by siRNA against CREB (si-CREB; Santa Cruz) (Fig. 6E, right), directly implicating CREB as a downstream effector of leptin in inducing NOR1 expression. Finally, in HEK293 cells, while forskolin strongly activated the luciferase activity of a reporter construct whose expression is driven by a 1.7-kb NOR1 promoter fragment containing the three copies of CREs, this activation was abolished by Dex (Fig. 6F).

Overall, our results suggest a gene regulatory network consisting of NOR1, leptin, CREB, and Gc/GR in energy balance. In this circuitry, leptin-elevated levels of NOR1 lead to downregulation of AgRP/NPY expression, while the orexigenic cue Gc reduces NOR1 levels via antagonizing the action of CREB, a positive regulator of NOR1 expression. The latter likely represents a critical component of the multifaceted actions of fasting in increasing the expression of AgRP/NPY in AgRP neurons (1, 2).

Leptin resistance of NOR1-null mice. Our findings led us to predict that NOR1-null mice would show relative resistance to the anorexigenic cue, leptin, in downregulating the AgRP/NPY expression. To test this idea, we examined the effect of intraperitoneally injected leptin on AgRP/NPY levels in the ARC. In strong support of our idea, leptin injection significantly suppressed AgRP/NPY expression in wild-type mice, while it showed no significant effect on AgRP/NPY expression in NOR1-null mice (Fig. 7A and B). Consistent with these results, leptin treatment led to a significant decrease in percent body weight changes in wild-type male and female mice but not in NOR1-null male and female mice (Fig. 7C and D). Leptin treatment also led to a significant decrease in percent food intake per body weight in wild-type male mice but not in NOR1-null male mice (Fig. 7C). These results lead us to conclude that NOR1 plays a critical role for the central action of leptin to suppress the expression of AgRP and NPY.

DISCUSSION

NRs regulate a diverse array of physiological processes in the human body, including the CNS control of energy balance (3). However, the detailed molecular basis for their roles in POMC and AgRP neurons, the first two groups of neurons that run into pe-

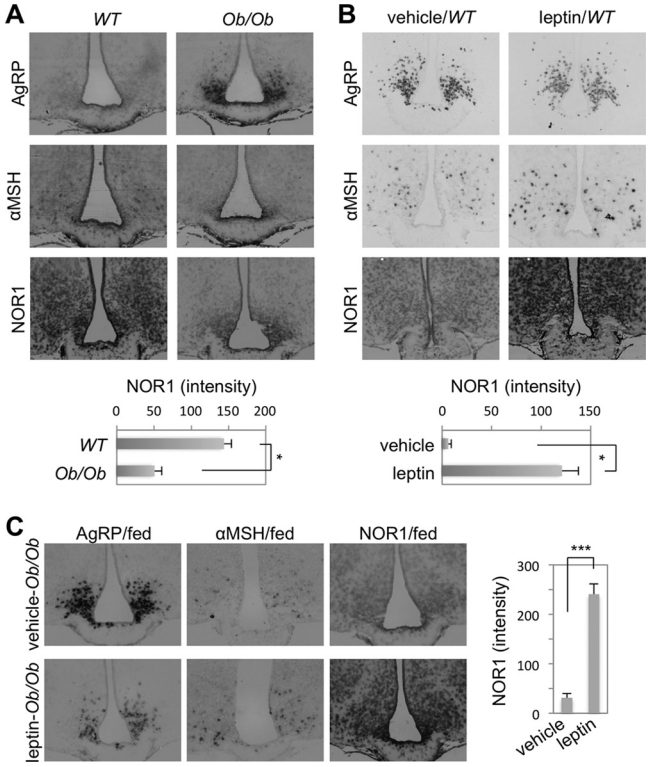

FIG 5 Regulation of NOR1 expression by leptin. (A) Representative ISH images for AgRP, αMSH, and NOR1 expression in fed wild-type and *Ob/Ob* male mice (2 mice per each group), as well as quantification of the NOR1 results using ImageJ software. (B) Representative ISH images for AgRP, αMSH, and NOR1 expression in wild-type male mice fasted for 24 h, followed by intraperitoneal injection of either vehicle or leptin (3 mg/kg) and perfusion 3 h after leptin injection (2 mice per each group). The NOR1 results were quantified using ImageJ. (C) Representative ISH images for AgRP, αMSH, and NOR1 expression in fed *Ob/Ob* male mice sacrificed 3 h after intraperitoneal injection of either vehicle or leptin (3 mg/kg) (2 mice per each group) as well as the quantification of the NOR1 results using ImageJ. *, $P < 0.05$; ***, $P < 0.0001$.

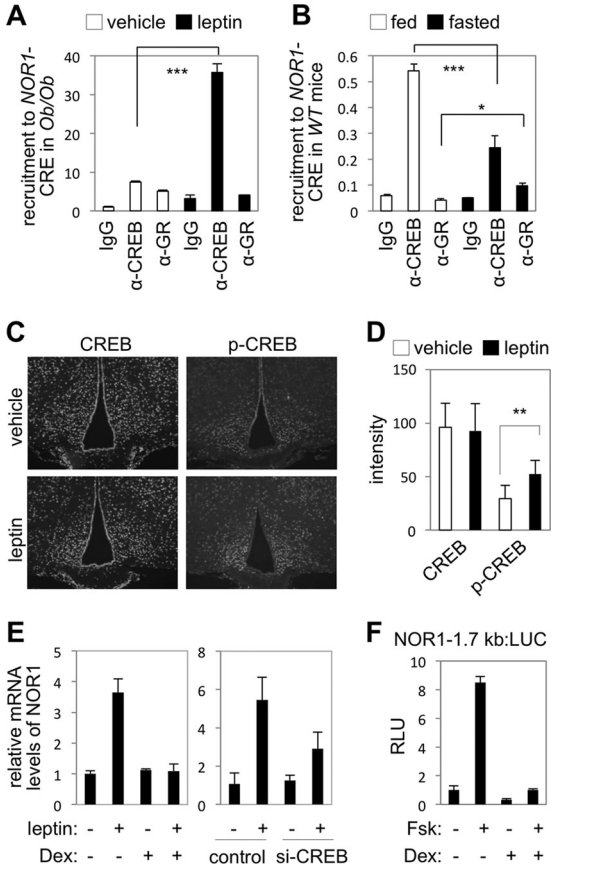

FIG 6 Involvement of CREB in the regulation of NOR1 expression by leptin. (A, B) ChIP and qRT-PCR to assess the levels of CREB and GR recruited to the NOR1-CRE region in fed *Ob/Ob* male mice 2 h after intraperitoneal injection of either vehicle or leptin (3 mg/kg) (A) and in wild-type male mice either fed or fasted for 24 h (B). Six mice were used per each treatment group, and their hypothalamus samples were pooled and subjected to ChIP with IgG, α-CREB, and α-GR. Significance was determined by one-way ANOVA, followed by the Bonferroni *post hoc* test. (C) Immunofluorescence microscopy was carried out on the ARC samples prepared from fed Ob/Ob male mice 2 h after intraperitoneal injection of either vehicle or leptin (3 mg/kg). (D) Quantification of the signal intensity for the results in panel C using ImageJ software. (E) Quantification by qRT-PCR of NOR1 levels in N42 cells treated with vehicle, leptin (60 nM), and/or Dex (10 nM) for 1 h (left) and in N42 cells treated with leptin (60 nM) for 1 h with the lentiviral particles for control siRNA or si-CREB from Santa Cruz (right). (F) Luciferase reporter assays in HEK293 cells for a reporter driven by a 1.7-kb NOR1 promoter fragment in the absence or presence of vehicle, forskolin (Fsk; 10 μM), and/or Dex (10 nM). *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

ripheral metabolic signals in the CNS, has remained relatively poorly understood. Our results in this report uncover a novel metabolic regulatory network in AgRP neurons which is built around a mutually antagonistic relationship between two NRs, NOR1 and GR (Fig. 8). In this circuitry, NOR1, whose expression is induced by leptin-activated CREB, inhibits the positive action of Gc on the expression of the orexigenic neuropeptides AgRP/NPY, while Gc-activated GR antagonizes the action of CREB on NOR1 expression. The resulting decrease in NOR1 levels leads to higher levels of AgRP/NPY because of less interference by NOR1 with the Gc action to induce AgRP/NPY expression. The consequent changes in AgRP/NPY levels in turn transduce the anorexigenic and orexigenic actions of leptin and Gc into alterations in energy expenditure and food intake (Fig. 8). Of note, NOR1 is widely expressed in the ARC; and this study is focused on AgRP neurons, only a small population of NOR1-positive cells in the ARC (Fig. 1B). Therefore, future studies should be directed at elucidating the metabolic roles of NOR1 in other types of neurons of the ARC.

Gc has been well characterized to mediate the fasting-dependent induction of AgRP/NPY expression (24–27). We have recently shown that GR in AgRP neurons directly mediates the positive action of Gc on AgRP expression by binding to a novel GRE in the AgRP promoter that we named the AgRP-GRE (7). Interestingly, inactivation of AgRP neuronal GR did not appear to affect the fasting-dependent induction of NPY expression (7), suggesting that Gc regulates NPY expression via a non-GR-dependent mechanism or that fasting activates additional, redundant pathways that can independently upregulate the expression of NPY. Importantly, although the molecular basis for how NOR1 suppresses NPY expression remains unclear, our findings presented in this report show that NOR1 inhibits AgRP expression at least in part by interfering with the DNA/AgRP-GRE-binding activity of GR (Fig. 3E).

Expression of NOR1 as well as its closest homologues, Nurr1 and Nur77, is similarly regulated by a wide array of signaling cues

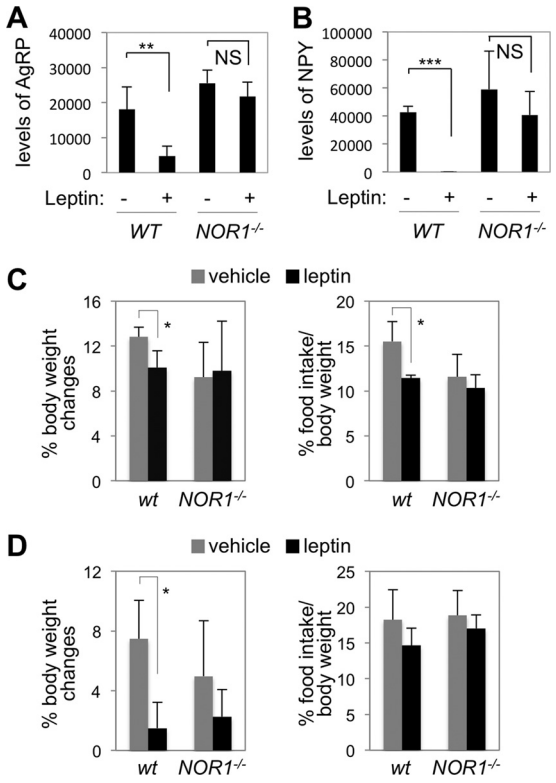

FIG 7 Leptin resistance of NOR1-null mice. (A, B) The ISH signal intensity for AgRP and NPY in wild-type and NOR1-null female mice sacrificed 3 h after intraperitoneal injection of leptin (3 mg/kg) following 24 h of fasting was quantified using ImageJ software. (C) Acute effects of leptin on fasting-induced changes in food intake and body weight were tested in wild-type and NOR1-null male mice (3 to 4 mice per each group). Prior to intraperitoneal injection of either vehicle or leptin (3 mg/kg), mice were fasted for 12 h, and percent body weight changes and percent food intake per body weight were measured 6 h after leptin injection. (D) Nonfasted wild-type and NOR1-null female mice (3 to 4 mice per each group) were injected intraperitoneally with either vehicle or leptin (3 mg/kg). Percent body weight changes and percent food intake per body weight were measured 15 h after leptin injection. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$; NS, not significant.

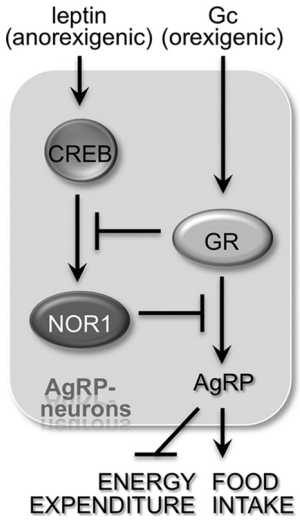

FIG 8 Working model (see the text).

(28), which raises two interesting possibilities. First, Nurr1 and Nur77 may also play similar roles in AgRP neurons. Indeed, we found that both Nurr1 and Nur77 are expressed in the ARC and the expression pattern of Nurr1 in the ARC is particularly similar to that of NOR1 (Fig. 4A). The leptin/CREB-mediated upregulation feature may also be conserved with Nurr1/Nur77 expression, as CREB is capable of directly upregulating Nurr1 and Nur77 expression (29–31). Consistent with an idea that the ability of CREB to induce Nurr1/Nur77 expression may also be inhibited by GR, the ARC expression of Nurr1 and Nur77 was strongly repressed by fasting (Fig. 4A). Like NOR1, Nurr1 and Nur77 are expected to repress GR transactivation, resulting in suppression of AgRP expression, because Nurr1 and Nur77 are also known to antagonize GR transactivation (15). Second, NOR1, Nurr1, and Nur77 may function as effectors not only for leptin but also for other anorexigenic cues in energy balance. The anorexigenic cue insulin is an obvious candidate, as insulin is also known to stimulate both phosphorylation and transcriptional activation of CREB (32). In this regard, it is interesting to note that Nur77 has been identified as a positive target gene of insulin in skeletal muscle (33). Other anorexigenic cues may also utilize NOR1/Nurr1/Nur77 as an effector of their action to curb the expression of the orexigenic neuropeptides AgRP/NPY.

Leptin activates multiple signal transduction pathways (34), and thus, it is possible that transcription factors downstream of these pathways also mediate the action of leptin to upregulate NOR1 expression, although our results strongly implicate CREB as a critical factor in this process (Fig. 6). Interestingly, leptin has been shown to stimulate nuclear factor κB (NF-κB) and hypoxia-inducible factor 1α (HIF1α) (35, 36), two transcription factors that have also been shown to be capable of upregulating NOR1 expression (37, 38). In the future, it will be interesting to determine whether leptin also utilizes NF-κB and HIF1α to upregulate NOR1 expression in AgRP neurons. In particular, the putative leptin–NF-κB–NOR1–AgRP/NPY axis may also underlie at least in part the recently reported hypothalamic link of overnutrition and chronic inflammation to energy imbalance (39).

Overall, our newly defined regulatory network is believed to play critical roles for both orexigenic (e.g., Gc) and anorexigenic (e.g., leptin) cues to exert their action in energy balance through regulating the expression of orexigenic peptides AgRP and NPY (Fig. 8). This prediction is strongly supported by our results that NOR1-null mice showed higher levels of AgRP and NPY expression (Fig. 1C and D), a significant decrease in the ability of leptin to lower AgRP/NPY levels (Fig. 7A and B), increased refeeding behavior upon fasting (Fig. 2), and relative resistance to leptin in body weight loss (Fig. 7C and D). Although our results identify NOR1 as a critical player in energy balance, it is important to note that Nurr1 and Nur77 may also function redundantly as pivotal players in the network, ensuring the intact function of this network even amid a problem in one pathway. This will be an interesting subject to address in future studies.

In conclusion, we present a novel gene network consisting of two NRs, GR and NOR1, in AgRP neurons which directs a regulated expression of orexigenic neuropeptides AgRP and NPY in response to peripheral signals. In this network, mutual antagonism between NOR1 and GR serves as a key rheostat for peripheral metabolic signals to centrally control energy balance. It will be

interesting to further examine whether defects in this circuitry may at least in part contribute to development of leptin resistance.

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