Gene regulation of the hypothalamic arcuate nucleus during development and postnatal energy balance function

By

Bora Lee

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CERTIFICATE OF APPROVAL

This is to certify that the PhD dissertation of

Bora Lee

has been approved

Advisor, Jae W Lee, PhD

Member and Chair, Anthony P Barnes, PhD

Member, Stephen A Back, MD, PhD

Member, Kevin Wright, PhD

Member, Magdalena Petryniak, MD

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List of Abbreviations

αMSH	alpha melanocyte stimulating hormone
ACTH	Adrenocorticotropin
ADX	Adrenalectomized
AgRP	agouti-related peptide
AMPK	AMP-activated protein kinase
AP 1	activator protein 1
ARC	arcuate nucleus
bHLH	basic helix-loop-helix
BSX	brain specific homeobox factor
CART	cocaine- and amphetamine-regulated transcript
ChIP	chromatin immunoprecipitation
CNS	central nervous system
CRE	cyclic AMP response element
CREB	cyclic AMP response element-binding protein
CRH	corticotropin-releasing hormone
db/db	leptin receptor deficiency
Dex	Dexamethasone
DMH	dorsomedial nucleus
E	embryonic day
ERα	estrogen receptor alpha
GABA	γ-aminobutyric acid
GA D	glutamic acid decarboxylase

Gc	Glucocorticoid
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHRH-R	growth hormone-releasing hormone receptor
GHSR	growth hormone secretagogue receptor
GR	glucocorticoid receptor
GR^{dim}	dimerization defective mutant glucocorticoid receptor
GRE	glucocorticoid response element
GnRH	gonadotropin releasing hormone
HIF1a	hypoxiainducible factor 1 alpha
HPA	hypothalamic-pituitary-adrenal
HSPs	heat shock proteins
ICV	Intracerebroventricular
IGF-1	insulin like growth factor 1
InsR	insulin receptor
IRS	insulin receptor substrate
ISH	in situ hybridization
Isl1	Islet 1
JAK	Janus kinase
LepR	leptin receptor
LHA	lateral hypothalamus
LIM-HD	LIM-homeodomain
MCR	melanocortin receptors

MC3R	melanocortin-3 receptor
MC4R	melanocortin-4 receptor
MMTV	mouse mammary tumor virus
MSHs	melanocyte-stimulating hormones
MR	melanocorticoid receptor
mTOR	mammalian target of rapamycin
NF-kB	nuclear factor kappa B
Ngn	Neurogenins
NOR1	neuron-derived orphan receptor 1
NPY	neuropeptide Y
NRs	nuclear hormone receptors
NTS	nucleus tractus solitarius of the brainstem
ob/ob	leptin deficiency
OTP	Orthopeida
P	postnatal day
PBN	parabrachial nucleus
PeV	periventricular nucleus
PI3K	phosphatidylinositol 3-kinases
POMC	pro-opiomelanocortin
PR	progesterone receptor
PVN	paraventricular nucleus
SHH	sonic hedgehog
SST	Somatostatin

TH	tyrosine hydroxylase
VMH	ventromedial hypothalamus
WAT	white adipose tissue

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ABSTRACT

We have witnessed a dramatic expansion of metabolic pathologies including obesity, diabetes, and cardiovascular diseases during the last several decades. The fundamental cause of obesity and many metabolic disorders is an energy imbalance between caloric input and energy output. The hypothalamic arcuate nucleus harbors several types of neurons that are crucial for controlling energy balance by sensing and translating various peripheral cues into the central nervous system action. Among these neurons, agouti-related peptide (AgRP) neurons and proopiomelanocortin (POMC) neurons regulate energy balance by producing orexigenic neuropeptides, AgRP and neuropeptide Y (NPY), and anorexigenic neuropeptide αMSH, respectively. Despite great advances in characterizing the physiological roles of these neurons, the molecular basis underlying how these neurons develop and function at later postnatal stages is still poorly understood. Here we present our discoveries that reveal two key molecular mechanisms for how the expression of the orexigenic neuropeptides, AgRP and NPY, are controlled, and also identify a critical transcription factor (Isl1) that regulates embryonic development of various arcuate neuronal cell types.

First, I present a novel glucocorticoid response element (GRE) in the AgRP promoter region (AgRP-GRE). The unique sequence of this GRE enables glucocorticoid receptor (GR) and brain-specific homeobox factor (Bsx) to synergize for the direct transactivation of the gene encoding the orexigenic

neuropeptide AgRP. During fasting, both blood glucocorticoid level and Bsx expression in AgRP neurons are increased. In turn, ligand bound GR is translocated to the nucleus where it interacts with Bsx and binds to AgRP-GRE to activate AgRP expression. In contrast to AgRP-GRE, Bsx suppresses transactivation mediated by many conventional GREs and functions as a gene context dependent modulator of GR actions. These results provide an opportunity to identify novel common targets of GR and Bsx and to better understand the molecular basis underlying the orexigenic activity of glucocorticoid and Bsx.

Second, I present a new gene regulatory network involving GR and another nuclear hormone receptor, neuron-derived orphan receptor-1 (NOR1), which regulates the expression of AgRP and NPY in response to peripheral signals. The anorexigenic signal leptin induces NOR1 expression via the transcription factor cAMP response element binding protein (CREB) while the glucocorticoid bound GR inhibits NOR1 expression by antagonizing the action of CREB on NOR1 expression. Conversely, NOR1 suppresses glucocorticoid-dependent activation of AgRP. It appears that mutual antagonism between NOR1 and GR is critical for detecting peripheral metabolic signals and for modulating gene expression in the arcuate nucleus to centrally regulate energy balance.

Lastly, I show that the LIM-homeodomain transcription factor Islet1 (Isl1) plays a critical role in fate specification of several developing arcuate neurons. The loss of Isl1 in the developing hypothalamus results in striking deficits in both

feeding and linear growth. In line with these phenotypes, expression of the orexigenic neuropeptides AgRP, NPY, growth hormone-releasing hormone (GHRH) and somatostatin (SST), which represent the key identity markers of feeding and growth controlling arcuate neurons, is completely abolished by the loss of Isl1. Finally, Isl1 directly regulates AgRP expression by cooperating with GR and Bsx.

Collectively, this dissertation identifies several critical gene regulatory mechanisms in hypothalamic arcuate neurons during development and postnatal energy balance function.

Chapter 1

1. INTRODUCTION

The hypothalamus is a critical integrator of many homeostatic processes that regulate basic physiological functions such as food and water intake, energy expenditure, stress response, reproduction, and circadian rhythm. The hypothalamus is composed of a diverse collection of cell groups called nuclei. The arcuate nucleus (ARC) surrounds the ventral part of the third ventricle and resides right above the median eminence, a circumventricular organ where the blood-brain barrier is incomplete and blood-borne signals can easily reach the ARC neurons. The ARC contains many types of neurons that are essential for feeding, reproduction, and growth. Two of these neuron subtypes, the agouti-related peptide (AgRP) neurons and pro-opiomelanocortin (POMC) neurons play a major role in feeding behavior, energy balance, autonomic nervous system control, and substrate metabolism (Remmers and Delemarre-van de Waal 2011). Growth hormone-releasing hormone (GHRH) neurons stimulate growth hormone (GH) release from the anterior pituitary gland to control linear growth (Balthasar et al. 2003). Also, somatostatin (SST) expressing neurons are suggested to control linear growth by regulating GHRH neurons (Muller et al. 1999). Another subtype of ARC neurons expresses tyrosine hydroxylase (TH), a key enzyme in dopamine synthesis. These neurons release dopamine to inhibit prolactin secretion from the anterior pituitary gland (Phelps et al. 2003). Lastly, kisspeptin expressing neurons in the ARC regulate gonadotropin-releasing hormone (GnRH) secretion (Dungan et al. 2006). Among these neurons, my work is focused on

four types of neurons, AgRP, POMC, GHRH, and SST neurons.

1.1. Feeding control.

The circulating hunger or satiety signals arriving at the CNS are first perceived by two populations of ARC neurons, AgRP neurons and POMC neurons. These anatomically distinct populations of ARC neurons project to overlapping areas of hypothalamus including the paraventricluar (PVN), ventromedial (VMH), dorsomedial (DMH), and lateral hypothalamus (LH). They also project to other non-hypothalamic area such as the nucleus of tractus solitarius (NTS), the parabrachial nucleus (PBN) and the amygdala. These two population of neurons exert opposing functions: AgRP neurons are orexigenic—the release of AgRP increase food intake, whereas POMC neurons are anorexigenic—the activation of POMC neurons decreases food intake and body weight.

In a situation of negative energy balance, such as fasting, the expression of AgRP is increased, and the expression of αMSH (produced from POMC neurons) is decreased. However, when the energy source is abundant, AgRP expression is reduced, and αMSH level is elevated (Cone 2005; Elmquist et al. 2005).

1.1.1 POMC neuron

POMC is synthesized in the anterior pituitary gland, the ARC, brain stem, and several peripheral tissues. POMC is a precursor protein that produces many biologically active peptides via a series of enzymatic steps in a tissue-specific

manner. The melanocyte-stimulating hormones (MSHs), adrenocorticotrophic hormone (ACTH), and β-endorphin are derived from POMC. POMC neurons in the ARC produce POMC and cocaine- and amphetamine-related transcript (CART) and secrete ACTH and α, β, and γ-MSH. The MSHs and ACTH, collectively known as melanocortins bind to the extracellular G-protein coupled melanocortin receptors (MCRs). Among five types of MCRs, MC3R and MC4R are expressed most widely in the central nervous system (CNS) and are believed to mediate the anorexigenic effect of melanocortins (Cone 2005). The release of α-MSH by POMC neurons and its binding to MCRs has been particularly well characterized and leads to decreased food intake and increased energy expenditure while AgRP blocks the binding of α-MSH to its receptor to inhibit the melanocortin-induced anorexic pathway (Tolle and Low 2008). One of the major phenotype of mouse knockouts for POMC, MC4R and MC3R, and humans expressing mutations of POMC and MC4R is obesity associated with increased food intake (Huszar et al. 1997). Also, defects in enzymes that cleave POMC precursors lead to obesity in both human and mouse (Coll et al. 2004) indicating the importance of POMC in regulating energy balance.

1.1.2 AgRP neurons

AgRP was first discovered as an endogenous inverse agonist for the melanocortin receptors, MC3R/MC4R (Fan et al. 1997; Tolle and Low 2008). Shortly after its discovery it was found that AgRP is co-expressed with neuropeptide Y (NPY), another peptide that stimulates food intake and increases

body weight in the hypothalamic ARC (Tatemoto et al. 1982). The neurons that co-express AgRP and NPY are now referred to as AgRP neurons, because they are the only neurons expressing AgRP in the CNS whereas NPY is widely expressed in the nervous system.

There are several lines of evidence showing that AgRP neurons are critical regulators of feeding and food seeking behavior. AgRP neurons inhibit the action of POMC neurons through tonic γ-aminobutyric acid (GABA)-ergic inhibition and by releasing NPY. NPY inhibits the activity of POMC neurons through interactions with the NPY-Y1 receptors expressed on POMC neurons . Acute ablation of AgRP neurons in adult mouse causes a dramatic decrease in food intake and leads to life-threatening anorexia (Luquet et al. 2005; Xu et al. 2005). Intact AgRP signaling in adult mice is also essential for maintaining normal lipid and glucose homeostasis in peripheral tissues, such as the liver, muscles and the pancreas (Joly-Amado et al. 2012; Varela and Horvath 2012). It is also noteworthy that acute photoactivation of AgRP neurons, in adult mice, promotes feeding.

Unlike adult mice, ablation of AgRP neurons in neonatal mice does not lead to starvation and mice continue to eat adequately (Luquet et al. 2005). This experiment shows that during development, arcuate nucleus can adapt to the loss of AgRP neurons in order to achieve metabolic homeostasis.

Interestingly, co-stimulation of both POMC and AgRP neurons leads to a rapid feeding response indicating that the inhibition of POMC neuron is neither necessary nor sufficient to trigger feeding behavior (Atasoy et al. 2012).

1.1.3. Signaling pathways that modulate AgRP and POMC neuron function

1.1.3.1. Leptin

Leptin is an anorexigenic adipokine, which is secreted by white adipose tissue and is positively correlated with the total amount of body fat. Central administration of leptin inhibits food intake and decreases body weight (Woods and Seeley 2000). Leptin receptor (LepR) is expressed within the hypothalamus including the ventromedial (VMH), dorsomedial (DMH), and arcuate nucleus (ARC). Among arcuate neurons, LepR is densely expressed in both AgRP and POMC neuron (Cowley et al. 2001).

Mice with leptin deficiency (ob/ob) or deficits in its receptor LepR (db/db) show increased body weight, increased food intake, and impaired glucose homeostasis. These phenotypes indicate a crucial role of leptin signaling in feeding and energy expenditure regulation (Zhang et al. 1994; Pelleymounter et al. 1995; Chen et al. 1996). Intracerebroventricular leptin injections in these mice result in reduced body weight and food intake and also ameliorate glucose metabolism suggesting that the central nervous system (CNS) is a major target of anorexigenic leptin action (Brown et al. 2006).

Leptin activates various signaling cascades. When leptin binds to its receptor (LepR), LepR recruits and activates Janus kinase (JAK) (Vaisse et al. 1996). In turn, activated JAK phosphorylates and activates LepR and a transcription factor Stat3. Phosphorylated Stat3 binds to POMC and AgRP

promoters, which results in increased expression of POMC and reduced expression of AgRP (Mesaros et al. 2008; Ernst et al. 2009). Leptin also activates phosphatidylinositol 3-kinases (PI3K) signaling cascades. This signaling pathway is critical for the regulation and activation of many proteins and transcription factors including forkhead protein O1 (FoxO1), 5' AMP-activated protein kinase (AMPK), and mammalian target of rapamycin (mTOR) (Minokoshi et al. 2004; Cota et al. 2006; Kim et al. 2006).

1.1.3.2. Insulin

Insulin is a hormone, which is made and secreted by pancreatic beta-cells to regulate blood glucose. An elevated level of blood glucose stimulates pancreatic beta-cells to secrete insulin. This increase in circulating insulin promotes glucose uptake into peripheral tissues, which is essential to maintain glucose homeostasis. Insulin functions as a strong anorexigenic factor as intracerebroventricular administration of insulin inhibits food intake and reduces body weight (Brown et al. 2006). Insulin receptors (InsR) are expressed in several areas of the CNS including AgRP and POMC neurons in the hypothalamic ARC (Konner et al. 2007). After insulin binds to InsR and phosphorylates the insulin receptor substrate (IRS), PI3K is activated to modulate body weight and glucose homeostasis. Activation of PI3K leads to phosphorylation and inactivation of a transcription factor FoxO1 through PDK1 and AKT activation. Phosphorylation of FoxO1 leads to its nuclear export and allows STAT3 to bind to POMC promoters to promote POMC transcription.

Conversely, FoxO1 phosphorylation inhibits AgRP expression (Kim et al. 2006; Ren et al. 2012).

1.1.3.3. Ghrelin

Ghrelin is an orexigenic hormone that promotes positive energy balance. In both humans and rodents, circulating ghrelin levels are decreased in the states of positive energy balance such as obesity and calorie intake, whereas plasma levels of ghrelin are increased by fasting (Cummings et al. 2001; Tschop et al. 2001).

Ghrelin was first identified as an endogenous ligand for the growth hormone secretagogue receptor (GHSR). It is synthesized and secreted predominantly by endocrine cells in the stomach (Kojima et al. 1999). Initially, ghrelin was known as a potent GH-releasing agent. Soon, however, it became evident that ghrelin has a big impact on food intake and metabolism. There are multiple reports demonstrating that peripheral or intracrebroventricular administration of ghrelin stimulates appetite, increases body weight, and decreases energy expenditure in rodents (Tschop et al. 2000; Wren et al. 2000; Nakazato et al. 2001). GHSR, a receptor for ghrelin, is expressed in several hypothalamic nuclei, including the ARC and the P
VN and ghrelin binds directly to GHSR in these regions (Cowley et al. 2001). GHSR is predominantly expressed in AgRP neurons, the orexigenic cell population in the ARC, but in only few POMC neurons (Nogueiras et al. 2008). Ghrelin increased both electrical activity and c-fos immunoreactivity, an index of

increased transcriptional activation, in AgRP neurons (Cowley et al. 2003; Andrews et al. 2008). Ghrelin does not stimulate feeding in AgRP/NPY double knockout mice or mice with AgRP neuronal ablation. These findings indicate that ghrelin signaling in AgRP neurons is required for controlling feeding behavior (Chen et al. 2004; Bewick et al. 2005).

1.1.3.4. Glucocorticoid

Glucocorticoid, cortisol in humans and corticosterone in rodents, is synthesized and secreted from the adrenal cortex under the control of the hypothalamic-pituitary-adrenal (HPA) axis. Corticotropin-releasing hormone (CRH) is produced by hypothalamic paraventricular neurons in response to a number of psychological and physiologic stressors, including hunger. CRH triggers the release of ACTH from the anterior pituitary gland. Circulating ACTH binds to its receptors in the adrenal cortex where it stimulates glucocorticoid synthesis and secretion into the systemic circulation. Glucocorticoid regulates the stress response and inhibits further HPA axis activation through intracellular receptors that are widely distributed throughout the brain and peripheral tissues (Chrousos and Gold 1992).

There are two types of receptors that bind to glucocorticoid, the mineralocorticoid receptors (MR) and the glucocorticoid receptors (GR). The physiological effects of glucocorticoid are mainly mediated by GR, a member of the nuclear hormone receptor superfamily. In its inactive state, GR resides primarily in the cytoplasm in association with a multi-protein complex including

several heat shock proteins (HSPs). Glucocorticoid binding induces a conformational change in the GR, resulting in the dissociation of the receptor from the heat shock protein complex and the translocation into the nucleus.

GR binds as a homodimer to a specific DNA motif termed glucocorticoid response element (GRE) in the regulatory region of its target genes to modulate their expression (Stahn et al. 2007). GR can also regulate gene expression independent of GRE through direct protein-protein interactions with other transcription factors. For instance, GR is tethered to the binding sites for pro-inflammatory transcription factor activator protein 1 (AP1) and nuclear factor B (NFκB) and represses their transactivation through protein-protein interaction (Adcock and Caramori 2001; Stahn et al. 2007).

Glucocorticoid is a well-known peripheral orexigenic signal, and its blood level is increased by fasting (Makimura et al. 2003; Fehm et al. 2004; Coppola and Diano 2007). Central infusion of dexamethasone (a synthetic GR agonist) stimulates food intake and body weight gain, and peripheral administration of dexamethasone or corticosterone increases anorexigenic neuropeptide NPY expression in the ARC (Akabayashi et al. 1994; Zakrzewska et al. 1999). Studies in adrenalectomized rodents have shown that glucocorticoid is important for fasting-dependent induction of NPY/AgRP expression (Makimura et al. 2003; Savontaus et al. 2002; Zakrzewska et al. 1999).

1.1.4. Neuron-derived orphan receptor 1

Nuclear receptors (NRs) are transcription factors that act as sensors of

hormones and nutrients, and also control development, metabolism, proliferation, apoptosis, circadian rhythms, and behavioral responses (Mangelsdorf et al. 1995). There are several NRs that have important roles in CNS control of energy balance. For example, estrogen receptor-alpha (ERα) expressed in POMC neurons exerts its anorexigenic function by regulating AgRP and POMC expression (Olofsson et al. 2009; De Souza et al. 2011).

Neuron-derived orphan receptor 1 (NOR1) is a member of NR4A subfamily that contains Nur77 and Nurr1 as well as NOR1. The expression of receptors in this family is rapidly and transiently induced by various stimuli including inflammatory mediators and growth factors (Li et al. 2006; Pei et al. 2006). They are also “true orphan” nuclear receptors that do not require ligand binding to regulate transcription. There is a report showing that all three members of NR4A subfamily, Nurr1, Nur77, and NOR1, bind to the Nur response element (NurRE) on POMC promoter to enhance POMC gene expression in response to CRH in the pituitary gland (Martens et al. 2005). GR antagonizes this NurRE-dependent transcription through the protein-protein interaction between Nur factors and GR (Martens et al. 2005).

1.1.5. Brain-specific homeobox factor (Bsx) in regulation of feeding behavior

Bsx is an evolutionarily conserved brain- specific homeobox transcription factor. It binds to the consensus homeodomain binding sequence ATTA and activates the transcription of its target genes (Chu and Ohtoshi 2007).

Bsx is widely expressed in the developing and postnatal hypothalamus

including the ARC, DMH, and LH (Chu and Ohtoshi 2007; Sakkou et al. 2007). Among ARC neurons, virtually all AgRP neurons express Bsx, but Bsx was not detected in POMC neurons. Bsx expression is up-regulated by fasting and ghrelin and down-regulated by leptin, suggesting that it has orexigenic roles (Sakkou et al. 2007; Nogueiras et al. 2008).

Bsx knockout mice revealed that Bsx is required for normal AgRP/NPY expression, locomotor activity, and food seeking behavior in response to food deprivation. Bsx was also shown to associate with other transcription factors such as CREB and FoxO1 to stimulate NPY and AgRP expression in response to fasting (Sakkou et al. 2007).

1.2. Hypothalamic regulation of growth

One of the major regulators of linear growth during development is growth hormone (GH) secreted from somatotrophs in the anterior pituitary gland. Activation of GH signaling stimulates the hepatic production of insulin-like growth factor-1 (IGF-1), which in turn controls the development of bone epiphyses and growth plates. Circulating IGF-1 is also considered the major factor that mediates the stimulatory effects of GH on linear growth (Cohen and Rosenfeld 1994).

Regulation of GH synthesis is under the control of two hypothalamic hormones: GHRH and somatostatin (SST). GHRH expressed in the arcuate nucleus stimulates GH synthesis, and somatostatin (SST) synthesized in the periventricular nucleus (PeV) (Bluet-Pajot et al. 2001) inhibits GH synthesis. SST can modulate GH secretion directly by regulating pituitary somatotroph function

as well as indirectly through central regulation of GHRH-neurons (Muller et al. 1999). The physiological roles of the arcuate SST neurons are not clear. In growth control, however, these neurons have been suggested to activate GHRH neurons likely through somatostatin receptors in GHRH neurons (Slama et al, 1993; Lanneau et al, 2000; Bluet-Pajot et al, 2001; Tannenbaum et al, 1998).

In both human and rodent, GHRH-positive neurons are located in the multiple hypothalamic regions including ARC, PVN, DMH, VMH, and LH. Among these neurons, only arcuate GHRH neurons are involved in GH secretion through the anterior pituitary gland. Other GHRH-positive neurons likely contribute to the extrahypophysiotropic projection (Muller et al. 1999).

GHRH released from GHRH-neurons in the ARC stimulates GH production and secretion through a membrane bound G-protein coupled receptor in the pituitary somatotrophs. The importance of GHRH in regulating GH production is demonstrated by the fact that mutations in the GHRH receptor (GHRH-R) cause isolated GH deficiency (IGHD) both in humans and in rodents (Muller et al. 1999). The *littl*e mouse, a naturally occurring dwarf mouse strain, is an animal model for IGHD with a recessive missense mutation in the extracellular domain of the GHRH-R. This mutation prevents ligand binding and renders the receptor un-responsive to GHRH. (Godfrey et al 1993). Similarly, humans with mutated GHRH-R gene also show deficits in growth (Baumann and Maheshwari 1997). Lastly, the GHRH knockout mouse showed significant growth retardation, reduced pituitary growth hormone expression, reduced level of serum IGF-1, and reduced hepatic IGF-1 transcript (Alba and Salvatori 2004b).

1.3. Transcriptional regulation of arcuate nucleus development

The hypothalamic primordium is morphologically evident from around embryonic day (E) 9.5 in the mouse. Several signaling pathways, especially sonic hedgehog (SHH), play important roles in the induction and early patterning of the hypothalamus (Szarek et al. 2010). In response to these signaling cues, a pattern of regionally restricted transcription factors expression is induced and establishes "a transcription factor code" that directs the generation of distinct neuronal cell types (Szarek et al. 2010). Here I will focus on the transcriptional programs important for the development of the arcuate neurons discussed above.

1.3.1. AgRP and POMC neurons

POMC transcript is first detected in the ventral hypothalamus at E10.5, and NPY expression begins between E13.5-14.5 (Padilla et al. 2010). The number of POMC-positive neurons starts to decrease drastically around E13.5. This decrease is not due to apoptosis, and it is believed that these cells begin to assume an NPY/AgRP phenotype (Padilla et al. 2010). Clearly these two cell types are tightly linked during development.

There are a number of homeobox and basic helix-loop-helix (bHLH) transcription factors that are essential for ARC development. Expression of the Nkx2.1 homeobox gene in progenitor cells of the ventral hypothalamus is essential for specification of this region. Nkx2.1 mutant mice die at birth and exhibit profound abnormalities in the ventral hypothalamus including agenesis of

the ARC and VMH (Kimura et al. 1996). Nkx2.1 expression persists in postnatal hypothalamic neurons, and it serves to regulate the expression AgRP and POMC (Kim et al. 2011).

Most of ARC neurons are dependent upon the activity of a basic helix-loop-helix transcription factor called Mash1 (also known as Achaete-scute complex 1, Ascl1). Mash1 is a proneural factor that is important for the generation of neural progenitor cells. The loss of Mash1 leads to increased cell death, impaired neurogenesis in the ventral diencephalon, impaired expression of other bHLH transcription factors such as NeuroD, Ngn3, and Nhlh-2, and the failure of the ARC to develop as distinct anatomical structure. Not all lineages of ARC-neurons are equally affected by the loss of Mash1. For example, NPY and POMC neurons are substantially reduced and GHRH neurons are completely absent in Mash1 null mutant mice (McNay et al. 2006).

The Neurogenins, another family of bHLH transcription factors, are also important in ARC development. Ngn3 expression begins as early as E9.5 and disappears by E17.5. Fate mapping studies indicate that many of the earliest born POMC neurons as well as a large fraction of NPY neurons arise from Ngn3-expressing progenitors and that later born POMC neurons arise from non-Ngn3 expressing progenitors. In Ngn3 knockout mice, the number of early born POMC neurons (E10.5-13.5) is reduced, but NPY-neurons are drastically increased (Pelling et al. 2011). Interestingly, deletion of Ngn3 specifically in the developing hypothalamus results in early onset obesity associated with hyperphagia and reduced energy expenditure. This phenotype is likely caused by the loss of

POMC expression (Anthwal et al 2013).

1.3.2. GHRH and SST neurons

The arcuate GHRH neurons first appear at E11.5 in the rat, but GHRH expression begins to start around E13.5 (Markakis and Swanson 1997). The GHRH lineage is completely dependent upon Mash1 activity because Mash1 null embryos show no hypothalamic GHRH expression (McNay et al. 2006). These mutant embryos also have very low expression of GS homeobox 1 (Gsx1, also known as Gsh1), a homeobox domain transcription factor necessary for expression of GHRH (Mutsuga et al. 2001). Gsx1 binds to the promoter region of GHRH gene to promote its expression (Mutsuga et al. 2001). Loss of Gsx1 leads to the complete absence of GHRH expression resulting in severe growth retardation (Li et al. 1996). Ikaros, a zinc finger-containing transcription factor expressed in GHRH neurons, is also essential for GHRH expression (Ezzat et al. 2006). In addition, two other homeobox transcription factors Hmx2 and Hmx3 are required for GHRH expression. GHRH and Gsx1 expression in the ARC of Hmx2/3 double knockout mouse brain is absent indicating that Hmx2 and Hmx3 regulate GHRH expression through Gsx1 (Wang et al. 2004).

Orthopedia (Otp) is a homeobox transcription factor expressed in neurons that give rise to the PVN, supraoptic nucleus (SON) and ARC throughout their development. OTP knockout mice die as neonates and have deficits in many neurons in the paraventricular, supraoptic, arcuate nucleus in the hypothalamus. These mice also failed to express SST, but retain a normal level of GHRH in

ARC (Acampora et al. 1999).

1.3.3. The function of Islet1 (Isl1) during development

The LIM-homeodomain (LIM-HD) transcription factor Islet 1 (Isl1) plays essential roles in cell differentiation and cell fate determination during embryogenesis (Gill 2003). Isl1 was originally identified as a protein that binds to an insulin gene enhancer and regulates its expression (Karlsson et al. 1990). Isl1 regulates gene expression by binding to consensus homeodomain-binding elements that contain core TAAT motif (Karlsson et al. 1990). Isl1 is expressed in multiple tissues and cell types, including the pancreas, the central and peripheral nervous system, the retina, the thyroid gland, the pituitary gland, and heart progenitor cells (Zhuang et al. 2013). Because of the essential role of Isl1 in heart formation, Isl1 knockout mice die at E9.5-10.5 (Cai et al. 2003). One interesting aspect of Isl1 is that it can form cell type-specific transcriptional complexes with various other transcription factors to regulate cell fate specification and differentiation during development. For instance, Isl1 interacts with the LIM-HD factor Lhx3 in spinal cord motor neurons, Lhx8 in forebrain cholinergic neurons, the bHLH transcription factor Beta2 in the pancreas and the POU-domain transcription factor Pou4f2 in retinal ganglion cells (Pfaff et al. 1996; Peng et al. 2005; Cho et al. 2014; Li et al. 2014).

Expression of Isl1 has been reported in several regions of the hypothalamus inducing ARC (Thor et al. 1991; Davis et al. 2004). Very recently, Isl1 was reported to promote the expression of the anorexigenic neuropeptide POMC in the ARC by directly binding to two related Isl1RE-containing enhancer

elements in the *POMC* gene, demonstrating that Isl1 directly specifies the identity of the arcuate POMC-neurons (Nasif *et al*, 2015), However, the function of Isl1 in other types of arcuate neurons remains unknown.

1.4. Summary

Despite the recent advances in our knowledge of how arcuate neurons control central energy balance, the underlying mechanisms by which peripheral metabolic signals such as glucocorticoid regulate the central neuropeptide expression are not completely understood. Furthermore, the gene regulatory programs required for the genesis of these critical neurons during development have not been well studied. In this thesis, I aim to characterize the gene networks involved in both the embryonic development and postnatal functions of arcuate neurons.

Chapter 2

Brain-specific homeobox factor as a target selector for glucocorticoid receptor in energy balance

Bora Lee[1,2], Sun-Gyun Kim[1], Juhee Kim[1], Kwan Yong Choi[5], Seunghee Lee[3], Soo-Kyung Lee,[12,3] and Jae W. Lee[1,2,5]

Neuroscience Section, Papé Family Pediatric Research Institute, Department of Pediatrics[1], Department of Cell and Developmental Biology[2] and Vollum Institute,[4] Oregon Health & Science University, Portland, Oregon, USA

College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, South Korea[3]

Division of Integrative Biosciences and Biotechnology, Pohang University of Science and Technology, Pohang, South Korea[5]

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2.1. Abstract

The molecular basis underlying the physiologically well-defined orexigenic function of glucocorticoid (Gc) is unclear. Brain-specific homeobox factor (Bsx) is a positive regulator of the orexigenic neuropeptide, agouti-related peptide (AgRP), in AgRP-neurons of the hypothalamic arcuate nucleus. Here we show that, in response to fasting-elevated Gc levels, Gc receptor (GR) and Bsx synergize to direct activation of *AgRP* transcription. This synergy is dictated by unique sequence features in a novel Gc response element in *AgRP* (AgRP-GRE). In contrast to AgRP-GRE, Bsx suppresses transactivation directed by many conventional GREs, functioning as a gene context-dependent modulator of GR actions or a target selector for GR. Consistently, AgRP-GRE drives fasting-dependent activation of a target gene specifically in $GR^{+}Bsx^{+}$ AgRP-neurons. These results define *AgRP* as a common orexigenic target gene of GR and Bsx, and provide an opportunity to identify their additional common targets, facilitating our understanding of the molecular basis underlying the orexigenic activity of Gc and Bsx.

2.2. Introduction

Energy balance is directed by the relationship between the amount of consumed food and energy expenditure, and both processes are regulated by the central nervous system (CNS). The hypothalamus is a main CNS structure responsible for appetite control and energy expenditure (Fehm et al. 2004; Coppola and Diano 2007). The peripheral hunger or satiety signals arriving at the CNS are first perceived by two populations of neurons, agouti-related peptide (AgRP)-neurons and pro-opiomelanocortin (POMC)-neurons, in the hypothalamic arcuate nucleus (ARC) (Fehm et al. 2004; Coppola and Diano 2007). Sensing the peripheral signals, AgRP-neurons produce the orexigenic peptides, AgRP and neuropeptide Y (NPY), and POMC-neurons yield the anorexigenic peptide, α-melanocyte stimulating hormone (αMSH). AgRP and NPY increase food intake and decrease energy expenditure, while αMSH does the opposite, contributing to maintaining energy homeostasis centrally (Fehm et al. 2004; Coppola and Diano 2007).

Glucocorticoid (Gc) is a well-known peripheral orexigenic signal (Fehm et al. 2004; Coppola and Diano 2007). Fasting increases plasma levels of Gc and activates expression of NPY and AgRP in AgRP-neurons (Jeanrenaud and Rohner-Jeanrenaud 2000; Makimura et al. 2003; Fehm et al. 2004; Coppola and Diano 2007). Gc replacement studies in adrenalectomized mice have also revealed that Gc triggers fasting-dependent induction of NPY/AgRP expression (Savontaus et al. 2002; Dhillo et al. 2003; Makimura et al. 2003; Shimizu et al. 2008). These studies indicate that Gc-mediated upregulation of NPY/AgRP in the

hypothalamus contributes to the orexigenic activity of Gc. However, both the molecular mechanism by which Gc controls expression of NPY/AgRP and the direct target genes of Gc in AgRP-neurons remain poorly understood.

Gc primarily functions through the transcription-dependent action of Gc receptor (GR), a member of the nuclear hormone receptor superfamily (Stahn et al. 2007). GR is a cytoplasmic protein that translocates to the nucleus upon binding Gc and interacts with Gc response element (GRE) of GR target genes to regulate their expression (Stahn et al. 2007). In addition to the genes with classical GREs, Gc also regulates expression of genes that contain response elements for GR-interacting transcription factors. For instance, via protein-protein interactions with the pro-inflammatory transcription factors AP1 and NFκB, GR is tethered to the binding sites for AP1 and NFκB and represses their transactivation (Adcock and Caramori 2001; Stahn et al. 2007).

Brain-specific homeobox factor (Bsx) is a transcription factor expressed in selective regions of the CNS, including the ARC, the dorsomedial nuclei (DMH), and the lateral hypothalamic area (LHA) of the hypothalamus (Cremona et al. 2004; McArthur and Ohtoshi 2007; Sakkou et al. 2007). Within the ARC, Bsx appears to be expressed exclusively in AgRP-neurons and functions as an orexigenic transcription factor (Sakkou et al. 2007). Consistently, Bsx levels are increased by fasting as well as ghrelin, an orexigenic signal, and decreased by leptin, an anorexigenic signal (Nogueiras et al. 2008). Bsx has also been suggested to link spontaneous locomotor activity and food intake, given that Bsx-null mice show impaired expression of NPY/AgRP and food-seeking locomotor

behavior (Sakkou et al. 2007). Bsx cooperates with FoxO1 to directly activate expression of AgRP through Bsx- and FoxO1-response elements in *AgRP* (Sakkou et al. 2007). However, the detailed molecular basis of Bsx action in regulating the expression of NPY/AgRP remains poorly understood.

In this report, we set out to address two specific questions: *i)* Does Gc directly regulate transcription of *NPY* and *AgRP* genes in the AgRP-neurons via GR. *ii)* Is there any mechanism that functionally couples the peripheral orexigenic signal Gc with the orexigenic transcription factor Bsx in controlling expression of NPY/AgRP. Our results demonstrate that, in AgRP-neurons, the peripheral Gc signal triggers a synergistic activation of AgRP expression by GR and Bsx via a novel Gc-response element in *AgRP*, named AgRP-GRE, identifying *AgRP* as the first direct and common orexigenic target gene of GR and Bsx. Interestingly, this synergy is dictated by unique sequence features in AgRP-GRE. Furthermore, we show that this sequence information can be used to identify additional common targets of GR and Bsx. Surprisingly, Bsx also represses the action of GR on many conventional GREs, suggesting that Bsx acts as a dual function target selector for GR. Together, our results provide critical insights into the molecular mechanisms for the central orexigenic action of Gc/GR and Bsx as well as for how GR targets distinct sets of genes in different tissues (Stahn et al. 2007).

2.3. Materials and Methods

2.3.1. Animals. 7-10 weeks old wild type C57BL/6 mice were maintained on a normal 12h light, 12h dark cycle with *ad libitum* access to normal chow and water, unless otherwise indicated. Mice were intraperitoneally or intracerebroventricularly injected with Dex (10mg/kg) or RU486 (50mg/kg).

2.3.2. Generation of transgenic mice. The transgenes were microinjected into one-cell stage embryo of C57BL/6 mice by the transgenic mouse core at Baylor College of Medicine. Founder lines for each eGFP reporter were generated and bred to wild-type C57BL/6.

2.3.3. ChIP. Mouse hypothalamus were dissected out and homogenized before cross-linked with 1% formaldehyde for 15-20 min. The cell lysates were sonicated, immunoprecipitated with anti-GR antibody (SC-8992, Santa Cruz), anti-Bsx antibody that we have generated, or control IgG (Santa Cruz), and incubated with protein A and G agarose overnight. DNAs were eluted and reverse cross-linked at 65°C over night. DNAs were purified with phenol chloroform extraction method. We also carried out ChIPs with P19 cells expressing either Flag-GR alone or Flag-GR and Bsx using anti-H3K4me3 and anti-H4Ac antibodies (AbCam). The primers used for the subsequent PCRs are as shown in Fig. S3A.

2.3.4. Immunostaining. Anaesthetized mice were perfused transcardially with 4% paraformaldehyde in PBS. Brains were removed and placed in 4% paraformaldehybe over night followed by incubation with 30% sucrose. Brain sections were prepared with cryostat, incubated with antibodies against GR (SC-1004, Santa Cruz), POMC (H-029-30, Phoenix Pharmaceutical), GFP (GFP-1020, Aves), and Bsx at 4°C overnight, and followed by 1-2h incubation with fluorescence conjugated secondary antibodies.

2.3.5. In situ hybridization. Digoxigenin labeled antisense RNA probes were hybridized to the brain sections at 68°C. Hybridized sections were washed, incubated with anti-digoxigenin-AP antibody (11093274910 Roche), and then subjected to color reaction.

2.3.6. Luciferase assays and co-immunoprecipitation. HEK293 or P19 cells were maintained in DMEM supplemented with 10% FBS. Cells were seeded into 48-well or 10cm plates, and transient transfections were performed using SuperFect (Qiagen) according to the manufacturer's instruction. Actin-β-galactosidase plasmid was cotransfected for normalization of the luciferase assay. 10 nM of Dex was used for all reporter assays.

2.3.7. Generation of hypothalamic cell line stably expressing si-Bsx. We adopted pSilencer™ 4.1-CMV puro vector (Ambion AM5775M) to stably express either control siRNA or siRNA against Bsx (si-Bsx) in hypothalamic cell line. The

mouse Bsx target sequences were AAT CTC AAC TTC ACT TCC CCT. We transfected N42 immortalized hypothalamic neurons (Cellutions Biosystems) with the modified vectors, and selected transformants against puromycin. The selected N42 cells were subsequently tested for specific down-regulation of Bsx using both RT-PCR and immunoblotting with anti-Bsx antibody (data not shown).

2.4. Results

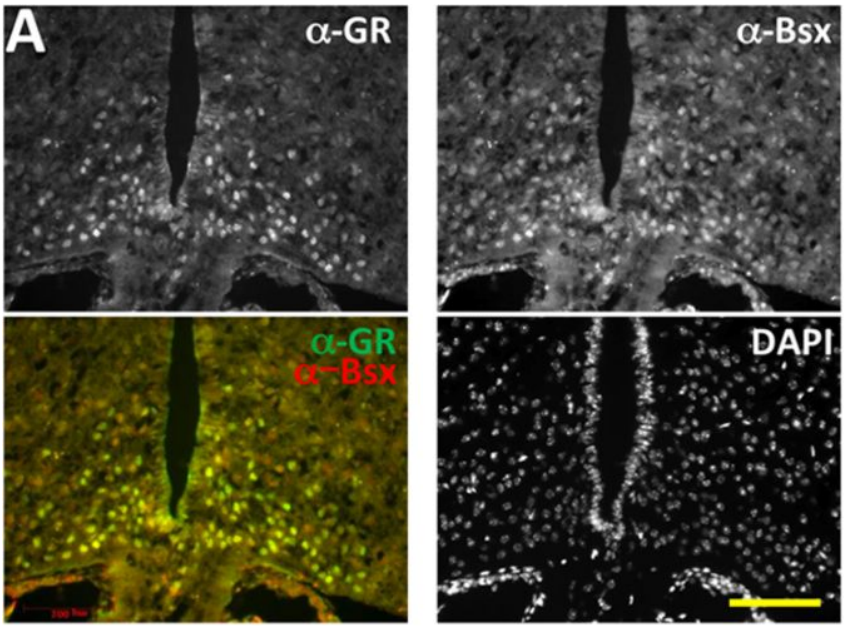
2.4.1. Specific expression of GR in AgRP-neurons.

To test whether Gc-bound GR directly controls the transcription of *NPY/AgRP* in AgRP-neurons, we examined the expression pattern of GR in the hypothalamus using immunohistochemical analysis. As reported (Ceccatelli et al. 1989), prominent GR-immunostaining signal was detected in the paraventricular nucleus (PVN) (Fig. S8C). GR was also enriched in the ARC (Fig. 1A and S1A). Double-immunostaining analyses with Bsx, a marker for AgRP-neurons (Sakkou et al. 2007; Nogueiras et al. 2008), revealed that GR is strongly co-expressed with Bsx in AgRP-neurons (Fig. 1A and S1A). Using transgenic mice expressing GFP in POMC-neurons (Cone et al. 2001), we also found that GFP^+ POMC-neurons displayed little to no GR expression (Fig. S1B). Our results show a relatively specific and strong expression of GR in AgRP-neurons, supporting the possible transcriptional regulation of *NPY/AgRP* genes by GR.

2.4.2. GR is required for upregulation of AgRP by fasting.

To test whether the function of GR is necessary for inducting NPY/AgRP expression upon fasting, we employed RU486, an antagonist of GR and progesterone receptor (PR) (Baulieu 1988). Intraperitoneal or intracerebroventricular injection of RU486 markedly suppressed fasting-mediated induction of AgRP levels, compared to the injection of vehicle alone (Fig. 1B, 1C, and S2). However, RU486 had no effect on NPY expression (Fig. 1B and S2).

These results indicate that the effect of GR/PR antagonism is relatively specific with *AgRP* gene, and that, based on intact NPY expression, AgRP-neurons are expected to remain unmarred after acute treatment of RU486. Notably, progesterone has been shown to have no effect on expression of AgRP in hypothalamic explants (Brewer et al. 2003). We also found that progesterone has no effect on the promoter activity of *AgRP* (Fig. S3A). These results suggest that RU486 interfered with the action of GR, not PR, in inducing AgRP expression. To further test the involvement of GR in AgRP expression, we generated mice in which GR is removed specifically in AgRP-neurons by mating $GR^{f/f}$ mice (Tronche et al. 1999) to a knock-in line expressing Cre recombinase in AgRP-neurons (Tong et al. 2008). The AgRP-neuron-specific removal of GR greatly blunted the fasting-dependent induction of AgRP expression in the hypothalamus (Fig. 1D). In contrast, NPY was still robustly induced upon fasting in the GR mutant mice (Fig. 1D). These results suggest that *AgRP,* but less likely *NPY,* is a direct target gene of GR.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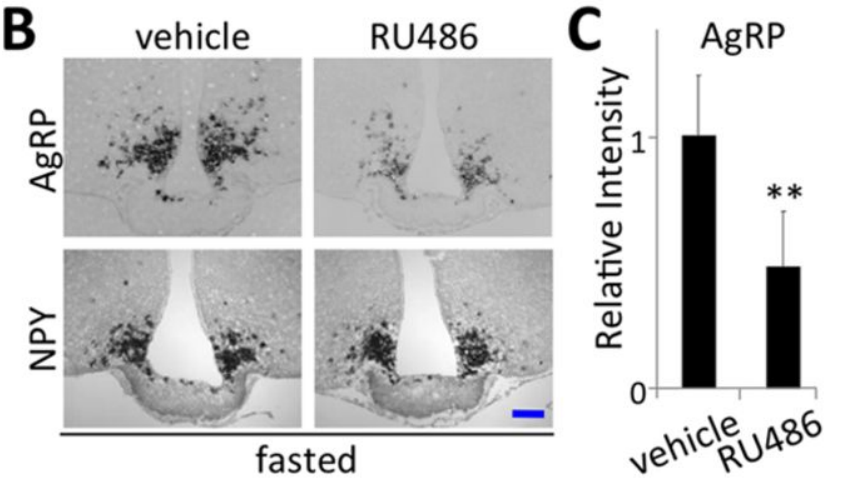
α-Bsx
α-GR
α-Bsx
DAPI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vehicle
RU486
AgRP
NPY
fasted
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Relative Intensity
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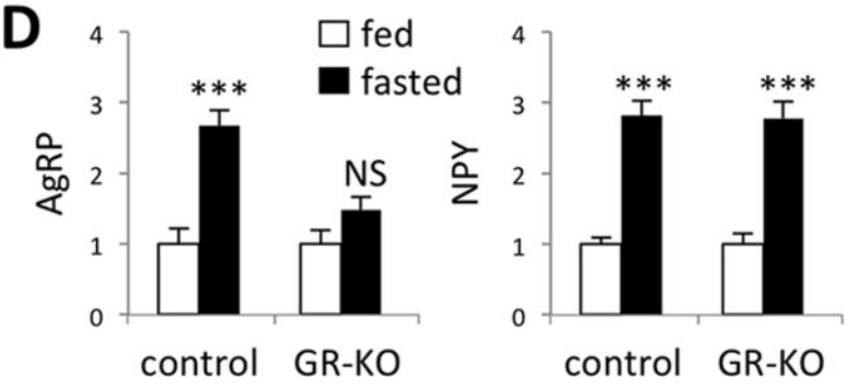
vehicle
RU486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4
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control
GR-KO
control
GR-KO

Figure 1. In vivo evidence for the involvement of GR in regulating expression of AgRP. (A) The coronal sections of the ARC region of mice fasted for 24 h were immunostained with antibodies against GR and Bsx. DAPI staining is included as a control for all of the nuclei in the section. The yellow scale bar indicates 100 μm. (B) In situ hybridizations for AgRP and NPY were carried out with serial coronal 12-μm sections of the ARC region of mice (n = 3 in each group) intraperitoneally injected with either vehicle or 50 mg/kg of RU486. Representative images are shown. (C) For all the images shown in panel B, the in situ signal intensity of AgRP was quantified using ImageJ and plotted against the intensity of samples injected with vehicle alone. (D) The in situ signal intensity of AgRP and NPY of serial coronal 12-μm sections of the ARC region of control (n = 3) and GRf/f:AgRP-Cre (GR-KO) mice (n = 5) was quantified using ImageJ and plotted against the intensity of fed control samples. P values of less than 0.001 and 0.0001 are denoted ** and ***, respectively (C and D).

2.4.3. Identification of AgRP-GRE.

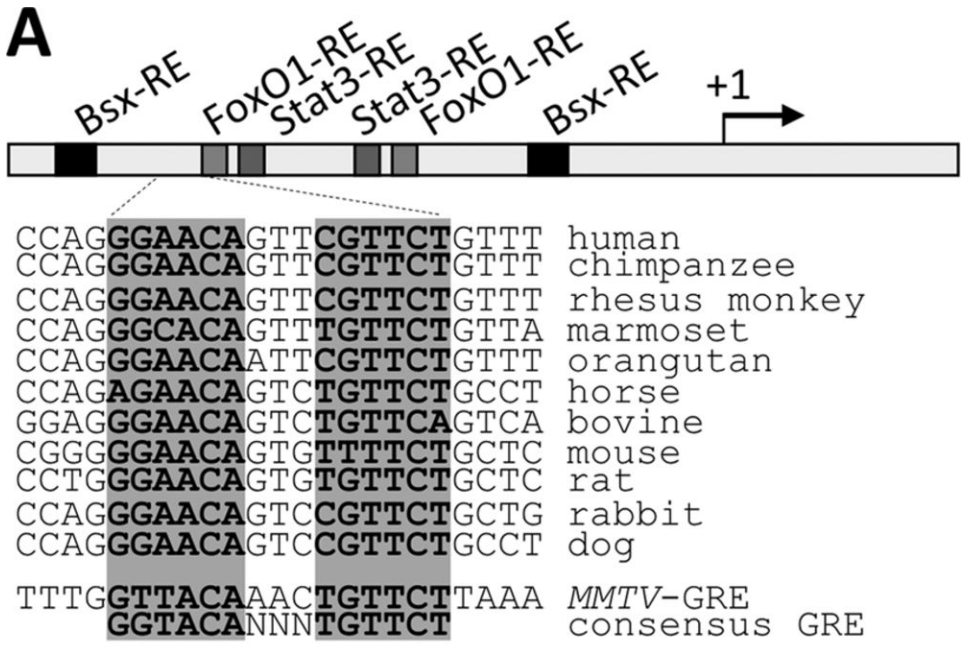
The *AgRP* promoter has the binding sites of transcription factors that respond to peripheral signals, such as leptin and insulin (Fig. 2A). FoxO1, whose activity is blocked by the insulin-PI3K-Akt pathway, binds to the AgRP promoter and upregulates AgRP levels, whereas Stat3, an effector of leptin signaling, blunts AgRP expression by inhibiting the action of FoxO1 (Muraoka et al. 2003; Kim et al. 2006; Kitamura et al. 2006). In addition, the 1 kb *AgRP* promoter has the binding sites for Bsx, a positive regulator of AgRP (Sakkou et al. 2007). To examine the Gc-responsiveness of the *AgRP* promoter, we tested whether a synthetic Gc, dexamethasone (Dex), is capable of activating the *AgRP* promoter using luciferase reporter assays. Dex strongly activated a luciferase reporter driven by the 1 kb *AgRP* promoter fragment containing two Bsx-, two FoxO1- and two Stat3-response elements (Fig. 2, A and B). It is possible that GR indirectly controls the activity of the 1 kb *AgRP* promoter by associating with Bsx, FoxO1 and/or Stat3. Alternatively, GR could directly regulate the AgRP promoter by binding to GRE. To distinguish between these possibilities, we first mutated each binding site for Bsx, FoxO1 and Stat3 in the *AgRP*-luciferase reporter, and monitored the reporter activation by Dex. This analysis revealed that the binding sites for Bsx, FoxO1 and Stat3 are dispensable for the Dex-response of the *AgRP* promoter (Fig. S3A). To test whether recognition of GRE by GR is important for Dex-dependent activation of the AgRP promoter, we used a human GR mutant with a point-mutation in the DNA binding domain (C438A), and found that this mutant GR failed to direct Dex-dependent activation of AgRP-1kb:LUC

(Fig. S3A). These results suggest that GR is recruited to the *AgRP* promoter via GRE rather than the binding sites for other transcription factors. Subsequent deletion analyses indeed mapped an evolutionarily conserved GRE-like motif, named AgRP-GRE, which exhibits a few nucleotide sequence variations from the consensus GRE (Fig. 2A and S3A). Deletion of AgRP-GRE or mutation of either half site of AgRP-GRE completely abolished the Dex response (Fig. 2C and S3A). Moreover, two copies of 27-mer containing the AgRP-GRE alone were sufficient to fully reproduce the Dex-response of the luciferase reporters driven by longer promoters of *AgRP* (Fig. 2D). AgRP-GRE also showed specific binding to GR in gel mobility shift assays (Fig. S4A). These results demonstrate that AgRP-GRE serves as a direct binding site for GR and it is necessary and sufficient in mediating Dex-dependent activation of the *AgRP* promoter.

Next, we tested whether Dex also regulates the promoter of *NPY*, encoding NPY, another orexigenic neuropeptide produced in AgRP-neurons. The *NPY* promoter has been shown to have binding sites for Bsx, FoxO1 and Stat3 (Muraoka et al. 2003; Kim et al. 2006; Kitamura et al. 2006; Sakkou et al. 2007) as well as a GRE-like motif (Zhang et al. 2012) (Fig. S3B). Dex exhibited no effect on a luciferase reporter driven by 1 kb promoter region of *NPY*, which includes the putative GRE-like motif (Zhang et al. 2012) (Fig. S3B). These results, along with the little effect of RU486 or AgRP-neuron-specific GR deletion on fasting-mediated NPY induction (Fig. 1B, D), suggest that NPY may not be a direct target of GR.

2.4.4. Recruitment of GR to AgRP-GRE in the hypothalamus.

To further test whether peripheral Gc signal controls AgRP expression via triggering GR binding to AgRP-GRE in vivo, we performed chromatin immunoprecipitation (ChIP) assays in the hypothalamus of adult mice and monitored the recruitment of GR to the *AgRP* promoter. The 235 bp PCR products from our primer set encompass not only the AgRP-GRE but also the neighboring Bsx-/FoxO1-/Stat3-sites (Fig. S3A). Interestingly, intraperitoneal injection of Dex, but not vehicle, induced a strong GR-binding to the *AgRP* promoter in the hypothalamus (Fig. 2E). Likewise, fasting, which increases plasma levels of Gc and activates expression of NPY/AgRP in AgRP-neurons (Jeanrenaud and Rohner-Jeanrenaud 2000; Makimura et al. 2003; Fehm et al. 2004; Coppola and Diano 2007), greatly enhanced GR binding to the *AgRP* promoter in the hypothalamus, as shown by ChIP analyses of the hypothalamic lysates of mice fasted for 24 h or fed chow diet (Fig. 2F). These results indicate that higher levels of circulating Gc (achieved by Dex injection or fasting) triggers direct recruitment of GR to AgRP-GRE in AgRP-neurons, likely contributing to a marked upregulation of AgRP upon fasting.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FoxO1-RE
Stat3-RE
Stat3-RE
FoxO1-RE
Bsx-RE
+1
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CCAGGGAACAGTTCGTTCTGTTT rhesus monkey
CCAGGGCACAGTTTGTTCTGTTA marmoset
CCAGGGAACAATTCGTTCTGTTT orangutan
CCAGAGAACAGTCTGTTCTGCCT horse
GGAGGGAACAGTCTGTTCAGTCA bovine
CGGGGGAACAGTGTTTTCTGCTC mouse
CCTGGGAACAGTGTGTTCTGCTC rat
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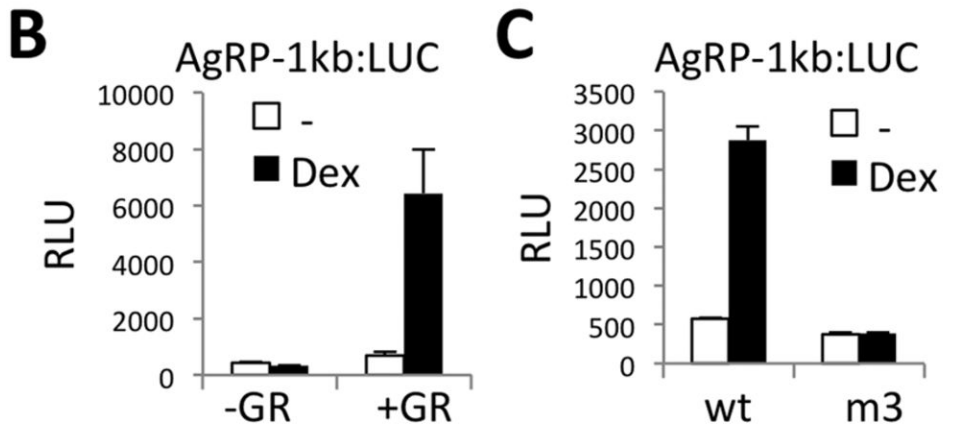
CCAGGGAACAGTCCGTTCTGCCT dog
TTTGGTTACAAACTGTTCTTAAA MMTV-GRE
GGTACANNNTGTTCT consensus GRE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RLU
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-GR
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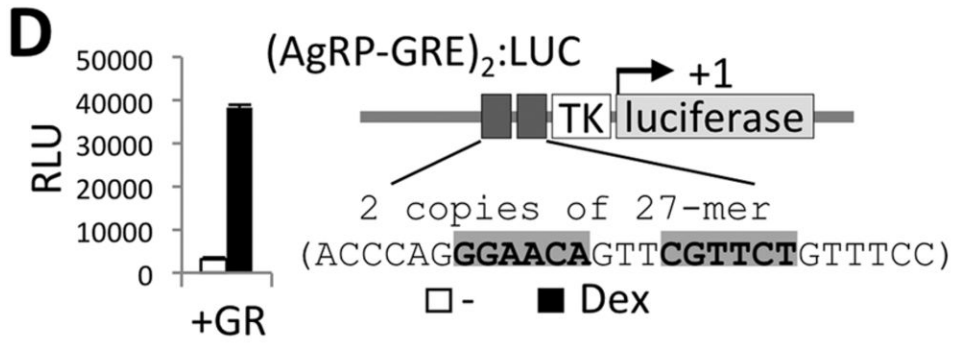
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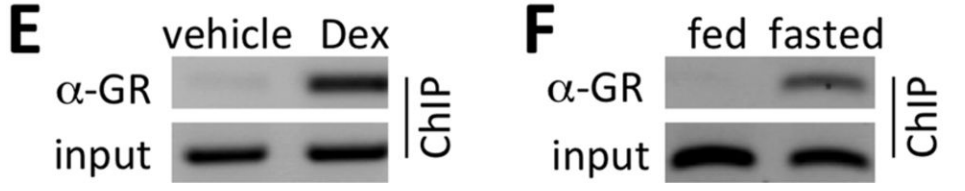
Dex


E
vehicle
Dex
α-GR
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ChIP
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fed
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α-GR
input
ChIP

Figure 2. Identification of AgRP-GRE. (A) Schematic representation of the AgRP promoter. AgRP-GRE is highly conserved in mammals, as indicated. The MMTV-GRE and the consensus GRE sequences are shown for comparison. (B and C) Luciferase reporter assays with AgRP-1kb:LUC reporter (B) and its derivative with a mutation in the first half site of the AgRP-GRE (m3) (C) in HEK293 cells. (D) Luciferase reporter assays with the (AgRP-GRE)2:LUC reporter in HEK293 cells. The 27-mer sequences, as well as a schematic representation of the reporter, are shown. (E and F) ChIP for GR binding in the hypothalamus lysates of mice intraperitoneally injected with either vehicle or 10 mg/kg of Dex (E) or in the hypothalamus lysates of mice either fed or fasted for 24 h (F).

2.4.5. Gc permits Bsx to synergize with GR in activating AgRP expression.

The proximal location of the AgRP-GRE and the upstream Bsx-binding site in *AgRP* and a common orexigenic function of Gc and Bsx (Sakkou et al. 2007) led us to investigate a possible cross-talk between Bsx and GR in upregulating AgRP expression in response to peripheral Gc signal. Although Bsx readily bound to Bsx-sites in the *AgRP* promoter as shown by gel mobility shift assays (Fig. S4C), it had minimal effect on activating the 1kb *AgRP* promoter containing Bsx-sites (Fig. 3A). Intriguingly, however, Bsx strongly synergized with GR in a Dex-dependent manner to activate the *AgRP* promoter (Fig. 3A). These results suggest that binding of Bsx to Bsx-sites alone is insufficient to activate expression of AgRP, and that Gc plays a permissive role for Bsx to activate expression of AgRP in synergy with GR.

To test whether the synergy between Bsx and Dex requires Bsx-binding sites in the *AgRP* promoter, we utilized the $(AgRP\text{-}GRE)_2$:LUC reporter, which has AgRP-GRE but no Bsx-binding sites. The stimulatory effect of Bsx on GR transactivation was significantly weakened in this reporter although the Dex-dependent synergy between GR and Bsx was still observed; i.e., ~7-fold enhancement over GR alone with AgRP-1kb:LUC vs. ~2-fold enhancement with $(AgRP\text{-}GRE)_2$:LUC (Fig. 3, A and B). Consistently, a DNA-binding defective mutant Bsx, which failed to bind to Bsx sites in *AgRP* promoter region (Fig. S4C), was much less potent in synergizing with GR than wild type Bsx (Fig. 3C). These results suggest that AgRP-GRE alone is capable of launching a synergistic activation of *AgRP* promoter by GR and Bsx and that the Dex-dependent synergy

between GR and Bsx becomes much more robust when both GR and Bsx bound to GRE and Bsx-binding sites, respectively, in the *AgRP* promoter. Our results also establish that the *AgRP* promoter functions as a central sensor for the fasting-elevated peripheral Gc levels in inducing AgRP expression.

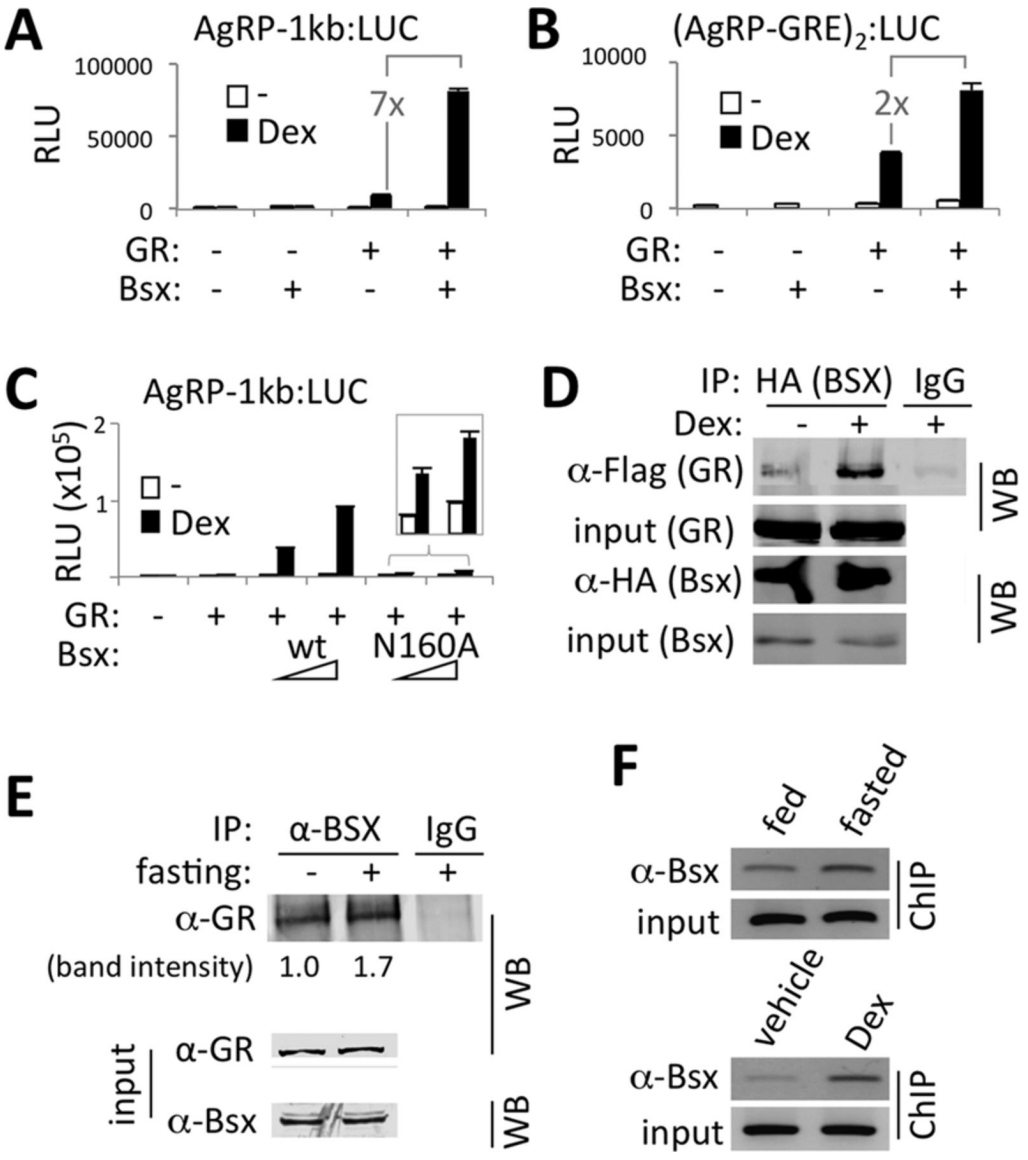
2.4.6. Gc-activated GR facilitates Bsx-recruitment to the AgRP promoter in the ARC.

To understand the molecular basis underlying Dex-dependent transcriptional synergy between GR and Bsx on the *AgRP* promoter, we considered the possibility that Dex promotes the association between GR and Bsx and controls the recruitment of the GR/Bsx complex to the *AgRP* promoter. Interestingly, Bsx was co-immunoprecipitated with GR in a Dex-stimulated manner in HEK293T cells transfected with Flag-tagged GR and HA-tagged Bsx (Fig. 3D), indicating that Gc signal facilitates the association between GR and Bsx in cells. Importantly, we also observed co-immunoprecipitation of endogenous GR and Bsx in the mouse hypothalamus (Fig. 3E).

Next, to investigate whether Bsx recruitment to the *AgRP* promoter is enhanced by peripheral signals that activate GR in the AgRP-neurons, such as fasting or Dex injection, in vivo, we performed ChIP experiments with the mouse hypothalamic lysates and the primer set used for the above GR-recruitment study (Fig. 2E, F). Fasting substantially enhanced the recruitment of Bsx to the *AgRP* promoter in the hypothalamus (Fig. 3F, upper panel). Given that fasting increases Bsx levels in the ARC (Sakkou et al. 2007; Nogueiras et al. 2008), the

enhanced binding of Bsx upon fasting could be simply due to the increased protein levels of Bsx. Notably, while we observed an increase in Bsx levels in the hypothalamic ARC region upon fasting in our immunostaining results (see Fig. S5 in the supplemental material), we failed to see a significant difference in our immunoblotting results with the whole hypothalamic lysates (Fig. 3E). This is likely due to the fact that fasting affects Bsx levels only in the ARC (i.e., AgRP neurons) but not in other hypothalamic neurons expressing Bsx (Nogueiras et al. 2008). Importantly, Bsx binding to the AgRP promoter was also enhanced only 2 h after intraperitoneal injection of Dex (Fig. 3F, lower). We also found that Bsx levels in mice with AgRP neuron-specific deletion of GR were similar to those in control mice (data not shown), suggesting that Dex injection, unlike fasting (Nogueiras et al. 2008), would not induce the expression of Bsx in AgRP neurons. Overall, these results suggest that the peripheral Gc signal facilitates Bsx recruitment to the AgRP promoter.

Together, our results suggest that the gain of protein-protein interactions between Bsx and Gc-bound GR contribute to the fasting-enhanced binding of Bsx to the *AgRP* promoter. Furthermore, our results support a model in which the peripheral Gc signal triggers an assembly of a transcriptionally active complex composed of Bsx and GR on the *AgRP* promoter, which leads to a robust induction of AgRP.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2x
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Bsx: - + - +
C
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RLU (x10^5)
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1
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Bsx: wt N160A
D
IP: HA (BSX) IgG
Dex: - + +
α-Flag (GR)
input (GR)
α-HA (Bsx)
input (Bsx)
WB
WB
E
IP: α-BSX IgG
fasting: - + +
α-GR
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WB
input
α-GR
α-Bsx
WB
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fed
fasted
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vehicle
Dex
α-Bsx
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Figure 3. Synergy of GR and Bsx in activating expression of AgRP. (A to C) Luciferase reporter assays with AgRP-1kb:LUC reporter (A and C) and (AgRP-GRE)2:LUC reporter (B) in HEK293 cells transfected with expression vectors as indicated. Bsx-N160A is a DNA binding-defective mutant form of Bsx (C). (D) Anti-HA antibody-coimmunoprecipitated HA-tagged Bsx and Flag-tagged GR from HEK293 cells transfected with expression vectors for HA-Bsx and Flag-GR. Immunoprecipitation with IgG was carried out as a negative control. IP, immunoprecipitation; WB, Western blotting. (E) Coimmunoprecipitation of endogenous GR and Bsx in the hypothalamus lysates of mice either fed or fasted for 24 h. Association of GR and Bsx observed in fed mice was further enhanced in fasted mice by approximately 1.7-fold. (F) ChIP for Bsx binding in the hypothalamus lysates of mice either fed or fasted for 24 h (top) or of mice intraperitoneally injected with either vehicle or 10 mg/kg of Dex (lower).

2.4.7. The opposite action of Bsx on AgRP-GRE and conventional GREs.

To test whether Gc-dependent synergy between Bsx and GR is a universal feature of all GR target genes, we monitored the effect of Bsx on multiple GRE-dependent reporters. Surprisingly, Bsx blunted Dex transactivation of a reporter driven by the well-characterized classical GREs defined in MMTV (MMTV:LUC) (Stahn et al. 2007) (Fig. 4A) as well as in other known GR target genes (Fig. S6). These results point to an intriguing possibility that Bsx functions as a binding-site-dependent dual regulator of Gc/GR target genes within the AgRP-neurons; i.e., an activator of AgRP-GRE-like motif and, at the same time, an inhibitor of other GREs. This would allow Bsx to effectively limit the spectrum of activated GR target genes in AgRP-neurons upon arrival of peripheral Gc signal, by strongly activating a group of orexigenic genes with AgRP-GRE-like motifs while inhibiting unwanted GR target genes with conventional GREs.

In our effort to understand the molecular mechanisms by which Bsx evokes the opposite effects on Dex-dependent GR transactivation in AgRE-GRE and conventional GREs, we employed a dimerization defective mutant GR (GR-A458T, named GR^{dim}) (Reichardt et al. 1998). As expected, GR^{dim} was inefficient in transactivating conventional GREs such as MMTV-GRE (Fig. 4A). Surprisingly, it fully supported GR transactivation of AgRP-GRE and furthermore synergized with Bsx (Fig. 4B). These results prompted us to ask whether the dual function of Bsx is dictated by the sequences in GREs and their flanking regions. A unique sequence feature in AgRP-GRE that is not shared by conventional GREs may be responsible for the unexpected responsiveness of AgRP-GRE to GR^{dim} as well

as for the synergistic activation of AgRP-GRE by Bsx and GR. To identify the critical sequences in AgRE-GRE, we generated a series of five hybrid constructs, in which some sequences of AgRP-GRE were swapped by those of MMTV-GRE (Fig. S3C), and tested their responsiveness to GR^{dim} and Bsx. AgRP-GRE-m6, in which the well-conserved GGA in the first half site of AgRE-GRE were replaced by the sequences in the comparable positions in MMTV-GRE, was not activated by GR^{dim}, like MMTV-GRE, while this mutant behaved like AgRP-GRE against wild type GR (Fig. 4C). In AgRP-GRE-m9, in which the sequences downstream of the second half site were changed to those in MMTV-GRE, Bsx did not synergize with GR and, moreover, suppressed transactivation by GR^{dim} (Fig. 4D). To further test the roles of "GGA" and "GTTTC" in AgRP-GRE, we created a new mutant (m10), in which both motifs were replaced by the sequences in the comparable positions in MMTV-GRE (Fig. 4E). This mutant showed the properties of both m6 and m9 mutants; i.e., m10 was similar to m6 for the diminished ability to respond to GR^{dim}, while m10 was not able to support the synergy of GR and Bsx, like m9 (Fig. 4E). Moreover, like MMTV-GRE, transactivation of m10 by wild type GR was suppressed by Bsx (Fig. 4E). Overall, m10 behaved like MMTV-GRE. Together, our mutational analyses revealed that the sequences in the first half site and the region flanking the second half site in AgRP-GRE play critical roles for the two unique properties of AgRP-GRE (i.e., the synergy with Bsx and the responsiveness to GR^{dim}).

2.4.8. Identification of AgRP-GRE-like GREs.

To identify additional common target genes of GR and Bsx in AgRP-neurons, we searched for genomic GREs containing sequences similar to the first half site (GGA) and the flanking region of the second half site (GTTTC) of AgRP-GRE. Our initial bioinformatics analysis identified approximately 20 AgRP-GRE-like sequences that are conserved in both human and mouse and located within 10 kb of gene-coding regions, including the previously reported GRE in *Per1 (Yamamoto et al. 2005; So et al. 2009)* and a new AgRP-GRE-like motif that we identified *in Asb4* (Fig. 4F). Notably, *Per1* has been demonstrated to be expressed in the ARC in a manner commensurate with Gc levels (Matsui et al. 2005; Shieh et al. 2005; Feillet et al. 2008; Minana-Solis et al. 2009) and *Asb4* has been found to be induced in AgRP-neurons by fasting (Li et al. 2005).

To test whether *Per1* and *Asb4* are common targets of GR and Bsx that are regulated via AgRP-GRE-like motifs, we made luciferase reporters driven by genomic regions encompassing their GRE motifs. Both reporters were synergistically activated by Dex and Bsx (Fig. 4G). These results suggest that AgRP-GRE-like motifs may direct upregulation of Per1 and Asb4 in the ARC, and also support the validity of our approach to use AgRP-GRE as a prototypic GRE to further identify common targets of GR and Bsx in AgRP-neurons.

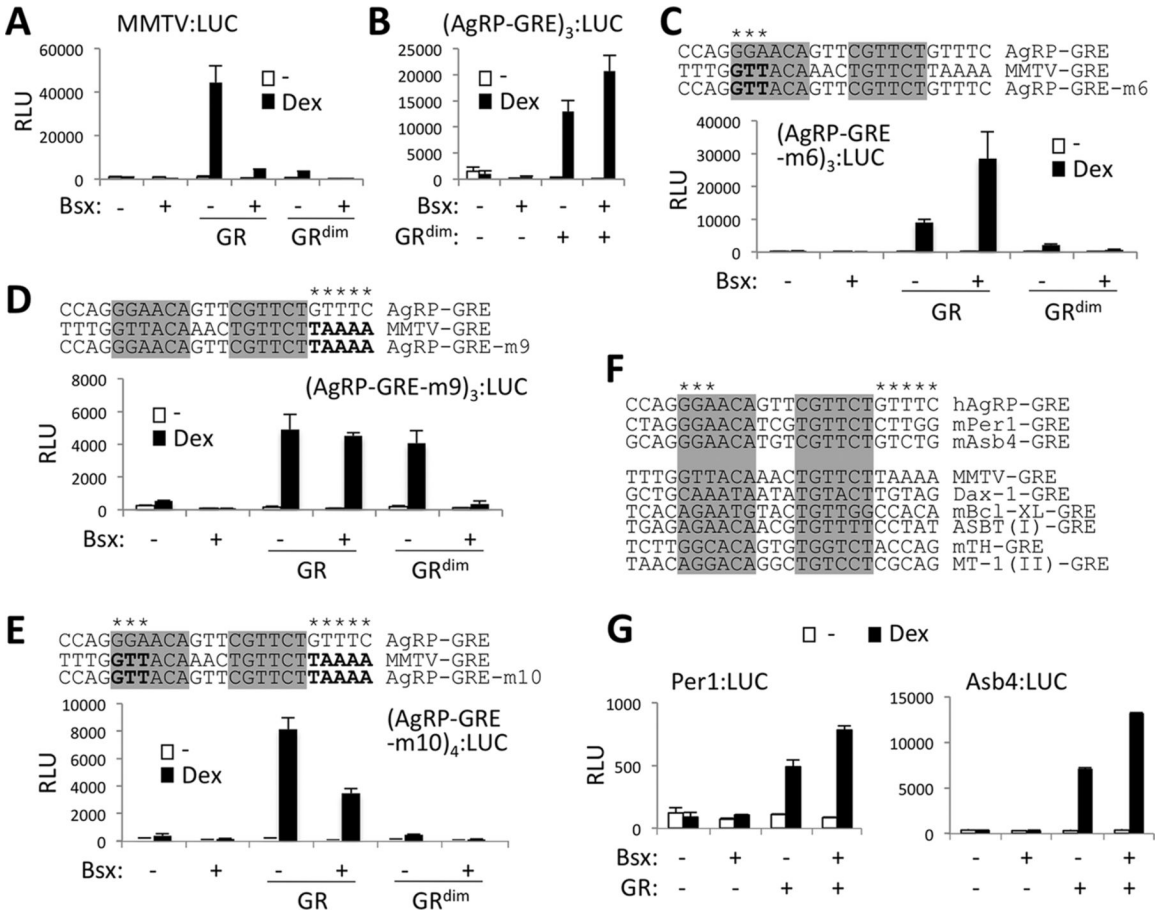

Figure 4. AgRP-GRE as a prototypic tool to find additional targets of GR and Bsx. (A to E and G) Luciferase reporter assays with MMTV:LUC (A), (AgRP-GRE)3:LUC (B), (AgRP-GRE-m6)3:LUC (C), (AgRP-GRE-m9)3:LUC (D), and (AgRP-GRE-m10)4:LUC (E) in HEK293 cells and Per1:LUC and Asb4:LUC (G) reporters in P19 cells transfected with the indicated expression vectors. Sequences of each hybrid GRE are shown (C to E). (F) Sequences of AgRP-GRE, Per1-GRE, and Asb4-GRE as well as of MMTV-GRE and other known GREs that are negatively regulated by Bsx are shown. The unique sequences in AgRP-GRE and AgRP-GRE-like motifs are indicated with an asterisk.

2.4.9. Bsx as an in vivo target selector of GR and Gc-triggered assembly of active chromatin on the *AgRP* promoter.

To test whether Bsx acts as a target selector for GR-regulated genes in vivo, we established N42 immortalized hypothalamic neurons (Cellutions Biosystems) that stably express either control siRNA or siRNA against Bsx (si-Bsx). In N42 cells expressing control siRNA, AgRP expression was induced in a Dex-dependent manner but Dex failed to induce AgRP expression in N42 cells expressing si-Bsx (Fig. 5A). In contrast, Dex-induced expression of Sgk1, whose expression is directed by a conventional GRE (and thus negatively regulated by Bsx), was significantly improved in N42 cells expressing si-Bsx relative to N42 cells with control siRNA. These results confirm our luciferase reporter assays and demonstrate that Bsx is indeed an in vivo target selector of GR.

P19 cells expressed a low level of GR but no Bsx (data not shown). P19 cells weakly supported Dex-induced expression of AgRP even after transient expression of additional GR (Fig. 5B). However, Dex-dependent AgRP expression was significantly enhanced in P19 cells transiently transfected with both GR and Bsx expression vectors (Fig. 5B). Based on these results, we examined our model for an assembly of a transcriptionally active complex composed of Bsx and GR on the *AgRP* promoter. Consistent with this idea, Dex was able to establish higher levels of two open chromatin marks, trimethylated histone H3 lysine 4 (H3K4me3) and acetylated H3 (H3Ac), in P19 cells expressing GR and Bsx relative to P19 cells expressing GR alone (Fig. 5C). These results, together with our results shown in Fig. 2 and 3, demonstrate that

the peripheral Gc signal recruits Bsx and GR and triggers formation of a transcriptional active enhanceosome on the *AgRP* promoter.

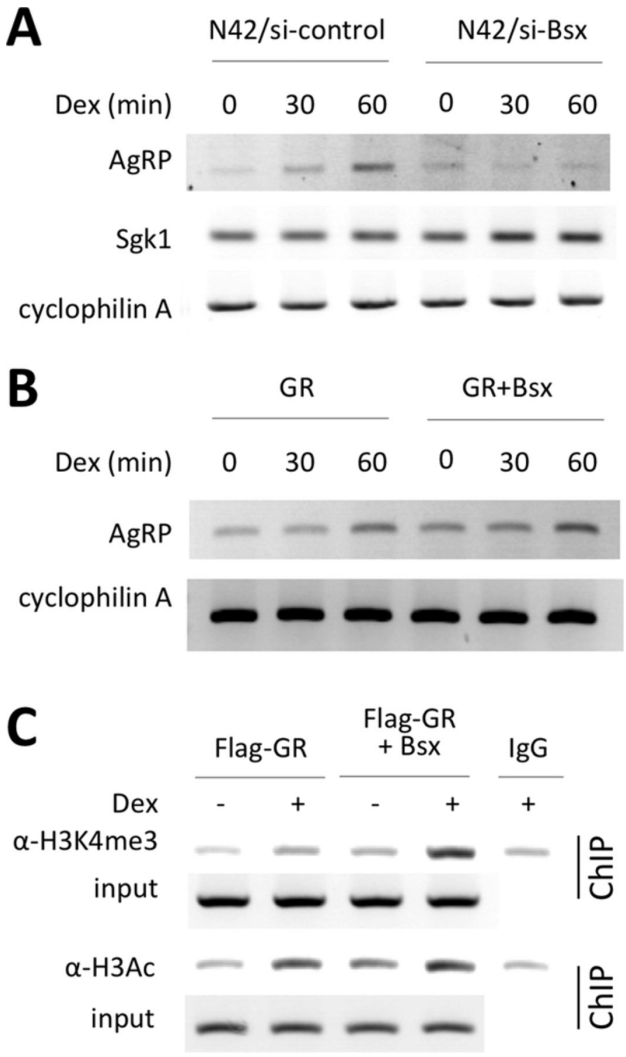

Figure 5. Bsx as an in vivo target selector of GR- and Gc-triggered assembly of active chromatin on the AgRP promoter. (A) Dex-dependent expression of AgRP and Sgk1, which are positively and negatively regulated by Bsx, respectively, were compared between N42 cells expressing either control siRNA or si-Bsx using RT-PCR. (B and C) Dex-dependent expression of AgRP (B) and formation of active chromatin on the AgRP promoter (C) were compared between P19 cells expressing either GR alone or both GR and Bsx using RT-PCR and ChIP, respectively. H3K4me3 and H3Ac indicate two open chromatin marks, trimethylated histone H3 lysine 4 and acetylated H3, respectively (C).

2.4.10. AgRP-GRE directs fasting-induced gene expression to AgRP-neurons.

Our data strongly suggest that the AgRP-GRE can function as a central sensor for the fasting-elevated peripheral Gc levels in triggering transactivation of *AgRP* in animals. To further test this idea in vivo, we made two enhanced GFP (eGFP) (Hechler et al. 2006) reporters, which are driven by the CMV minimal promoter linked to either the *AgRP* 1kb promoter fragment or seven copies of the 27-mer containing the AgRP-GRE alone (Fig. 6A, S7A). The reporter cassettes were flanked by insulators (Reddi et al. 2007). Both reporters were activated weakly by Dex, and this activation was further facilitated by Bsx in a Dex-dependent manner in HEK293 cells (Fig. S7B). Next, to investigate the in vivo response of the *AgRP* 1kb promoter and AgRP-GRE to fasting, we made transgenic mouse lines for both GFP reporters and analyzed the pattern of GFP expression in adult mice with or without 24h fasting. The *AgRP* 1kb promoter directed GFP expression weakly yet specifically in the ARC, but not in other brain areas (Fig. 6, B-D, and S8). Intriguingly, fasting led to a significant upregulation of GFP specifically in the ARC (Fig. 6B). Closer examination of GFP^+ cells revealed that GFP is expressed in AgRP-neurons expressing Bsx and GR (Fig. 6, C and D), but not in POMC-neurons (Fig. S8A). In addition, GFP was expressed neither in the DMH, which expresses Bsx but not GR, nor in the PVN, which expresses GR but not Bsx (Fig. S8, B and C). Remarkably, the transgenic mouse line with the $(AgRP\text{-}GRE)_7$:eGFP reporter also exhibited fasting-directed GFP induction in the Bsx^+ AgRP-neurons although overall GFP expression level was lower than that in

the transgenic mice with the *AgRP* 1kb promoter (Fig. S7, C and D), indicating that AgRP-GRE is capable of mediating fasting-triggered upregulation of *AgRP* gene. Our results suggest that *i)* AgRP-GRE alone is sufficient to recapitulate the endogenous fasting responsiveness of AgRP expression in AgRP-neurons, and that *ii)* for a robust fasting-dependent induction of AgRP, binding sites for both GR and Bsx as well as peripheral Gc signal are required.

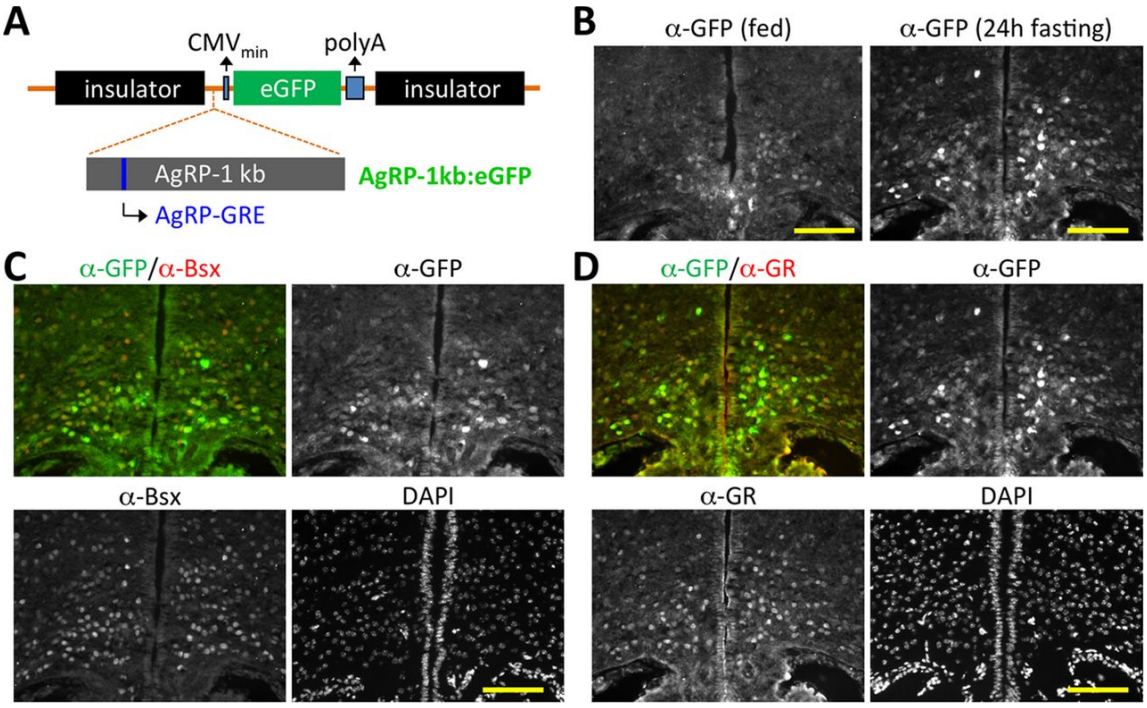

Figure 6. AgRP neuronal expression of GFP directed by 1-kb AgRP promoter in transgenic mice. (A) Schematic representation of an EGFP reporter flanked by two insulators and driven by a minimal CMV promoter fused to the 1-kb AgRP promoter. (B to D) The coronal sections of the ARC region of transgenic mice with the 1-kb AgRP promoter linked to EGFP, either fed (B) or fasted for 24 h (B to D), were immunostained with antibodies against GFP, Bsx, and GR. Yellow scale bars, 100 μm.

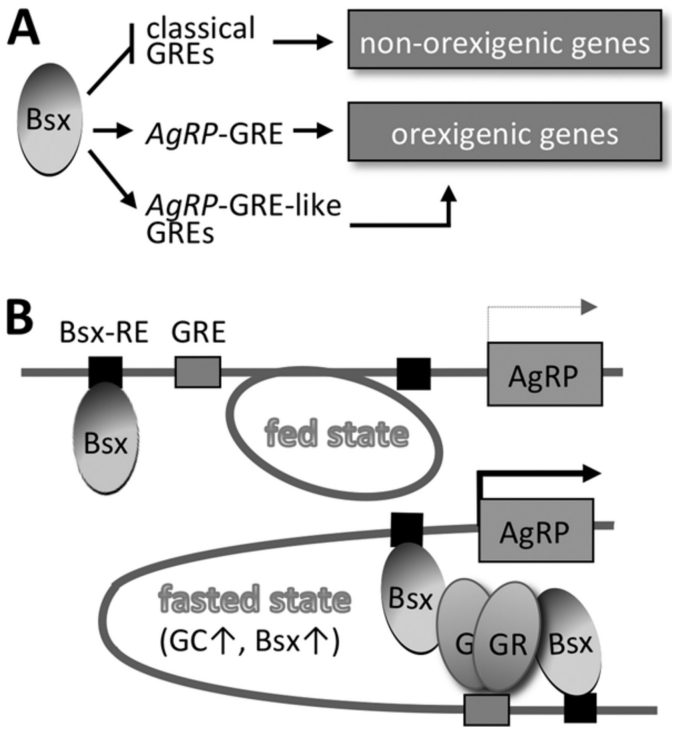

Figure 7. Roles of Bsx in AgRP neurons. (A) Dual function of Bsx segregates GREs into two groups, positive targets, such as AgRP-GRE, and negative targets. The positive targets can be associated with critical orexigenic genes in AgRP neurons. (B) The working model for the synergistic transactivation of the AgRP gene by GR and Bsx. Fasting increases nuclear GR due to increased Gc levels. Both GR and Bsx likely are required to form a transcriptionally active enhanceosome at the AgRP promoter. Protein-protein interactions between GR and Bsx, as well as binding sites for both GR and Bsx, likely play critical roles in the synergy.

Supplemental figure S1-S8

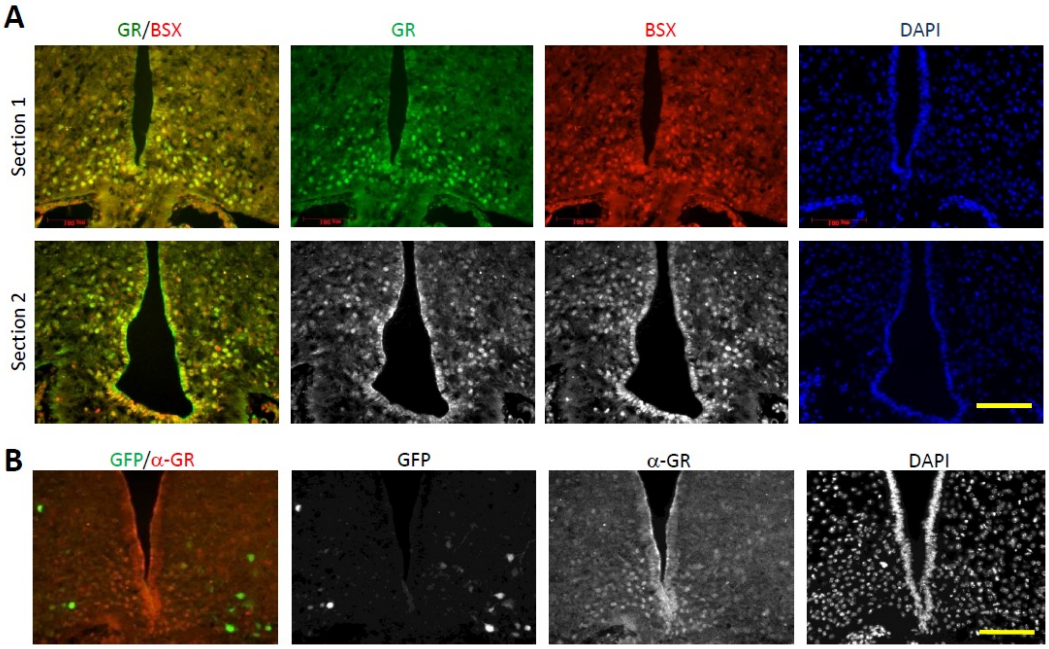

Figure S1. GR Expression in AgRP-neurons but not in POMC-neurons. (A) The color images shown in Fig. 1A are shown here in section 1. The images in section 2 are for an ARC location more rostral to the section 1. Although images similar to the section 2 images are typically used as representative AgRP-neuron-enriched images, more cuadal locations (like the above section 1 images) contain even more AgRP-neurons, as apparent in these images. (B) The ARC sections of GFPPOMC transgenic mice were immunostained with anti-GR antibody. GFP+ POMC neurons did not express GR. POMC- and AgRP-neurons are known to be located in the lateral part of the ARC and near the 3rd ventricle, respectively. In support of the fact that GFP+ cells are indeed POMC-neurons and GR+ cells are AgRP-neurons, GFP+ and GR+ cells are located in the lateral part of the ARC and near the 3rd ventricle, respectively. The yellow scale bar in the DAPI panel is for 100 μm.

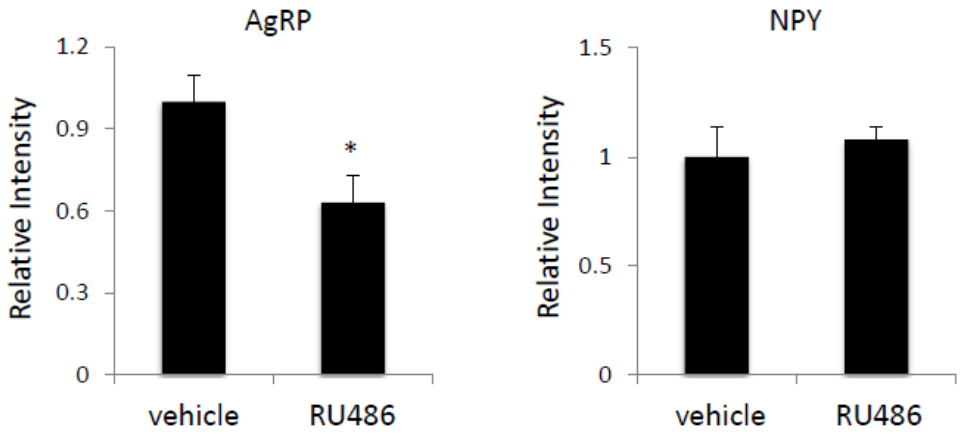

Figure S2. Intracerebroventricular (ICV) injection of GR antagonist, RU486. RU486 was introduced to the ARC via ICV injection (n=3 for each group), followed by in situ hybridization (ISH) for AgRP an NPY. The ISH signal intensity of serial coronal 12 μm sections of the ARC region was measured using ImageJ and plotted against the intensity of vehicle control samples.

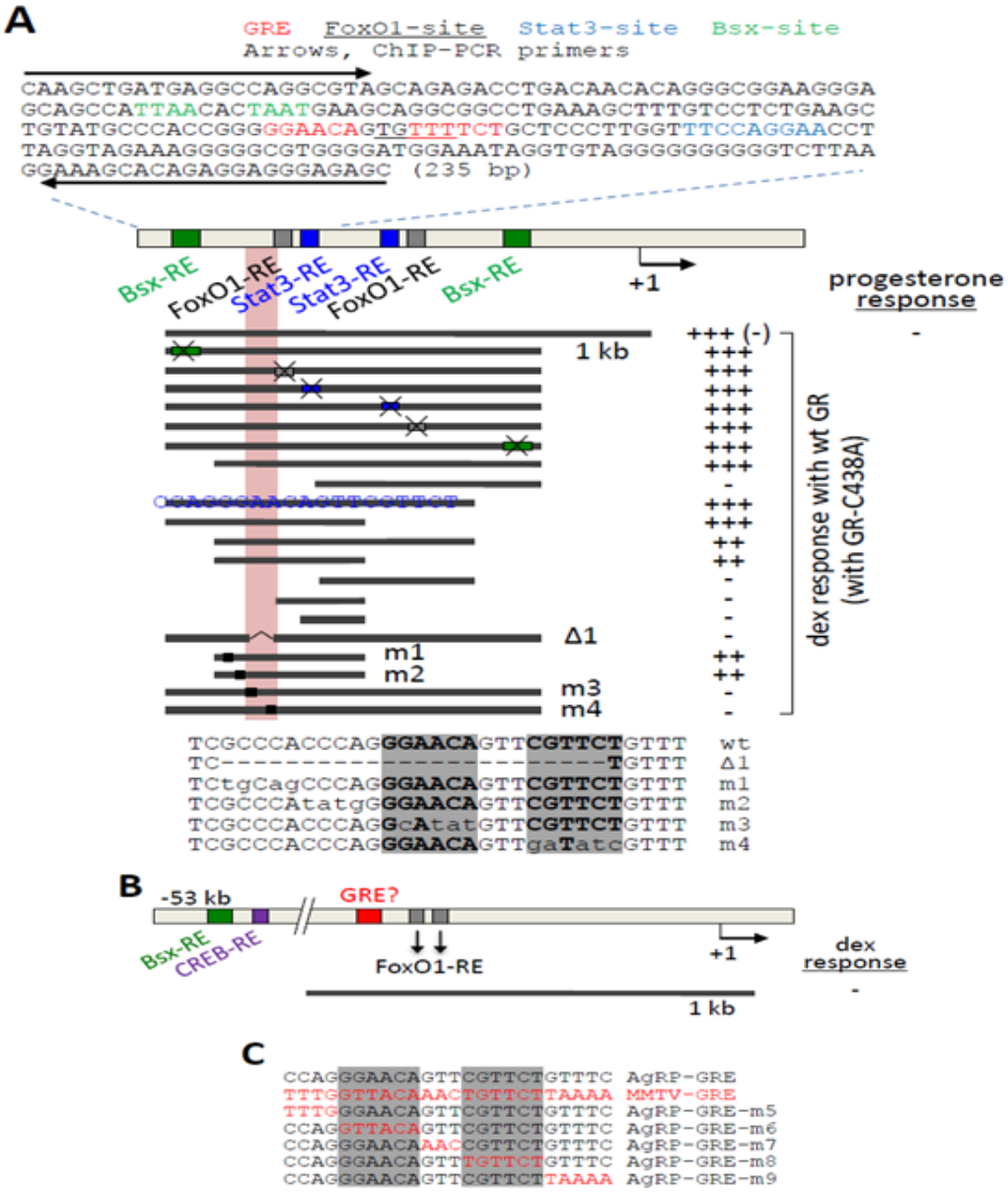
A
GRE FoxO1-site Stat3-site Bsx-site
Arrows, ChIP-PCR primers
CAAGCTGATGAGGCCAGGCGTAGCAGAGACCTGACAACACAGGGCGGAAGGGA
GCAGCCATTAACACTAATGAAGCAGGCGGCCTGAAAGCTTTGTCCTCTGAAGC
TGTATGCCCACCGGGGGAACAGTGTTTTCTGCTCCCTTGGTTTCCAGGAACCT
TAGGTAGAAAGGGGGCGTGGGGATGGAAATAGGTGTAGGGGGGGGGGTCTTAA
GGAAAGCACAGAGGAGGGAGAGC (235 bp)
Bsx-RE
FoxO1-RE
Stat3-RE
Stat3-RE
FoxO1-RE
Bsx-RE
+1
progesterone response
1 kb
Δ1
m1
m2
m3
m4
dex response with wt GR (with GR-C438A)
TCGCCCACCCAGGGAACAGTTCGTTCTGTTT wt
TC----------T----------T-----TGTTT Δ1
TCtgCagCCCAGGGAACAGTTCGTTCTGTTT m1
TCGCCCAtatgGGGAACAGTTCGTTCTGTTT m2
TCGCCCACCCAGGcAtatGTTCGTTCTGTTT m3
TCGCCCACCCAGGGAACAGTTgaTatcGTTT m4
B
-53 kb
GRE?
Bsx-RE
CREB-RE
FoxO1-RE
+1
dex response
1 kb
C
CCAGGGAACAGTTCGTTCTGTTTC AgRP-GRE
TTTGGTTACAAACTGTTCTTAAAA MMTV-GRE
TTTGGGAACAGTTCGTTCTGTTTC AgRP-GRE-m5
CCAGGTTACAGTTCGTTCTGTTTC AgRP-GRE-m6
CCAGGGAACAAACGTTCTGTTTC AgRP-GRE-m7
CCAGGGAACAGTTTGTTCTGTTTC AgRP-GRE-m8
CCAGGGAACAGTTCGTTCTTAAAA AgRP-GRE-m9

Figure S3. Analysis of AgRP/NPY Promoters. (A) Deletions and pointmutations were introduced to human AgRP promoter, which enabled us to map the AgRP-GRE (sequences shaded in gray). Both the Dex response and the synergy with Bsx were inseparable and required the AgRP-GRE. Dex failed to activate AgRP 1kb promoter in the presence of GR-C438A, a mutant GR incapable of binding to GRE. Progesterone also failed to activate AgRP 1kb promoter. Two primers for mouse hypothalamic ChIP assays are as shown, which produce a PCR product of 235 bp containing not only the AgRP-GRE but also the upstream Bsx-, FoxO1- and Stat3-sites. (B) Schematics for NPY promoter and the known response elements. The 1 kb NPY promoter containing a putative GRE did not respond to Dex. (C) Hybrid AgRPGRE motifs to map critical nculeotides in AgRP-GRE (see text).

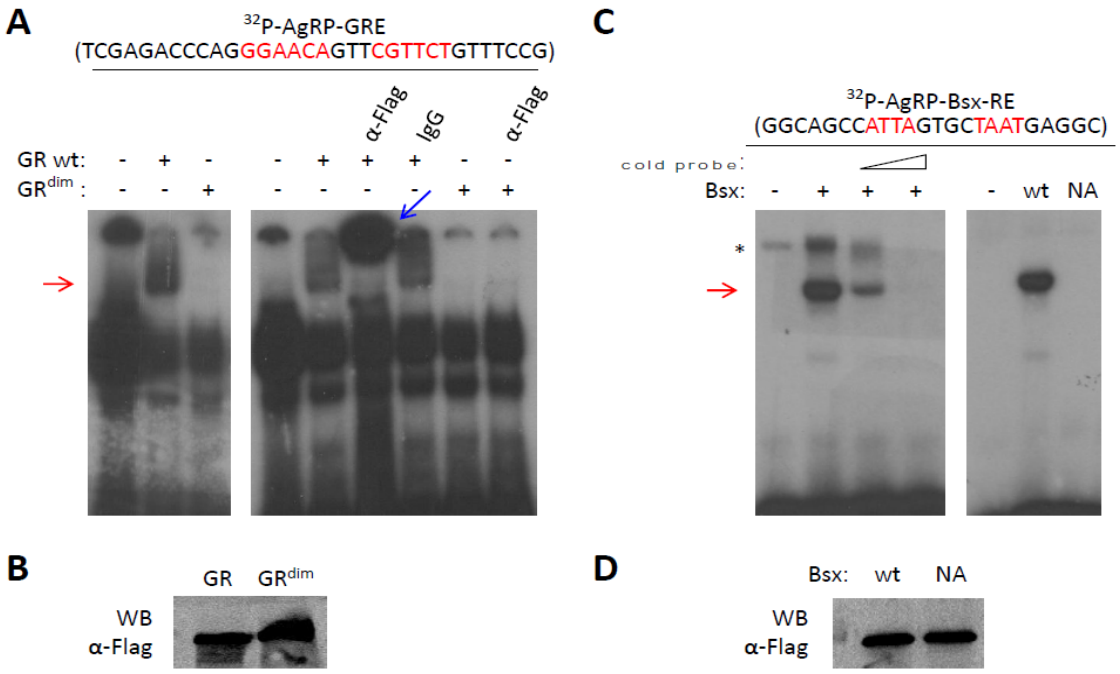

Figure S4. Binding of AgRP promoter by GR and Bsx. Gel shift assays were carried out to show binding of GR and Bsx to AgRPGRE (A) and Bsx-response elements (C), respectively. * indicates a non-specific binding. Red and blue arrows indicate specific GR or Bsx band (red) and super-shifted GR band (blue), respectively. GRdim indicates a dimerization-defective (hence a DNAbinding defective) mutant GR, GR-A458T. NA indicates N160A, a DNA-binding defective mutant Bsx protein with an 'N to A' mutation at the 160th amino acid in the DNA binding domain. The sequences of the probes are as shown (response elements highlighted in red). (B, D) Western blotting of in vitro translated Flag-tagged GR and Bsx proteins used in the gel shift assays.

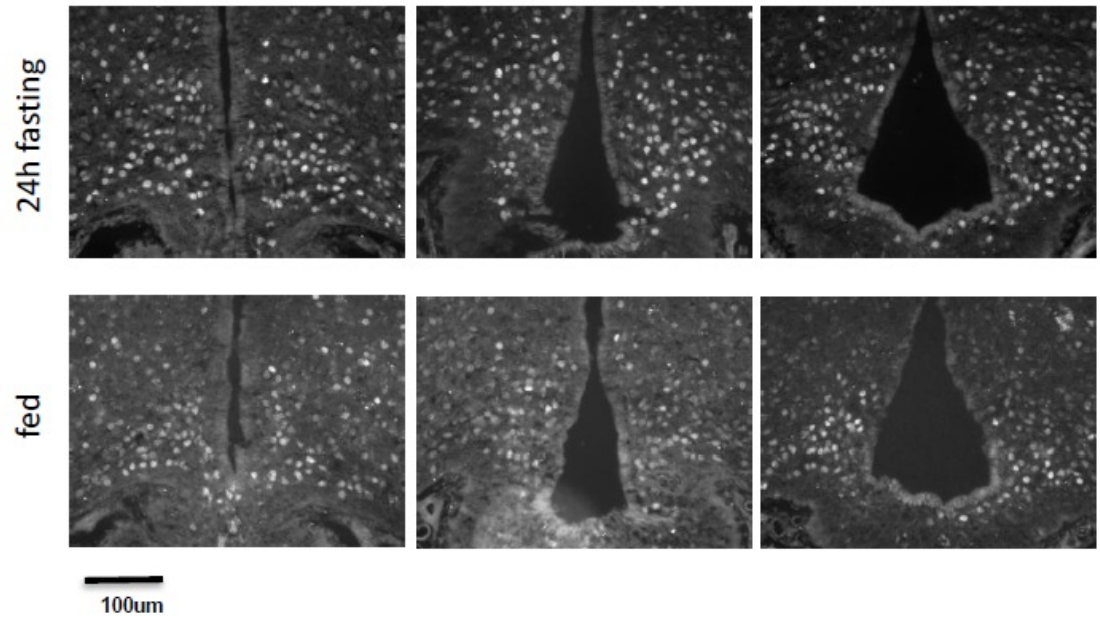

Figure S5. Immunostining of Bsx in the ARC. The coronal sections of the ARC region of wild-type mice, either fed (lower row) or fasted for 24h (upper row), were immunostained with antibody against Bsx. Bsx levels were enhanced upon fasting as reported (Nogueiras et al. 2008).

Ref.	GREs	+Dex	BSX+ DEX
1	mBcl-X_L (I)	+	-
	mBcl-X_L (II)	-	NA
	mBcl-X_L (III)	-	NA
2	TAT	+	-
3	Dax-1	+	-
4	MuRF1	+	-
5	ASBT (I)	+	-
	ASBT (II)	+	-
6	G6Pase (I, II)	-	NA
	G6Pase (III)	-	NA
7	β2-AR	+	-
8	TH	+	-
9	Sgk1	-	NA
10	ENaCα	-	NA
11	MT-1 (I)	+	-
	MT-1 (II)	+	-

Figure S6. Bsx as a Negative Regulator of GREs. 2-4 copies of the known GREs from the genes referenced below were cloned into the TK-LUC reporter. The response of these reporters to Dex alone or Dex+Bsx was examined in HEK293 cells. 10 out of 16 GREs directed robust Dex response (indicated as + in +Dex column) and all of these Dex responses were potently suppressed by Bsx (indicated as – in Bsx+Dex column). GREs that did not respond to Dex were not affected by the presence of Bsx (NA).

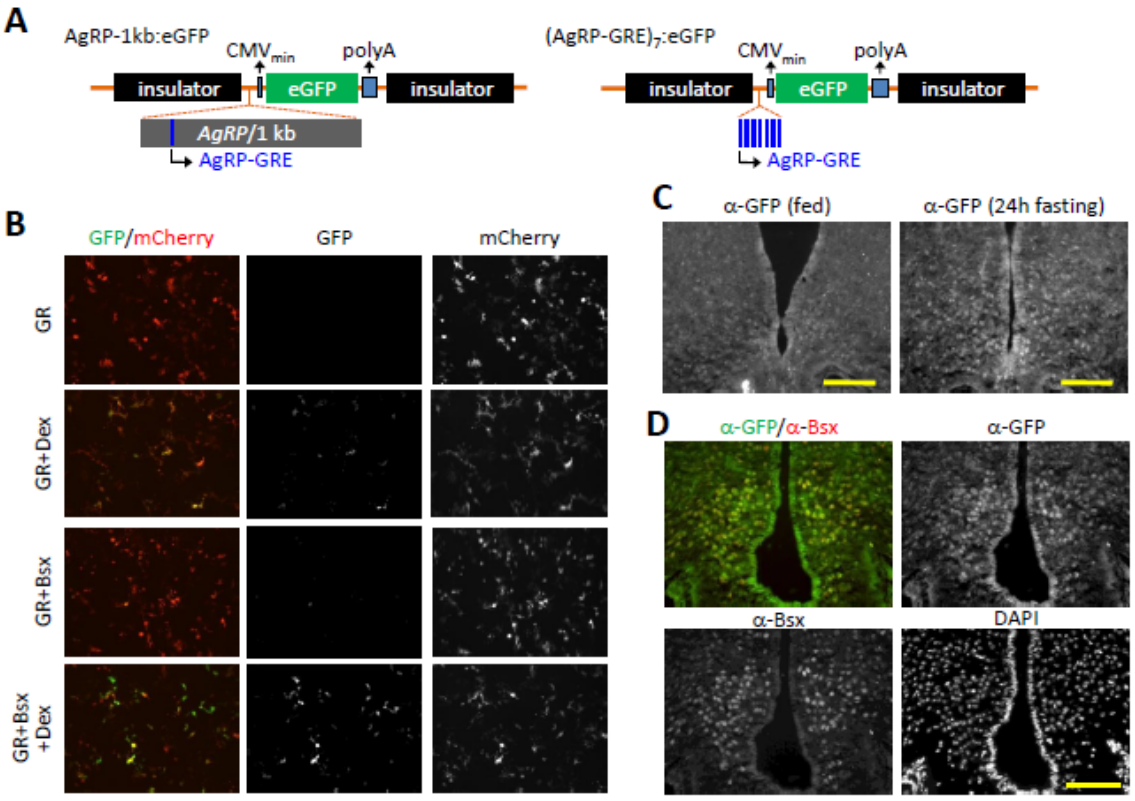

Figure S7. AgRP-GRE-directed Expression of GFP in HEK293 and AgRP-neurons. (A) Schematics of AgRP-1kb:eGFP and $(AgRP\text{-}GRE)_7$:eGFP reporters. (B) In HEK293 cells transfected with GR-expression vector, AgRP-1kb:eGFP reporter directed GFP expression in a Dex-dependent manner. Coexpression of Bsx significantly potentiated the Dex response. mCherry was used as a transfection indicator. Similar results were also obtained with $(AgRP\text{-}GRE)_7$:eGFP reporter. (C, D) The coronal sections of the ARC region of transgenic mice with $(AgRP\text{-}GRE)_7$:eGFP, either fed (C) or fasted for 24h (C, D), were immunostained with antibodies against GFP and Bsx, as indicated

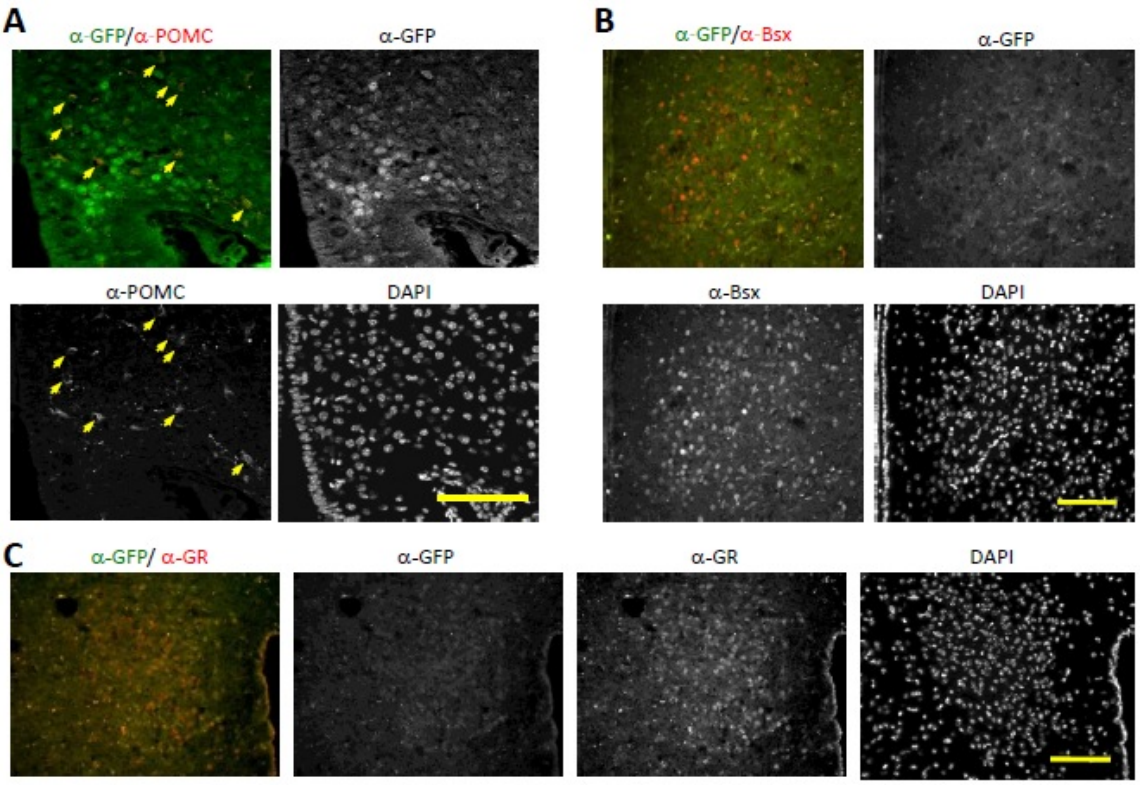

Figure S8. Lack of GFP Expression in POMC-neurons, the DMH, and the PVN. (A) GFP+ cells in the ARC of AgRP-1kb:eGFP transgenic mice were not immunostained with anti-POMC antibody. Of note, POMC antibody immunostained not only POMC-neuronal cell bodies (indicated as yellow arrows) but also axons and synaptic termini. (B, C) GFP expression was hardly detected either in the DMH (B) or in the PVN (C), while the DMH and the PVN expressed Bsx and GR, respectively. The yellow scale bars denote 100 μm (A-C).

2.5. Discussion

Gc is a physiologically well-defined peripheral orexigenic cue (Fehm et al. 2004; Coppola and Diano 2007). However, the underlying molecular mechanism for the appetite-stimulating action of Gc has been unclear. In this study, we show that the gene encoding the orexigenic neuropeptide AgRP is a direct target of GR in AgRP-neurons. Furthermore, we found that specific sequences in AgRP-GRE mediate the synergistic induction of the *AgRP* gene by Bsx and GR in response to Gc signal, which sets AgRP-GRE apart from conventional GREs. The unique feature of AgRP-GRE allowed us to identify other putative common target genes of GR and Bsx in AgRP-neurons (Fig. 7A), as exemplified by our discovery of functional AgRP-GRE-like motifs in two genes, *Per1* and *Asb4* that are expressed in ARC neurons (Fig. 4, F and G). Future genome-wide search for AgRP-GRE-like motifs (e.g. beyond the 10 kb limit that we initially used in this study), coupled with further dissection of critical nucleotides in AgRP-GRE, is expected to uncover additional, key target genes of GR/Bsx in AgRP-neurons that play important roles in central Gc actions. Studies of these genes would facilitate our understanding of the orexigenic function of the Gc signal and Bsx in AgRP-neurons. For instance, *Per1,* as a putative target of Gc and Bsx, might link circadian rhythm, which is tightly coupled to food intake (Sahar and Sassone-Corsi 2012), to the control of energy balance by AgRP-neurons. Asb4, another putative target of Gc and Bsx, could play a role in the cross-talk between insulin and Gc signals in CNS, considering that it mediates degradation of insulin

receptor substrate 4 in the hypothalamic AgRP-/POMC-neurons (Li et al. 2011).

GR is known to target distinct sets of genes in different tissues (Stahn et al. 2007). Our results suggest that Bsx determines the scope of GR target genes in AgRP-neurons through its GRE sequence-dependent dual function that activates some GREs (e.g., AgRP-GRE, Per1-GRE and Asb4-GRE) while simultaneously suppressing other GREs (Fig. 7A). For instance, Bsx inhibits the GRE in *tyrosine hydoxylase (TH)* (Fig. 4F and S6), which was recently shown to be downregulated in the ARC (Jovanovic et al. 2010). Active silencing of *tyrosine hydoxylase (TH),* a marker of dopaminergic neuron, by Bsx may be important for the function of AgRP neurons, in which synaptic release of GABA is required for normal regulation of energy balance (Tong et al. 2008). Overall, the dual function of Bsx would pose a distinct advantage in surviving food restriction, which increases levels of Gc (i.e., increased levels of activated, nuclear GR), by allowing GR to upregulate a specific set of targets while keeping unnecessary GRE-containing genes silent in AgRP-neurons (Fig. 7A). In other tissues or cell types, factors similar to Bsx may also act as a target selector by activating only a subset of GR target genes while actively suppressing other targets of GR.

Our results revealed that AgRP-GRE serves as a focal regulatory point that responds to fasting or peripheral Gc signal by dictating a synergy of Gc-bound GR and Bsx (Fig. 7B). First, both Bsx and GR are required for activating expression of AgRP in vivo, as evident by our finding that the 1 kb *AgRP* promoter drives the target gene expression to AgRP-neurons, which express both GR and Bsx, but not to other cell types expressing either GR or Bsx alone

(Fig. 6, S8). Second, Gc-activated GR is a permissive factor for Bsx in upreguating AgRP expression, given that Bsx alone failed to activate the 1 kb *AgRP* promoter construct containing the Bsx sites in the absence of Dex (Fig. 3A). It is notable that, while AgRP-GRE was sufficient to direct a synergy of GR and Bsx in reporter assays, the synergy between GR and Bsx was more robust in the 1 kb *AgRP* reporter containing AgRE-GRE and Bsx-binding sites than in AgRE-GRE alone (Fig. 3, A and B). Consistently, DNA-binding defective mutant Bsx was not as efficient as wild type Bsx in enhancing GR activity in the 1 kb *AgRP* reporter (Fig. 3C). These results suggest that Bsx-binding to its cognitive sites also contributes to a strong activation of AgRP. It is possible that, under physiological fasting condition, levels of Gc and Bsx may not be high enough to enable the synergy through AgRP-GRE alone and thus both AgRP-GRE and Bsx-sites are required for the maximal synergy. Overall, our results provide compelling evidence supporting that AgRP-GRE, together with the Bsx-binding sites, senses the rise in Gc levels upon fasting, thereby leading to upregulation of AgRP (Fig. 7B).

Given that Gc induces expression of both NPY and AgRP (Savontaus et al. 2002; Dhillo et al. 2003; Makimura et al. 2003; Shimizu et al. 2008), our results that GR directly regulates expression of AgRP, but likely not NPY, were unexpected. Neither RU486 injection nor AgRP-neuron-specific deletion of GR blocked fasting-dependent induction of NPY (Fig. 1, B-D, and S2). Dex failed to activate the NPY promoter (Fig. S3B). It is possible that Gc regulates expression of NPY using a different mechanism that does not involve GR. Alternatively,

inhibition of Gc/GR action alone may not be sufficient to shut down fasting-mediated induction of NPY expression due to redundant pathway that is also activated by fasting. Further investigation is warranted to clarify this issue.

In summary, we found that GR and Bsx synergistically activate expression of AgRP via AgRP-GRE. Moreover, our dissection of AgRP-GRE defined a new class of GREs that dictates a positive action of Bsx. Future identification and characterization of more genes with AgRP-GRE-like motifs would provide critical insights into the molecular understanding of the central orexigenic action of Gc/GR and Bsx in the AgRP-neurons.

Chapter 3

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

Sun-Gyun Kim,[1] Bora Lee, [1,2] Dae-Hwan Kim,[1] Juhee Kim, [1] Seunghee Lee, [3] Soo-Kyung Lee, [1,2,4] Jae W. Leea, [2,5]

Neuroscience Section, Papé Family Pediatric Research Institute, Department of Pediatrics[1], Department of Cell and Developmental Biology[2] and Vollum Institute,[4] Oregon Health & Science University, Portland, Oregon, USA

College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, South Korea[3]

Division of Integrative Biosciences and Biotechnology, Pohang University of Science and Technology, Pohang, South Korea[5]

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3.1. Abstract

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.

3.2. Introduction

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) (Ellacott and Cone 2004; Seeley et al. 2004). 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 (Ellacott and Cone 2004; Seeley et al. 2004). 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 (Ellacott and Cone 2004; Seeley et al. 2004).

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 (Bantubungi et al. 2012). 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 (Olofsson et al. 2009). 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 (de Souza et al. 2011). 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 (Varela et al. 2012). 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 (Lee et al. 2013). 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 expression 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.

3.3. Materials and Methods

3.3.1. 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.

3.3.2. 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) (Pearen et al. 2006) and lentiviral particles (as a control) and CREB short hairpin RNA from Santa Cruz.

3.3.3. 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 (Castro et al. 2001; Nilaweera et al. 2002). In situ hybridization (ISH) was performed essentially as described previously (Fishilevich et al. 2005; Thompson et al. 2006).

Double-fluorescent ISH was performed as described previously (Fishilevich et al. 2005) 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).

3.3.4. 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 (Lee et al. 2013).

3.3.5. 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) (Nomiyama et al. 2006) or two copies of the AgRP-GRE (nucleotides −691 to −665) (Lee et al. 2013) 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 (Lee et al. 2013).

3.3.6. 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).

3.3.7. 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.

3.4. Results

3.4.1. 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. 8A). 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. 7B 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. 8C and D; data not shown). In contrast, αMSH levels were similar between wild-type and NOR1-null mice under the fed condition (Fig. 8C and D). These results suggested an unanticipated role for NOR1 in suppressing the expression of AgRP/NPY in AgRP neurons.

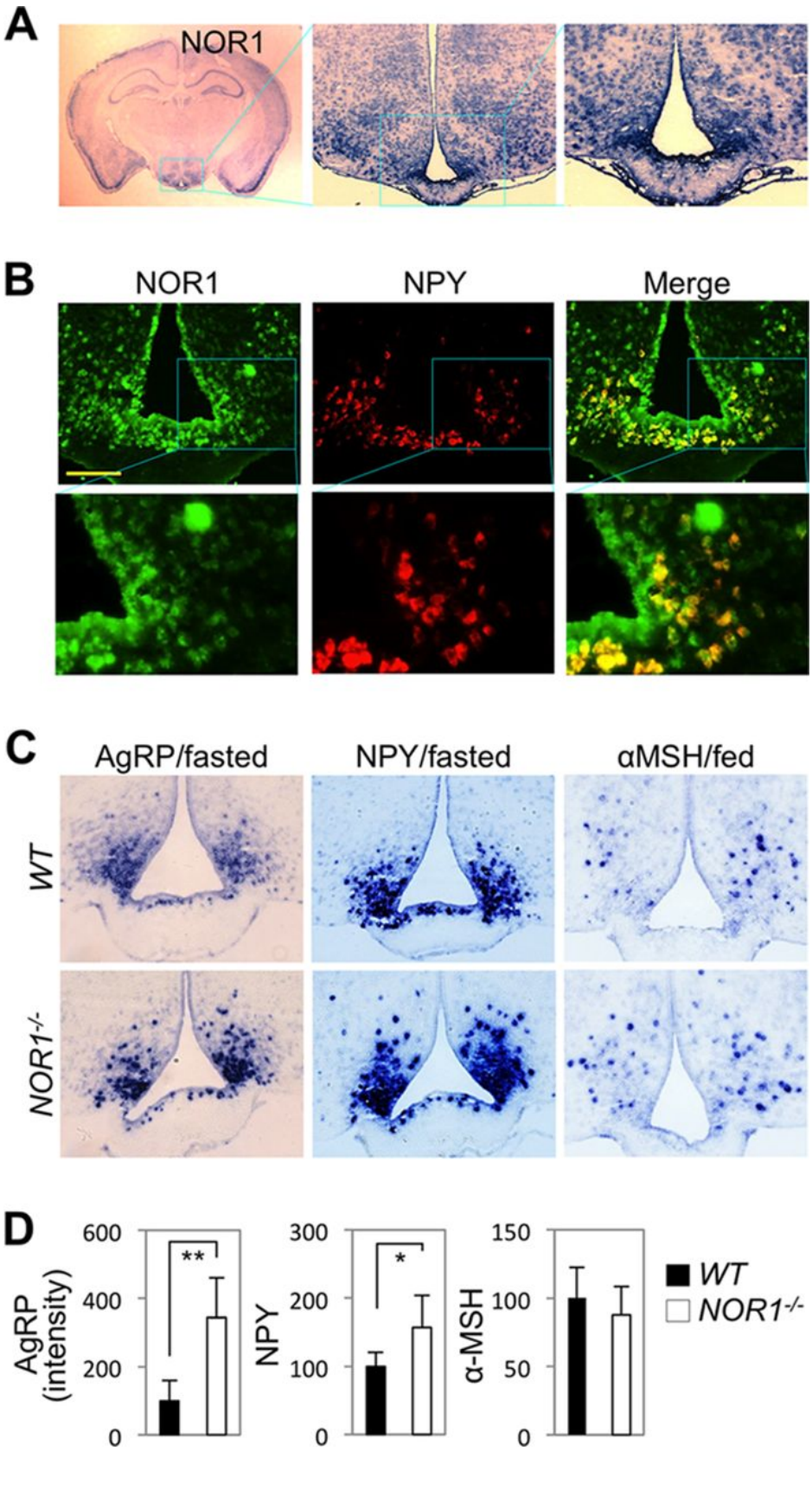

A
NOR1
B
NOR1
NPY
Merge
C
AgRP/fasted
NPY/fasted
αMSH/fed
WT
NOR1-/-
D
AgRP (intensity)
600
400
200
0
**
NPY
300
200
100
0
*
α-MSH
150
100
50
0
WT
NOR1-/-

Figure 8. 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. *, $P < 0.05$; **, $P < 0.001$.

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 (Ponnio et al. 2002). 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. 9A and E). Moreover, likely with the increased activity, NOR1-null mice lost more body weight than wild-type mice upon fasting (Fig. 9B, C, F, and G) but clearly consumed more food during refeeding (Fig. 9D 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. 9D 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.

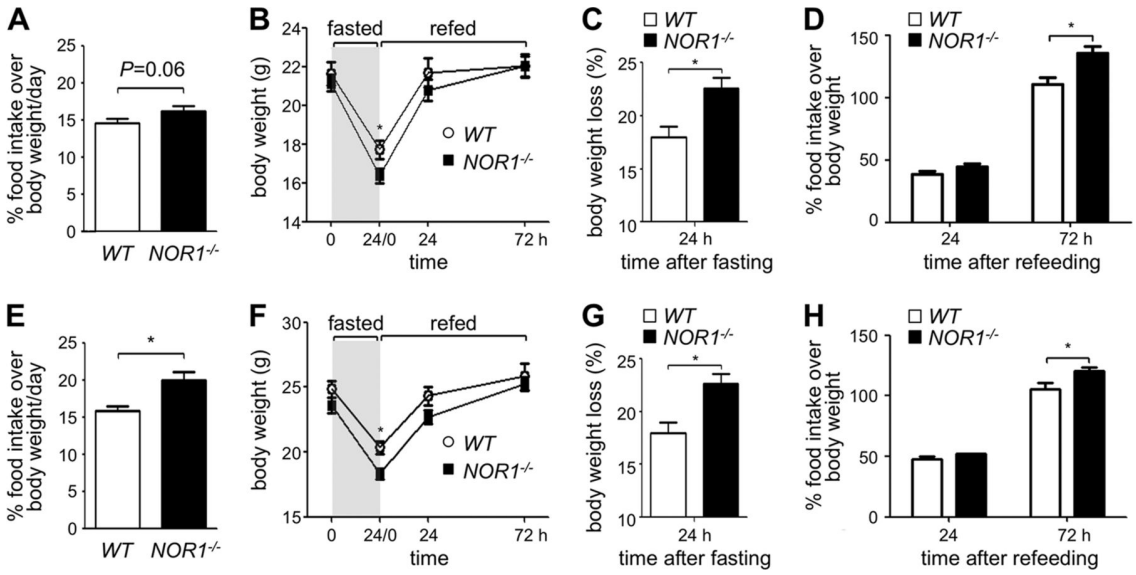


Figure 9. Increased food intake with NOR1-null mice. For male (A to D) and female (E to H) wild-type and NOR1-null mice (5 to 8 mice per each group), we measured daily food intake for 6 days and calculated the percent food intake over body weight per day (A and E), finding that NOR1-null mice consume 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. *, $P < 0.05$.

3.4.2. 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 (Lee et al. 2013). Of note, NOR1 is known to antagonize GR transactivation, likely via protein-protein interactions with GR (Martens et al. 2005). 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. 10A). In contrast, AgRP expression was increased in the presence of si-NOR1 (Pearen et al. 2006) (Fig. 10B). In addition, the Dex-dependent transactivation of a luciferase reporter driven by two copies of the AgRP-GRE was significantly impaired by NOR1 in HEK293 cells (Fig. 10C). Moreover, our ChIP analysis with mouse hypothalamus lysates revealed that the AgRP-GRE is occupied by NOR1 (Fig. 10D). Interestingly, the amount of NOR1 on the AgRP-GRE under fasting conditions was similar to that under fed conditions (Fig. 10D), despite the fact that NOR1 levels were significantly lower under fasting conditions (Fig. 11). 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. 10E). 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. 10E). More importantly, the amount of GR bound to the AgRP-GRE was significantly decreased by ectopic expression of NOR1 (Fig. 10E; compare the results between Dex plus vector and Dex plus NOR1). These results, together with the reported interactions between GR and NOR1 (Martens et al. 2005), 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.

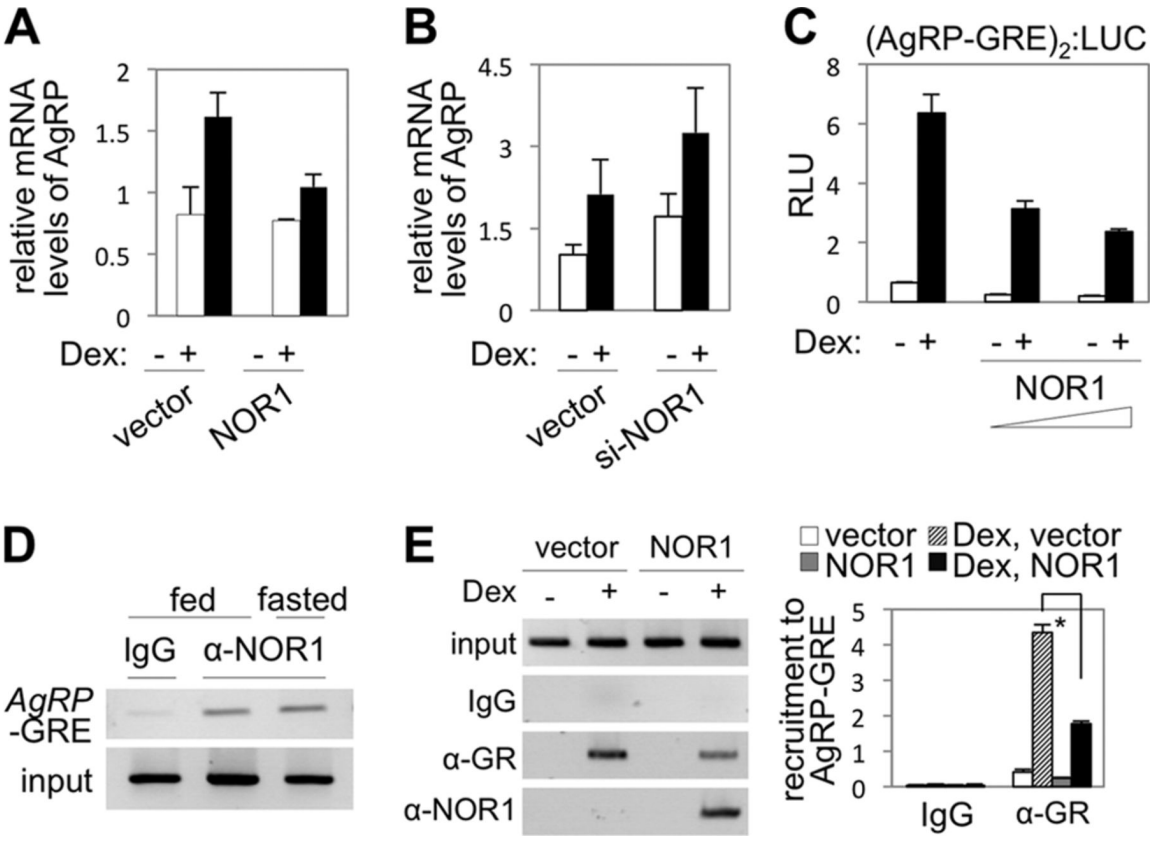


Figure 10. NOR1 interferes with Gc induction of AgRP. (A and B) Quantification of AgRP levels using qRT-PCR in P19 cells treated with either vehicle or 10 nM Dex for 4 h under the indicated conditions. (C) Luciferase (LUC) reporter assays in HEK293 cells for a reporter driven by two copies of the AgRP-GRE 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. *, $P < 0.05$.

3.4.3. Regulation of NOR1 expression by Gc.

Our finding that the expression of AgRP and NPY is suppressed by NOR1 (Fig. 8), 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. 11A to C). As expected, AgRP expression was induced by fasting, while αMSH levels were suppressed by fasting (Fig. 11A). 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 (Kobayashi et al. 1999), were increased by Dex, NOR1 expression was dramatically reduced by Dex (Fig. 11D 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 (Lee et al. 2013) (Fig. 11C; 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.

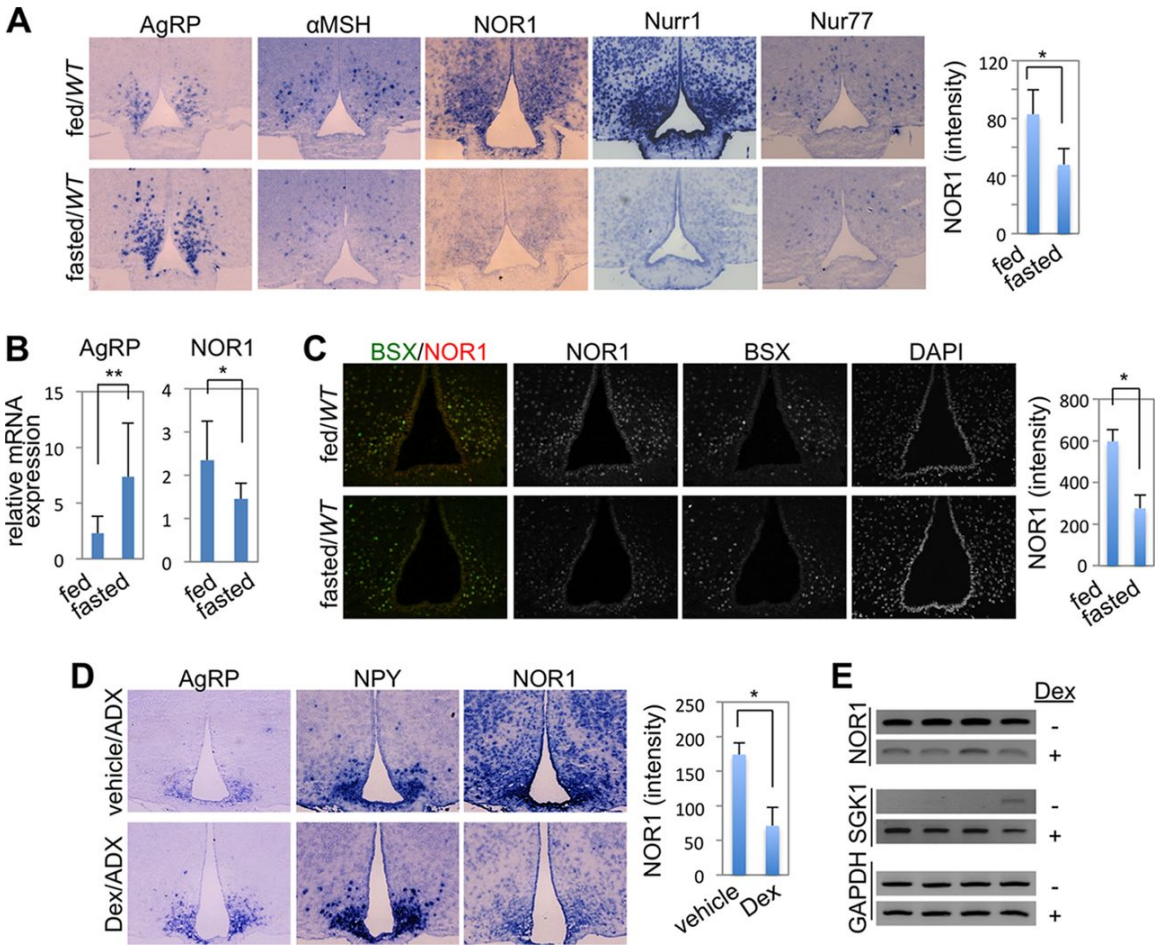

A
AgRP
αMSH
NOR1
Nurr1
Nur77
fed/WT
fasted/WT
NOR1 (intensity)
120
80
40
0
*
fed
fasted
B
AgRP
NOR1
relative mRNA expression
15
10
5
0
**
4
3
2
1
0
*
fed
fasted
C
BSX/NOR1
NOR1
BSX
DAPI
fed/WT
fasted/WT
NOR1 (intensity)
800
600
400
200
0
*
fed
fasted
D
AgRP
NPY
NOR1
vehicle/ADX
Dex/ADX
NOR1 (intensity)
250
200
150
100
50
0
*
vehicle
Dex
E
Dex
NOR1
SGK1
GAPDH
-
+

Figure 11. 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). *, $P < 0.05$.

3.4.4. 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 (Ellacott and Cone 2004; Seeley et al. 2004). 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. 12A). 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. 12B). 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. 12C).

Our results demonstrate that the expression of NOR1 in the ARC is regulated by both orexigenic (e.g., Gc) and anorexigenic (e.g., leptin) cues and raise an interesting possibility that NOR1 serves as their major effector in regulating the expression of AgRP/NPY.

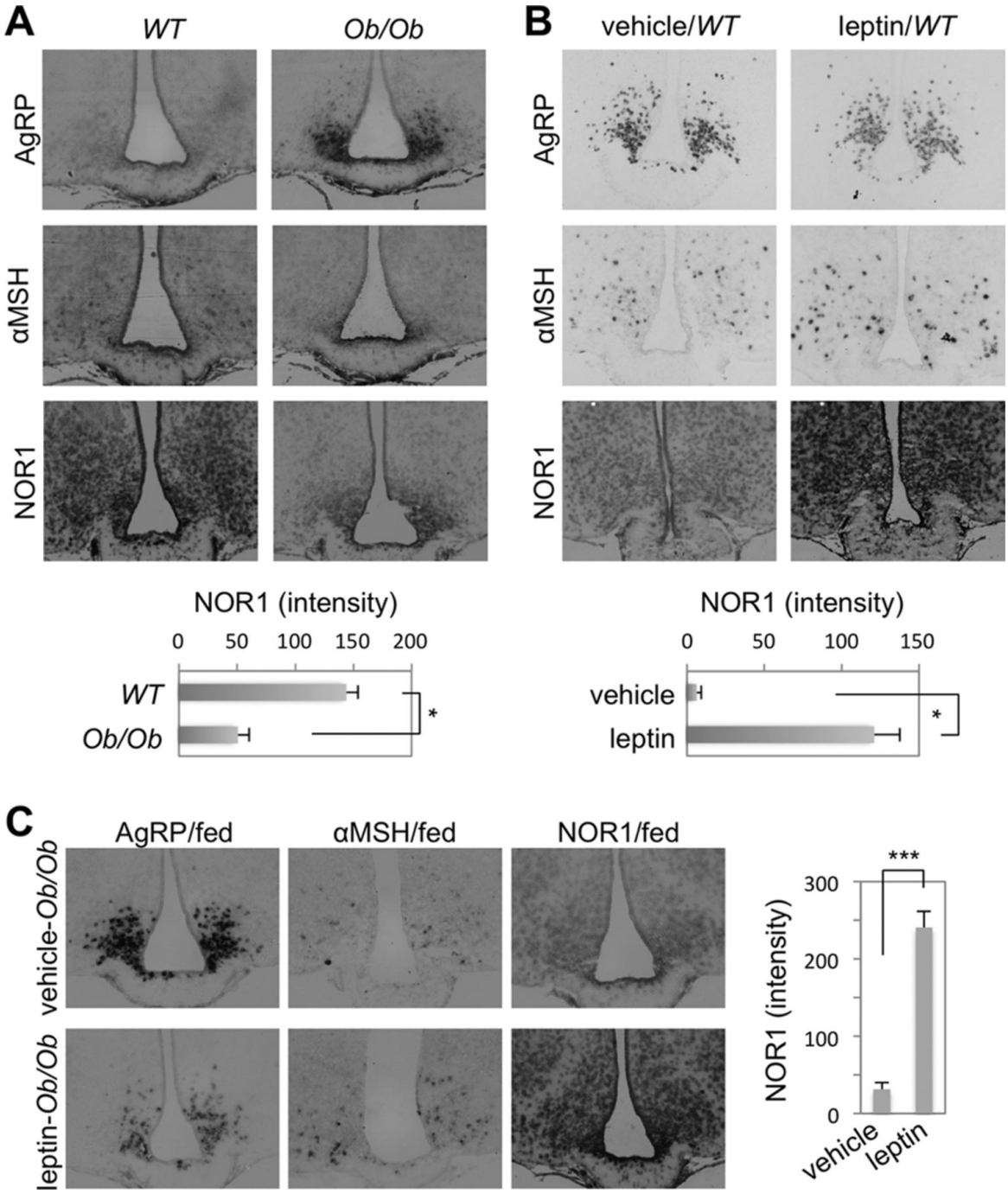

A
WT
Ob/Ob
AgRP
αMSH
NOR1
B
vehicle/WT
leptin/WT
AgRP
αMSH
NOR1
NOR1 (intensity)
0
50
100
150
200
WT
Ob/Ob
*
NOR1 (intensity)
0
50
100
150
vehicle
leptin
*
C
AgRP/fed
αMSH/fed
NOR1/fed
vehicle-Ob/Ob
leptin-Ob/Ob
***
NOR1 (intensity)
300
200
100
0
vehicle
leptin

Figure 12. 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. *, $P < 0.05$; ***, $P < 0.0001$.

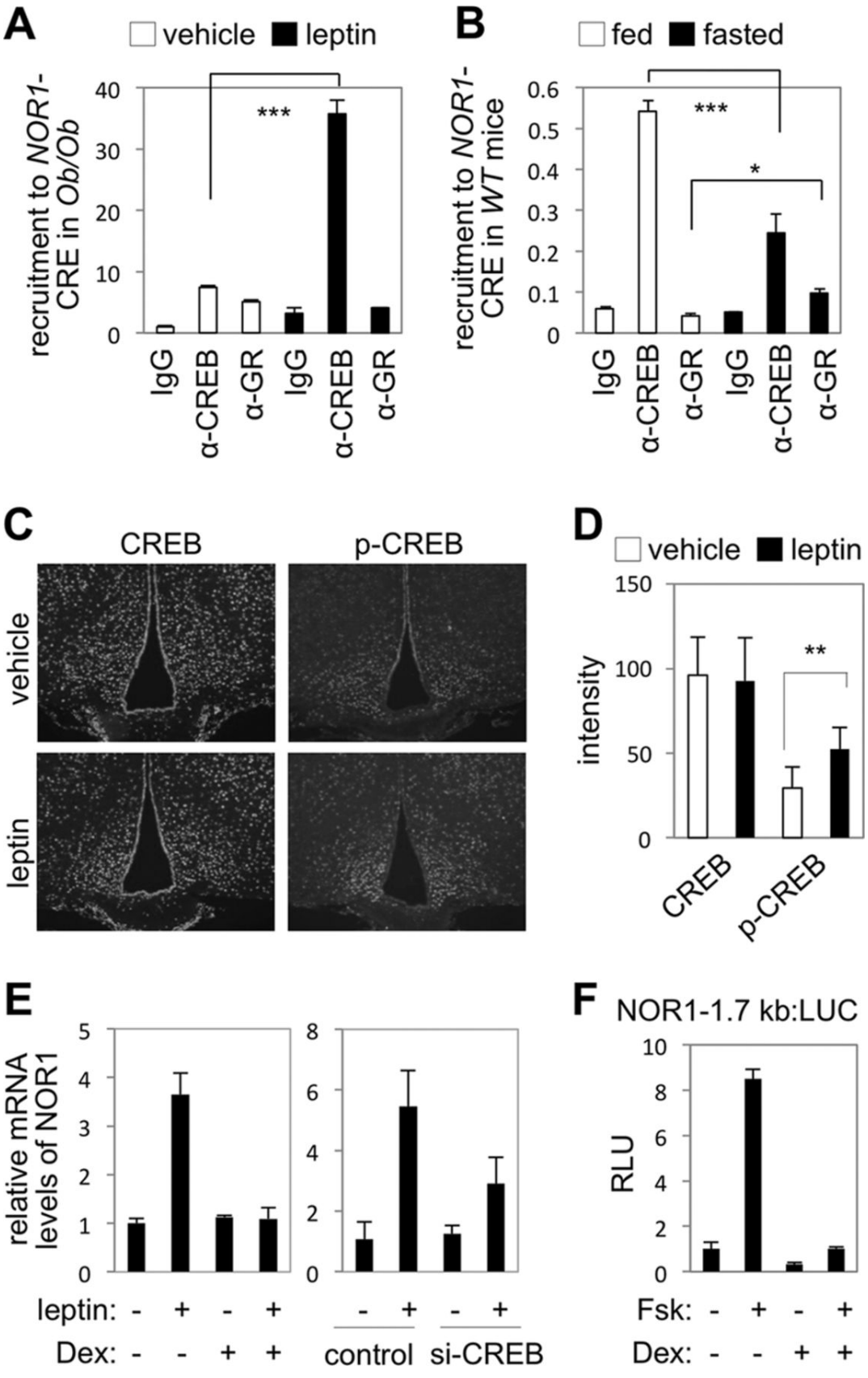
3.4.5. Interplays of leptin, CREB, and Gc in NOR1 expression.

Leptin has been shown to utilize the transcription factor cyclic AMP response element-binding protein (CREB) in enhancing the expression of cyclin D1 (Catalano et al. 2009) and an intestinal epithelial apical membrane transporter, PepT1 (Nduati et al. 2007). 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 (Duan et al. 2007). In addition, CREB has been reported to upregulate NOR1 expression via three copies of the cAMP response element (CRE) in the NOR1 promoter (Ohkubo et al. 2000; Rius et al. 2004; Nomiyama et al. 2006). Furthermore, mutual cross interference between GR and CREB has been observed (Stauber et al. 1992). 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.

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. 13A). 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. 13B), 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. 13B), 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. 13C 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 (Weng et al. 2007), 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. 12B 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 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. 13E, left). Moreover, leptin induction of NOR1 expression in N42 cells was impaired by siRNA against CREB (si-CREB; Santa Cruz) (Fig. 13E, 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. 13F).

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 (Ellacott and Cone 2004; Seeley et al. 2004).


A
vehicle
leptin
recruitment to NOR1-CRE in Ob/Ob
40
30
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***
IgG
α-CREB
α-GR
IgG
α-CREB
α-GR
B
fed
fasted
recruitment to NOR1-CRE in WT mice
0.6
0.5
0.4
0.3
0.2
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0
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*
IgG
α-CREB
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IgG
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C
CREB
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vehicle
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leptin
intensity
150
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50
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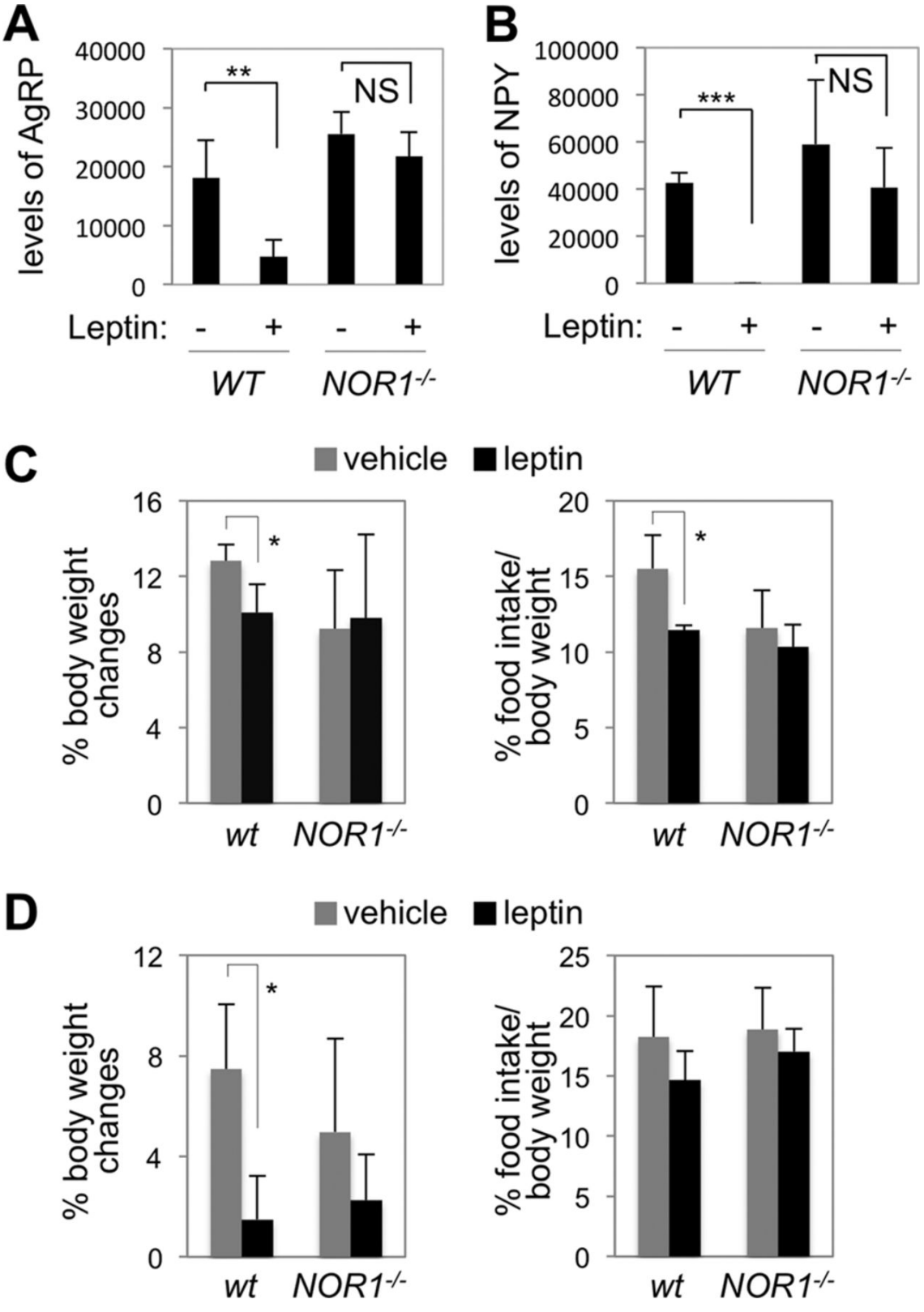
CREB
p-CREB
E
relative mRNA levels of NOR1
5
4
3
2
1
0
8
6
4
2
0
leptin: - + - +
Dex: - - + +
- + - +
control si-CREB
F
NOR1-1.7 kb:LUC
RLU
10
8
6
4
2
0
Fsk: - + - +
Dex: - - + +

Figure 13. 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). *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

3.4.6. 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. 14A 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. 14C 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. 14C). 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.


A
levels of AgRP
40000
30000
20000
10000
0
**
NS
Leptin: - + - +
WT
NOR1-/-
B
levels of NPY
100000
80000
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40000
20000
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NS
Leptin: - + - +
WT
NOR1-/-
C
vehicle
leptin
% body weight changes
16
12
8
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NOR1-/-
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vehicle
leptin
% body weight changes
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NOR1-/-
% food intake/ body weight
25
20
15
10
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0
wt
NOR1-/-

Figure 14. 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.

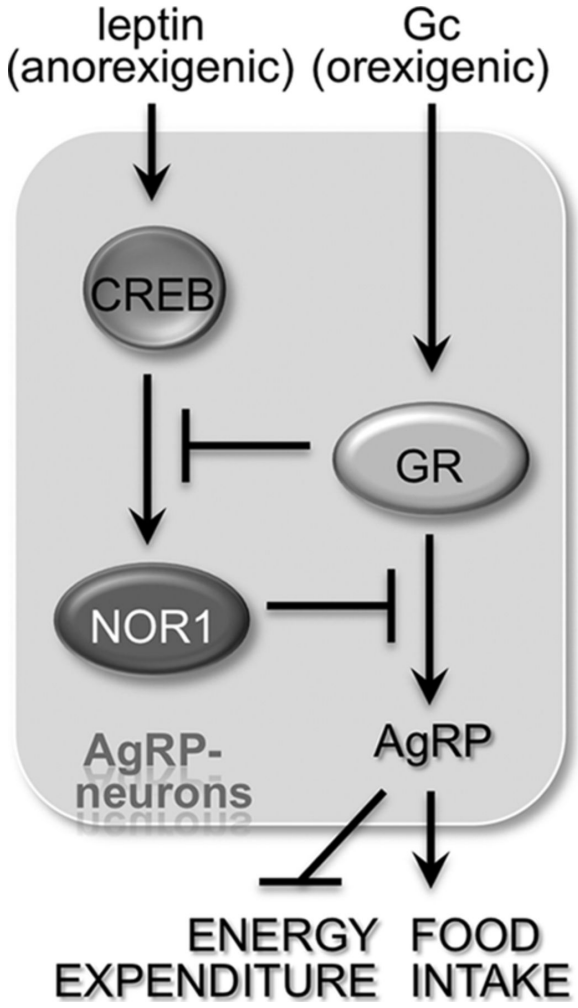


Figure 15. Working model (see the text)

3.5. Discussion

NRs regulate a diverse array of physiological processes in the human body, including the CNS control of energy balance (Bantubungi et al. 2012). However, the detailed molecular basis for their roles in POMC and AgRP neurons, the first two groups of neurons that run into peripheral 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. 15). 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. 15). 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. 8B). 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 (Savontaus et al. 2002; Dhillo et al. 2003;

Makimura et al. 2003; Shimizu et al. 2008). 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 (Lee et al. 2013). Interestingly, inactivation of AgRP neuronal GR did not appear to affect the fasting-dependent induction of NPY expression (Lee et al. 2013), 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. 10E).

Expression of NOR1 as well as its closest homologues, Nurr1 and Nur77, is similarly regulated by a wide array of signaling cues (Mohan et al. 2012), 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. 11A). 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 (Chen et al. 1998; McEvoy et al. 2002; Ohkubo et al. 2002). 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.

11A). 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 (Martens et al. 2005). 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 (Klemm et al. 1998). In this regard, it is interesting to note that Nur77 has been identified as a positive target gene of insulin in skeletal muscle (Wu et al. 2007). 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 (Wauman and Tavernier 2011), 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. 13). Interestingly, leptin has been shown to stimulate nuclear factor κB (NF-κB) and hypoxia-inducible factor 1α (HIF1α) (Bouloumie et al. 1999; Gonzalez-Perez et al. 2010), two transcription factors that have also been shown to be capable of upregulating NOR1 expression (Martorell et al. 2009; Zhao et al. 2010). 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(Zhang et al. 2008).

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. 15). This prediction is strongly supported by our results that NOR1-null mice showed higher levels of AgRP and NPY expression (Fig. 8C and D), a significant decrease in the ability of leptin to lower AgRP/NPY levels (Fig. 14A and B), increased refeeding behavior upon fasting (Fig. 9), and relative resistance to leptin in body weight loss (Fig. 14C 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.

Chapter 4

Fate specification of multiple arcuate nucleus neurons by the LIM-homeobox transcription factor Isl1

Bora Lee[1,2], Seunghee Lee[3], Soo-Kyung Lee[1,2,4] and Jae W. Lee[1,2]*

[1]Neuroscience Section, Papé Family Pediatric Research Institute, Department of Pediatrics, Oregon Health & Science University, Portland, Oregon; [2]Department of Cell, Developmental & Cancer Biology, Oregon Health & Science University, Portland, Oregon; [3]College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, Korea; [4]Vollum Institute, Oregon Health & Science University, Portland, Oregon

4.1. Abstract

Neurons in the hypothalamic arcuate nucleus relay and translate important cues from the periphery into the central nervous system. However, the gene regulatory program directing their development remains poorly understood. Here we report that the LIM-homeodomain transcription factor Isl1 is expressed in several subpopulations of developing arcuate neurons and plays crucial roles in their fate specification. Mice with conditional deletion of the Isl1 gene in developing hypothalamus display severe deficits in both feeding and linear growth. Consistently, their arcuate nucleus fail to express key fate markers of Isl1-expressing neurons that regulate feeding and growth. These include the orexigenic neuropeptides AgRP and NPY for specifying AgRP-neurons, the anorexigenic neuropeptide αMSH for POMC-neurons, and the two growth-stimulatory peptides, growth hormone-releasing hormone (GHRH) for GHRH-neurons and somatostatin (Sst) for Sst-neurons. Finally, we show that Isl1 directly enhances the expression of AgRP by cooperating with the key orexigenic transcription factors glucocorticoid receptor and brain-specific homeobox factor. Our results identify Isl1 as a critical transcription factor that directs the gene regulatory program specifying the fate of multiple arcuate neuronal subpopulations.

4.2. Introduction

In the central nervous system (CNS), the hypothalamus plays critical roles in centrally integrating and processing sensory inputs, and subsequently coordinating appropriate responses to regulate homeostatic processes that are essential to survival and species propagation (Hill et al, 2008). Among the multiple nuclei of the hypothalamus, the arcuate nucleus (ARC) in the mediobasal hypothalamus is particularly important in sensing and processing various peripheral cues. The ARC is a key regulator of these processes because its diverse array of neuronal subpopulations that control energy balance, growth and reproductive behaviors are located adjacent to the third ventricle and the median eminence, making them poised to interact with both the peripheral blood stream and numerous regions of the brain (Bluet-Pajot et al. 2001; Biebermann et al. 2012; Hrabovszky 2014). Despite recent advances in our understanding of the physiological roles of distinct arcuate neuronal subpopulations, the gene regulatory program that orchestrates the development of the ARC remains poorly understood.

GHRH-neurons in the ARC release growth hormone-releasing hormone (GHRH), which is then carried by the hypothalamo-hypophyseal portal system to the anterior pituitary gland. In turn, GHRH triggers secretion of growth hormone (GH) by stimulating the GHRH receptor (Bluet-Pajot et al. 2001). Activation of GH signaling leads to the hepatic expression of insulin-like growth factor-1 (IGF1), which controls bone epiphyses and growth plate development as well as muscle

and adipose tissue development (Cohen and Rosenfeld 1994). The actions of GHRH are antagonized by the growth-hormone-inhibiting hormone somatostatin (Sst), which is released from the neurosecretory nerve terminals of periventricular nucleus (PVN) Sst-neurons (Bluet-Pajot et al. 2001). These PVN Sst-neurons are distinct from the centrally projecting arcuate neurons that also express Sst. The physiological roles of the arcuate Sst-neurons or their Sst peptides remain poorly understood. Paradoxically, however, these neurons have been suggested to trigger activation of GHRH-neurons (Slama et al. 1993; Lanneau et al. 2000; Bluet-Pajot et al. 2001), likely through Sst-receptors in GHRH-neurons (Tannenbaum et al. 1998).

In addition, the ARC contains two subpopulations of neurons that control feeding behavior, pro-opiomelanocortin (POMC)-neurons and agouti-related protein (AgRP)-neurons (Biebermann et al. 2012). POMC-neurons co-express two anorexigenic peptides, cocaine and amphetamine-related transcript (CART) and the melanocyte-stimulating hormone-α (αMSH), which is produced from the large precursor peptide POMC. In contrast, AgRP-neurons co-express the orexigenic peptides neuropeptide Y (NPY) and AgRP. Fasting upregulates the expression of NPY and AgRP (Lewis et al. 1993; Stephens et al. 1995; Mizuno and Mobbs 1999) and decreases αMSH expression (Cowley et al. 2001). Additionally, fasting increases plasma levels of glucocorticoid, a well-known peripheral orexigenic signal, which in turn activates the expression of NPY and AgRP in AgRP-neurons (Fehm et al. 2004). Thus, POMC- and AgRP-neurons play essential roles in coordinating the CNS response to peripheral metabolic

cues by informing the brain on the peripheral status of energy homeostasis.

We have previously shown that glucocorticoid receptor (GR), the glucocorticoid-activated orexigenic transcription factor, is expressed in AgRP-neurons and directly regulates the expression of AgRP through a novel glucocorticoid response element (GRE) located in the promoter region of the AgRP gene (Lee et al. 2013). We have further demonstrated that, in response to fasting-elevated glucocorticoid levels, GR synergizes with another orexigenic transcription factor expressed in AgRP-neurons, brain-specific homeobox factor (Bsx) (Sakkou et al. 2007; Nogueiras et al. 2008), to direct the activation of AgRP transcription (Lee et al. 2013).

In this study, we investigated the gene regulatory program that governs the development of the ARC during embryogenesis. Islet-1 (Isl1), a member of the LIM-homeodomain (LIM-HD) transcription factors, has been shown to regulate cell fate specification in multiple tissues and species (Merchenthaler et al. 1984; Peng et al. 2005; Mazzoni et al. 2013; Cho et al. 2014; Li et al. 2014; Perdigoto et al. 2014). Here we report that Isl1 serves as a major fate specification determinant of ARC neurons, which control feeding and linear growth. We found that Isl1 is expressed in several developing arcuate neurons during their fate specification, and plays critical roles in inducing the expression of central fate markers of those neurons, including AgRP and NPY in AgRP-neurons, αMSH in POMC-neurons, GHRH in GHRH-neurons, and Sst in Sst-neurons. We further show that Isl1 directly controls the expression of AgRP likely by forming a complex with two partner orexigenic transcription factors expressed

in AgRP-neurons, GR and Bsx. Overall, our results demonstrate that Isl1 is an important regulator of the gene program that specifies the fate of feeding and growth-controlling arcuate neurons.

4.3. Materials and Methods

4.3.1. Animals. 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 noted. The generation of *Isl1*$^{f/f}$ and *Nkx2.1-Cre* mice has been described previously (Mu *et al*, 2008; Xu *et al*, 2008). *Isl1*$^{f/f}$ mice were crossed with *Isl1*$^{f/+}$*;Nkx2.1Cre* mice to generate *Isl1*$^{f/f}$*;Nkx2.1Cre* mice (*Isl1*^{cko} mice). Mice were intraperitoneally injected with Dex (10 mg/kg). For BrdU assay, pregnant female mice were intraperitoneally injected with BrdU (150mg/kg) and sacrificed 2 hrs later. All studies were approved by the Institutional Animal Care & Use Committee**.**

4.3.2. Chromatin Immunoprecipitation. Mouse hypothalamus were dissected out and homogenized before crosslinked with 1% formaldehyde for 10-15 min at room temperature, and quenched by 125 mM glycine. The cells were washed in Buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM Hepes, pH 6.5) and Buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Hepes, pH 6.5) sequentially, lysed with lysis buffer (0.5% SDS, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0, Protease inhibitor cocktail), and then subjected to sonication. Cell lysates were diluted in ChIP buffer (0.5% Triton X-100, 2 mM EDTA, 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, Protease inhibitor cocktail) and incubated with IgG and protein A agarose beads for one hour for immunoclearing. The supernatant was collected after quick spin-down, immunoprecipitated with anti-GR (SC-1004, Santa Cruz), our home-made anti-Isl1 antibody or control IgG

(Santa Cruz), and incubated with protein A agarose over night at 4°C. Next day, the beads were washed with TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), TSE II (same components as in TSE I except 500 mM NaCl) and Buffer III (0.25M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) sequentially for 10 minutes at each step. The beads were subsequently washed with TE buffer three times. The protein/chromatin complexes were eluted in elution buffer (1% SDS, 1 mM EDTA, 0.1M $NaHCO_3$, 50 mM Tris-HCl, pH 8.0) and reverse cross-linked by incubating at 65°C overnight. The eluates were incubated at 50°C for more than two hrs with Proteinase K. The DNA was purified with phenol/chloroform and ethanol precipitation and resolved in water. Following primers were used for PCR: 5'-CAA GCT GAT GAG GCC AGG CGT A-3' and 5'-GCT CTC CCT CCT CTG TGC TTT C-3'

4.3.3. Immunohistochemistry and In situ hybridization. Anaesthetized mice were perfused transcardially with PBS first and then with 4% paraformaldehyde. Brains were removed and placed in 4% paraformaldehybe over night, washed with PBS, and incubated with 30% sucrose. Embryos were fixed in 4% paraformaldehyde and incubated in 30% sucrose. The 2 um brain sections were prepared with cryostat and incubated with anti-GR (SC-1004, Santa Cruz), anti-Nkx2.1 (SC-13040, Santa Cruz), anti-Isl1 and anti-Bsx antibodies at 4°C over night and followed by 1-2 hrs incubation with fluoresence conjugated secondary antibodies. For ISH, antisense RNA probes were labeled with digoxigenin-UTP

(Roche Diagnostics) according to the manufacturer's protocol. Hybridization was performed at 68°C overnight. Hybridized sections were washed in 4X SSC and 0.2X SSC solution, and incubated with anti-digoxigenin-AP antibody (11093274910 Roche) overnight. The sections were subjected to color reaction with NBP/BCIP. The VECTASTAIN Elite ABC Kit (PK-6101, Vector labs) was used according to the manufacturer's instruction for immunohistochemistry assay following in situ hybridization. The cDNAs for mouse AgRP, somatostatin, CRH and TRH were cloned to pBluescript vector to generate digoxigenin-labeled riboprobes. Probes for for POMC, GHRH, Mash1 and DLX1 have been described previously (Nilaweera *et al*, 2002; McNay, 2005). The probes for SF1 and GAD1 are kind gifts from Dr. Holly Ingraham at UCSF and Dr. Jane E. Johnson at UT Southwestern, USA respectively. The NPY probe was obtained from Microarray core facility at BCM, USA.

4.3.4. Luciferase assay. HEK293 or 293T cells were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin. For luciferase assay, cells were seeded into 48-well and transiently transfected with AgRP 1kb-luc reporter (Lee *et al*, 2013) and combinations of rat Isl1/pcs2, mouse Bsx/pcs2 and rat GR/pcs2 as described, using SuperFect (Qiagen) according to the manufacturer's instruction. The actin-β-galactosidase plasmid was cotransfected for normalization of the luciferase assay. Data were shown in relative luciferase units (mean±SD).

4.3.5. RNA extraction and RT-PCR analysis. Total RNAs were extracted from a piece of mouse liver using the Trizol (Invitrogen) and reverse-transcribed using the Thermo Scientific Maxima H Minus Reverse Transcriptase. The following primers were used with SYBR green kit for quantitative PCR of IGF-1: 5'-TCA TGT CGT CTT CAC ACC TCT-3' and 5'-TCC ACA ATG CCT GTC TGA GG-3'

4.3.6. Electrophoretic mobility shift assay. Double stranded oligonucleotides were end labeled withg-^{32}P-ATP using T4 polynucleotide kinase. Isl1 protein was synthesized using the Promega TNT coupled transcription-translation kit. Binding mixtures for reaction included 10 mM HEPES (pH 7.7), 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1 μg of poly(dI-dC), labeled probe and in vitro translated protein. The reaction mixtures were incubated at room temperature for 30 min and analyzed by nondenaturing 5% polyacrylamide gel. Following oligos were used: Isl1RE, 5'-AGC CAT TAA CAC TAA TGA AGC AGG C-3'; non-specific competitor, 5'-TCG AGA CCC AGG GAA CAG TTC GTT CTG TTT CCG-3'

4.4. Results

4.4.1. Expression of Isl1 in developing arcuate nucleus neurons

Our search of the public databases www.brain-map.org and www.genepaint.org suggested that, among many LIM-HD factors, Isl1 is highly expressed in the mediobasal hypothalamus where the ARC is located. This finding prompted us to systematically monitor the expression pattern of Isl1 throughout the development of the ventral hypothalamus. Nkx2.1 is expressed in progenitor cells of the ventral hypothalamus and is required for development of this region (Marin et al. 2002). At E10.5, Isl1 is upregulated in a subset of Nkx2.1+ progenitor cells in the lateral diencephalon (Fig. 16A), suggesting that Isl1 expression is induced as neurons emerge from Nkx2.1+ progenitors in the ventricular zone. By E13.5, Isl1 is expressed relatively broadly in the mantle zone, in which postmitotic neurons reside (Fig. 16A). Isl1 becomes highly enriched in the ARC over time and continues to be expressed in the ARC across all postnatal stages (e.g., http://developingmouse.brain-map.org/gene/show/16165).

To test whether Isl1 is expressed in specific classes of the ARC neurons, we performed immunohistochemistry and in situ hybridization (ISH) analyses. To label AgRP-neurons, we used two transgenic mouse models. The NPY-hrGFP transgenic mouse is an ideal model to label AgRP-neurons with GFP in developing embryos, because GFP expression is driven by the promoter of the NPY gene, which is turned on in embryonic AgRP-neurons prior to the expression of AgRP (van den Pol et al. 2009). To label AgRP-neurons

postnatally, we generated AgRP-ires-Cre;Rosa26CAG-tdTomato mice, in which a red fluorescent protein (tdTomato) is specifically and postnatally expressed in AgRP-neurons due to expression of Cre driven by the AgRP promoter and the resulting Cre-mediated recombination only in AgRP-neurons (Tong et al. 2008; Madisen et al. 2010). Isl1 was expressed in almost all GFP+ AgRP-neurons in the ARC of NPY-hrGFP mice at E14.5 (Fig. 16B). Likewise, Isl1 was expressed in almost all tdTomato+ AgRP-neurons of AgRP-ires-Cre;Rosa26CAG-tdTomato mice at P28 (Fig. 16C). Consistently, immunostaining with anti-Isl1 antibody, combined with ISH for either NPY or AgRP probe, revealed the co-expression of Isl1 with NPY at E16.5 and with AgRP at P28 (Fig. 16, D and E). These results establish that Isl1 is expressed in the AgRP-neurons from embryonic to postnatal stages. In POMC-eGFP mice (Cowley et al. 2001), approximately 70-80% of GFP+ POMC-neurons co-expressed Isl1 in the ARC at E17.5 (Fig. 16F). Likewise, the combined analyses of ISH and immunostaining showed co-expression of αMSH and Isl1 at E16.5 and P28 (Fig. 16, G and H). The combined ISH and immunohistochemical analyses also revealed that Isl1 is expressed in most GHRH+ and Sst+ neurons in the ARC of E16.5 embryos (Fig. 16, I and J). Taken together, these results indicate that Isl1 is expressed in AgRP-, POMC-, GHRH- and Sst-neurons in the ARC during embryonic development and through subsequent postnatal stages.

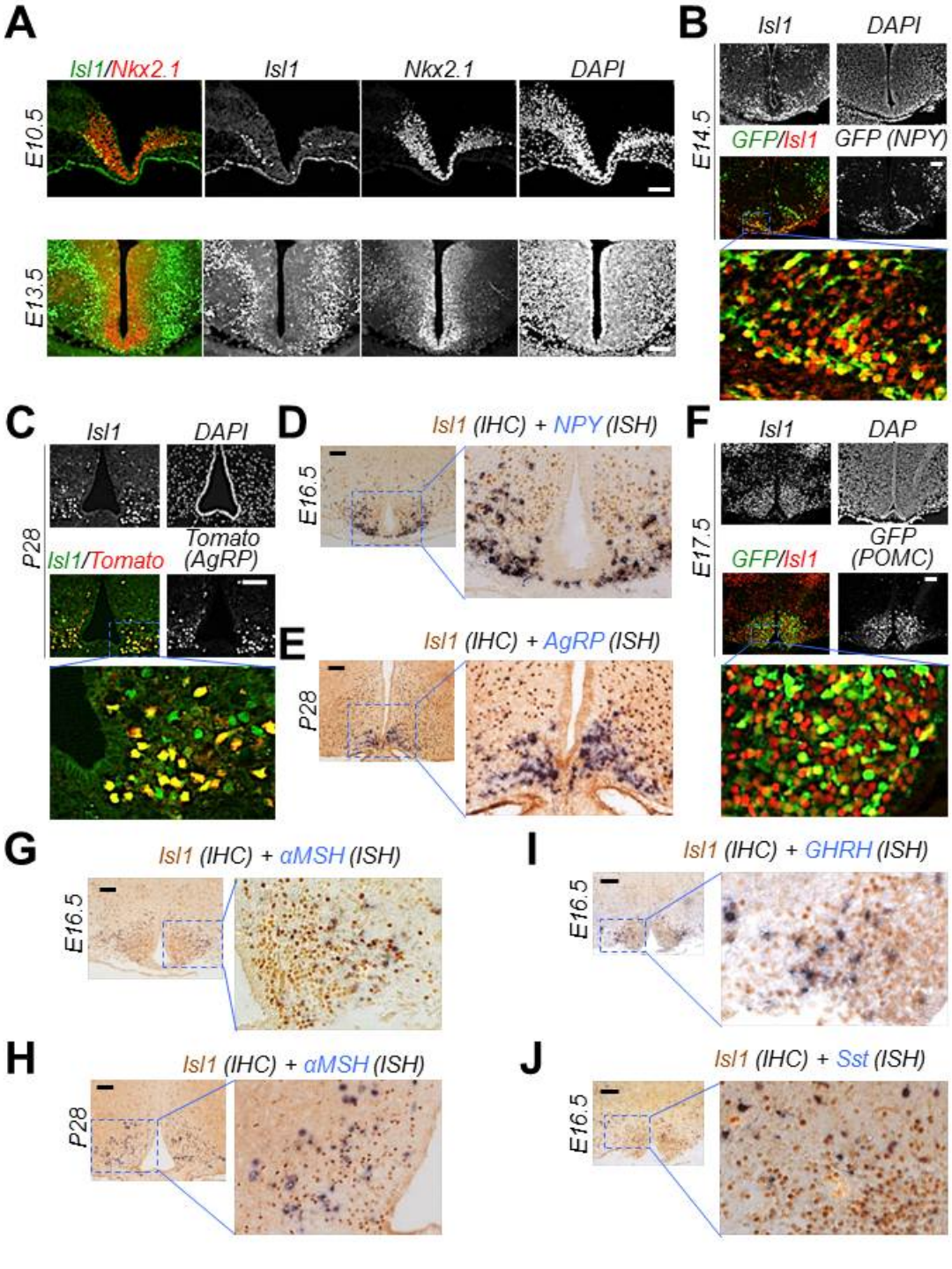

A
Isl1/Nkx2.1
Isl1
Nkx2.1
DAPI
E10.5
E13.5
B
Isl1
DAPI
E14.5
GFP/Isl1
GFP (NPY)
C
Isl1
DAPI
P28
Isl1/Tomato
Tomato (AgRP)
D
Isl1 (IHC) + NPY (ISH)
E16.5
E
Isl1 (IHC) + AgRP (ISH)
P28
F
Isl1
DAP
E17.5
GFP/Isl1
GFP (POMC)
G
Isl1 (IHC) + αMSH (ISH)
E16.5
H
Isl1 (IHC) + αMSH (ISH)
P28
I
Isl1 (IHC) + GHRH (ISH)
E16.5
J
Isl1 (IHC) + Sst (ISH)
E16.5

Figure 16. Expression of Isl1 in the arcuate neurons. (A) Wild-type mouse embryonic hypothalamus was immunostained with anti-Isl1 and anti-Nkx2.1 antibodies. DAPI staining marks all nuclei in the section. (B, C, F) Representative images of the ARC from E14.5 NPY-hrGFP embryos (B), P28 AgRP-ires-Cre;Rosa26CAG-tdTomato mice (C) and E17.5 POMC-eGFP embryos (F) were immunostained with anti-Isl1 antibody. (D, E, G-J) ISH (blue) for NPY (D), AgRP (E), αMSH (G, H), GHRH (I) and Sst (J) was performed with either E16.5 or P28 wild-type mouse ARC, followed by immunostaining with anti-Isl1 antibody (brown). All bars (A-F) indicate 100 μm.

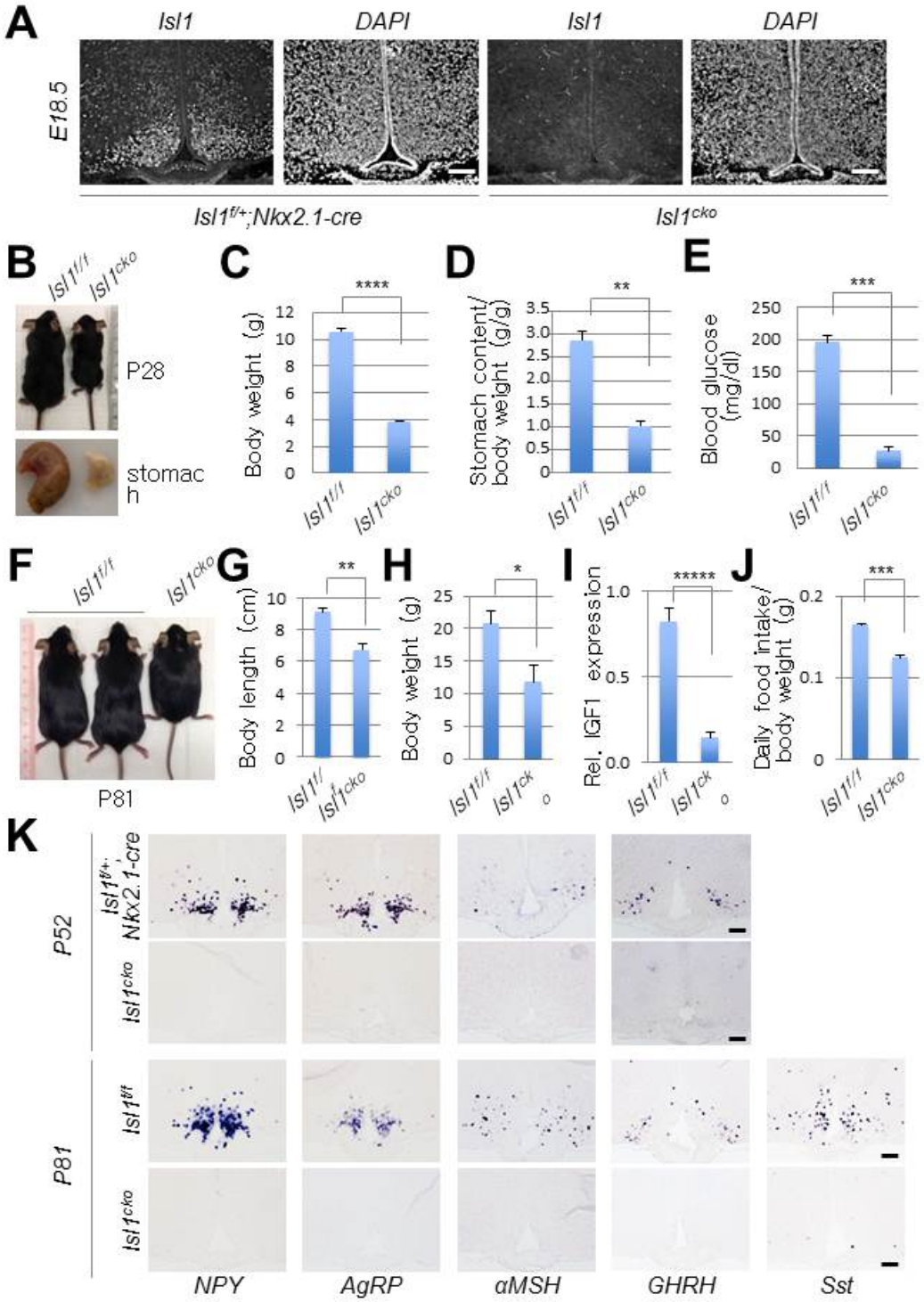
4.4.2. Isl1^{cko} mice show deficits in feeding and growth

To investigate whether Isl1 plays a role in development of AgRP-, POMC-, GHRH- and Sst-neurons, we generated *Isl1$^{f/f}$;Nkx2.1-Cre* mice (herein referred to as *Isl1^{cko}*), in which the *Isl1* gene is deleted in Nkx2.1^{+} neural progenitors, which give rise to Isl1^{+} neurons in the ARC (Fig. 16A). Confirming that Isl1^{+} cells indeed derive from Nkx2.1^{+} progenitors in developing ARC, Isl1 protein was no longer expressed in the ARC of *Isl1^{cko}* mice (Fig. 17A).

The body weight of *Isl1^{cko}* mice was similar to that of their littermate controls at birth. Interestingly, *Isl1^{cko}* mice began to show retardation in both linear growth and body weight gain around P10, which became progressively worse (Fig. 17, B and C). *Isl1^{cko}* mice also showed a marked reduction in the size and the content weight of their stomachs (Fig. 17, B and D) as well as in blood glucose levels (Fig. 17E). Approximately 80% of *Isl1^{cko}* mice died between P21 and P30, likely due to a decrease in feeding and the resulting low glucose levels. Notably, the remaining ~20% of *Isl1^{cko}* mice, which escaped the early death, continued to exhibit growth retardation at later stage of life, as shown by a significant decrease in the body length and weight (Fig. 17, F-H). These *Isl1^{cko}* escaper mice showed a drastic reduction in the hepatic IGF1 levels, a well-defined surrogate marker for the growth hormone produced in the pituitary (Fig. 17I), suggesting defects in the GHRH-GH-IGF1 axis. In addition, the escaper mice also exhibited a substantial reduction in food intake (Fig. 17J), indicating that the feeding circuitry is disrupted in *Isl1^{cko}* mice.

To investigate whether the hypothalamic dysregulation of growth and

feeding contributes to the severe growth/feeding phenotypes of *Isl1*^{cko} mice, we monitored the expression of key neuropeptides controlling growth and feeding in the ARC of adult *Isl1*^{cko} escaper mice. Strikingly, the ISH analyses revealed that the expression of NPY, AgRP, POMC, GHRH and Sst was eliminated in *Isl1*^{cko} mice at P52 and P81 (Fig. 17K). These results suggest that Isl1 is required for the development and/or survival of AgRP-, POMC-, GHRH- and Sst-neurons in the ARC, which control feeding and growth.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Isl1
DAPI
Isl1
DAPI
E18.5
Isl1f/+;Nkx2.1-cre
Isl1cko
B
Isl1f/f
Isl1cko
P28
stomach
C
Body weight (g)
****
Isl1f/f
Isl1cko
D
Stomach content/ body weight (g/g)
**
E
Blood glucose (mg/dl)
***
F
P81
G
Body length (cm)
**
H
Body weight (g)
*
I
Rel. IGF1 expression
*****
J
Daily food intake/ body weight (g)
***
K
P52
P81
NPY
AgRP
αMSH
GHRH
Sst

Figure 17. Feeding and growth deficits of *Isl1*^{cko} mice. (A) The ARC from E18.5 control or Isl1cko embryos was immunostained with anti-isl1 antibody. The bar indicates 100 μm. (B-J) Representative images of control and Isl1cko mice and their stomachs at P28 (B) and P81 (F). Their body weights were measured at P28 (C) and P81 (H). Their stomachs were dissected out to measure their contents (D) at P28. Blood glucose levels were measured from tail blood with a glucometer (E) and the body length was measured from the nose tip to the tail base (G). The expression level of IGF-1 mRNA at P81 was determined with qRT-PCR (I). Daily food intake was measured for 4 weeks from P80 (J). At least 3 mice were used for each group. All values are presented as means ± standard errors of the means. Differences between groups were analyzed by two-tailed Student's t test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; *****, $P < 0.00001$. (K) ISH with NPY, AgRP, αMSH, GHRH and Sst was performed on the ARC of surviving escaper Isl1cko and their littermate control mice at P52 and P81. All bars indicate 100 μm.

4.4.3. Lost expression of POMC, AgRP, NPY, GHRH and Sst in the ARC of *Isl1^{cko}* embryos

To test whether Isl1 is needed for development of arcuate neurons that control feeding and growth, we analyzed the expression of key neuropeptides that designate the identities of specific ARC subpopulations in Isl1cko embryos. NPY, αMSH, GHRH and Sst were not expressed in the ARC of E16.5 Isl1cko embryos, while their expression was readily detected in control littermate embryos (Fig. 18A), suggesting that Isl1 is required for the specification of neurons expressing these neuropeptides during embryogenesis. To test whether the specification of the arcuate neuronal subpopulations that express these key neuropeptides was delayed in the absence of Isl1 but eventually recovered postnatally, we monitored the expression of the neuropeptides at P21, including AgRP, whose expression is known to increase postnatally in AgRP-neurons (Nilsson et al, 2005). Interestingly, the expression of NPY and AgRP in AgRP-neurons, αMSH in POMC-neurons, GHRH in GHRH-neurons, and Sst in Sst-neurons remained severely compromised in the ARC of P21 Isl1cko mice in comparison to their littermate controls (Fig. 18B). Of note, normal levels of NPY expression were detected in the amygdala region of P28 Isl1cko brains (Fig. 18C), proving that the ISH procedure was successfully carried out.

To test the specificity of the loss of AgRP-, POMC-, GHRH-, and Sst-neurons in Isl1cko mice, we examined the expression of several well-established markers of hypothalamic neurons in Isl1cko mice; SF1, a marker for a subregion of the VMH that expresses Nkx2.1 but not Isl1 (Dellovade et al. 2000; Davis et al.

2004), CRH, a marker for the PVN that expresses neither Nkx2.1 nor Isl1 (Shimogori et al. 2010), and TRH, a marker for the DMH that expresses low levels of Isl1 but not Nkx2.1 (Davis et al. 2004). The expression of SF1, CRH, and TRH was not significantly altered in the hypothalamus of P28 Isl1cko mice (Fig. 18D), indicating that the SF1+, CRH+, and TRH+ neurons are normally formed in Isl1cko mice.

Together, these results suggest that Isl1 plays important roles in driving and maintaining the expression of feeding and growth-controlling neuropeptides NPY, AgRP, αMSH, GHRH and Sst in the ARC. Given that the loss of these key neuropeptides in the hypothalamus of Isl1cko mice would result in disruption of the central control of feeding and growth, the loss of NPY, AgRP, αMSH, GHRH and Sst is likely a major contributing factor to the severely compromised feeding and growth phenotypes of Isl1cko mice (Fig. 17).

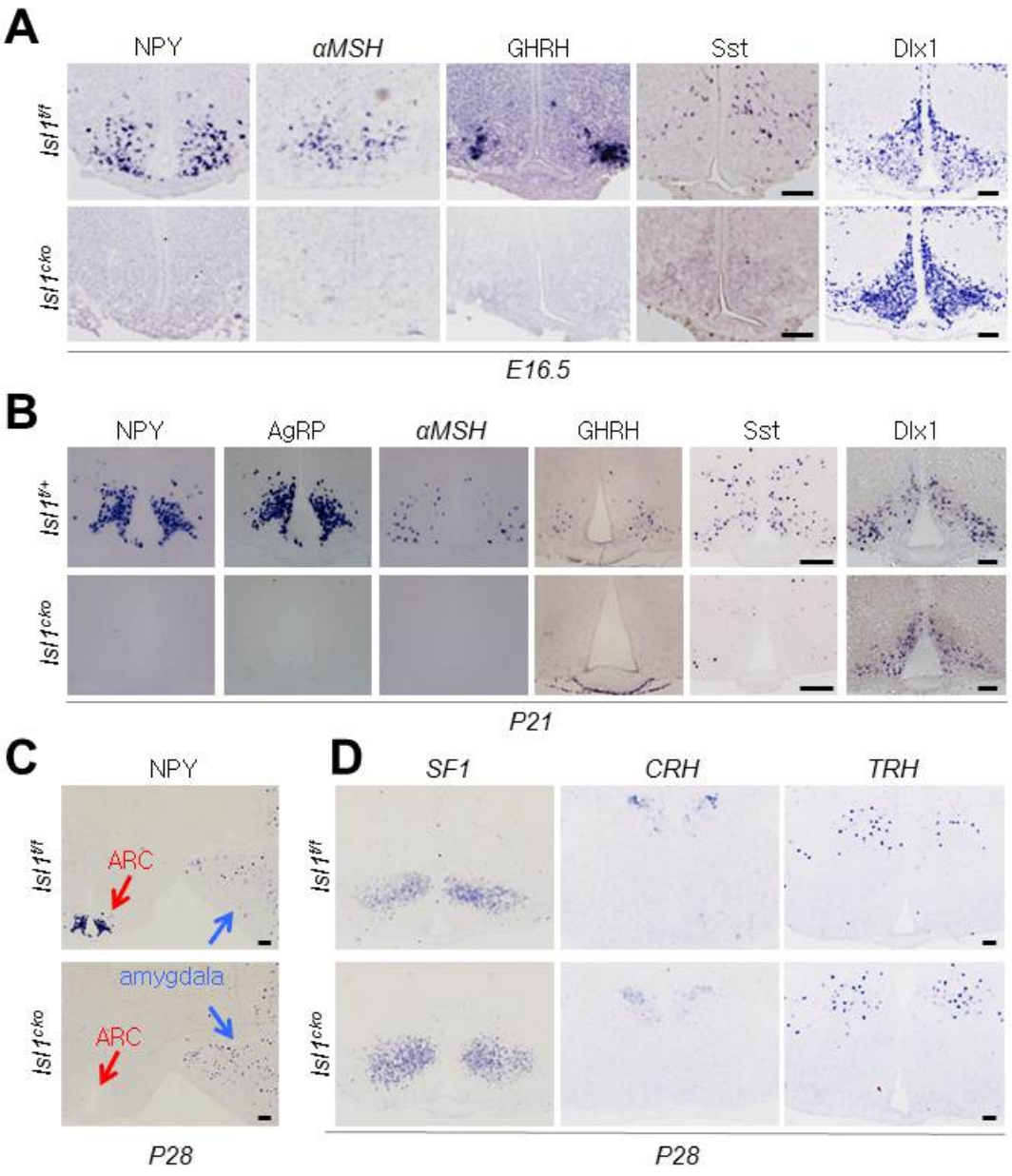


Figure 18. Impaired expression of αMSH, AgRP, NPY, GHRH and Sst in the ARC of *Isl1*^{cko} mice. (A-D) ISH was performed for NPY/AgRP, αMSH, GHRH, Sst and Dlx1 (A, B) as well as NPY (C) and SF1, CRH and TRH (D) on the ARC of control and Isl1cko mice at E16.5 (A), P21 (B) and P28 (C, D). All bars indicate 100 μm.

4.4.4. Isl1 directs the fate specification of the embryonic arcuate neuronal subpopulations.

The neuropeptides NPY/AgRP, αMSH, GHRH and Sst represent key markers of the cell fates of AgRP-, POMC, GHRH- and Sst-neurons, and also serve as central molecules that determine the functionality of these neurons. Thus, the loss of NPY, AgRP, αMSH, GHRH and Sst in the ARC of *Isl1*^{cko} mice points to the possibility that Isl1 serves as a critical factor that directs the fate-determination of these arcuate neuronal subpopulations. Alternatively, Isl1 may be needed for the proliferation or neurogenesis of neural progenitors giving rise to these neurons or their survival. To monitor the proliferation status of neural progenitors, we performed BrdU labeling assays in E12.5 embryos, which were exposed to BrdU for 2 hrs in utero. The number of BrdU^{+} cells was comparable between *Isl1*^{cko} and control embryos (Fig. 19A). To investigate ARC neurogenesis, we examined the expression of a proneural basic helix-loop-helix factor Mash1, which is a well-known marker for neural progenitors that is also functionally important for neurogenesis (Lo et al. 1991; McNay et al. 2006). The number of Mash1^{+} cells was comparable between E12.5 *Isl1*^{cko} and littermate control mice (Fig. 19B).

To investigate the role of Isl1 in neuronal differentiation in the ARC, we monitored the presence of GABAergic neurons using ISH with a probe detecting *glutamate decarboxylase-1 (GAD1)*, which encodes an enzyme that converts glutamate to γ-aminobutyric acid (GABA) and CO_2. Of note, most NPY^{+} AgRP-neurons are known to be GABAergic, whereas most POMC neurons are non-

GABAergic (Horvath et al. 1997; Ovesjo et al. 2001; Hentges et al. 2004). There are also other types of GABAergic neurons in the ARC, including a subset of Kisspeptin-neurons (Hrabovszky 2014). Interestingly, more GABAergic neurons were observed in E14.5 *Isl1*^{cko} mice in comparison to control mice (Fig. 19C). These results indicate that GABAergic neurons are still formed in *Isl1*^{cko} mice, although they fail to upregulate NPY on time.

To evaluate cell death, we performed immunostaining for activated caspase 3, an apoptotic cell marker, and found that very few cells die in either control or *Isl1*^{cko} mice at E18.5 (Fig. 19D). The gross morphology of the ARC was also comparable between control and *Isl1*^{cko} mice, as determined by the DAPI staining (Fig. 19D).

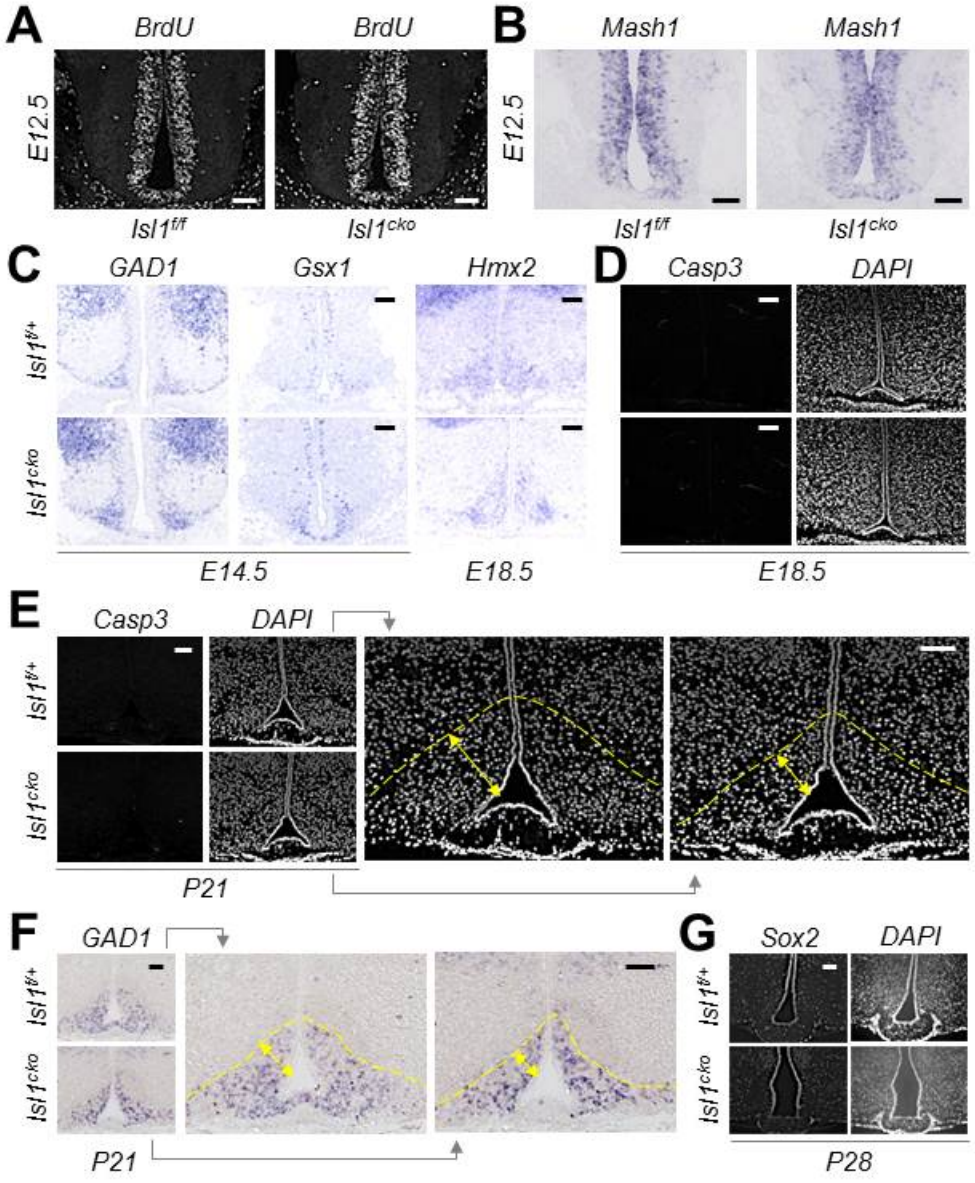
To examine additional markers of differentiated arcuate neurons, we monitored the expression of the homeobox transcription factors Gsx1 and Hmx2/3, which have been shown to play essential roles in the expression of GHRH in the ARC (Li et al. 1996; Wang et al. 2004). Overall, the expression of Gsx1 and Hmx2 in the ARC was largely comparable between control and *Isl1*^{cko} mice (slightly more Gsx1 and slightly less Hmx2 in *Isl1*^{cko}) (Fig. 19C).

Finally, we examined the expression of Dlx1, a HD transcription factor expressed in a subset of dopaminergic neurons in the ARC (Yee et al. 2009). Interestingly, the number of Dlx1^{+} cells and the level of Dlx1 expression dramatically increased in E16.5 *Isl1*^{cko} brains (Fig. 18A) but not in P21 *Isl1*^{cko} brains (Fig. 18B). Taken together, these results suggest that AgRP-, POMC, GHRH- and Sst-neurons fail to be properly specified in the absence of Isl1.

Further, the loss of NPY/AgRP, αMSH, GHRH and Sst-expressing cells in *Isl1*^{cko} brains was not due to abnormal levels of cell death or survival. Also, interestingly, presumptive embryonic AgRP-, POMC-, GHRH- and Sst-neurons may show aberrant gene expression profiles such as upregulation of Dlx1 in *Isl1*^{cko} brains.

To examine the effects of cell fate specification failure and aberrant gene expression in presumptive AgRP-, POMC, GHRH- and Sst-neurons in the ARC, we examined P21-28 *Isl1*^{cko} mice. Interestingly, DAPI staining revealed that the ARC region becomes smaller in *Isl1*^{cko} mice relative to control mice at P21 (Fig. 19E), while the embryonic ARC structure was comparable between *Isl1*^{cko} and control mice (Fig. 19D). Similarly, the number of GABAergic neurons was significantly reduced in P21 *Isl1*^{cko} (Fig. 19F), although few apoptotic cells were detected in both control and *Isl1*^{cko} mice at P21 (Fig. 19E). The number of Sox2^{+} ependymal cells, which represent proliferating adult neural progenitor cells (Ming and Song 2011), did not change in the ARC of *Isl1*^{cko} mice at P28 (Fig. 19G), suggesting that the reduced ARC area in *Isl1*^{cko} mice was not caused by a loss of neural progenitors. These results suggest that at least some of the miss-specified presumptive AgRP-, POMC, GHRH- and Sst-neurons may be lost postnatally in *Isl1*^{cko} mice.

Overall, our data suggest that Isl1 plays an important role in the fate specification of AgRP-, POMC, GHRH- and Sst-neurons during hypothalamic development.


A
BrdU
BrdU
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Isl1cko
B
Mash1
Mash1
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C
GAD1
Gsx1
Hmx2
Isl1f/+
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E
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Isl1f/+
Isl1cko
P21
F
GAD1
Isl1f/+
Isl1cko
P21
G
Sox2
DAPI
Isl1f/+
Isl1cko
P28

Figure 19. No apparent defects in genesis and survival of the arcuate neurons in *Isl1*^{cko} mice. (A, B) Immunohistochemistry with anti-BrdU antibody (A) and ISH with Mash1 (B) were performed on the ARC of control and Isl1cko mice at E12.5. BrdU was intraperitoneally injected to pregnant females 2 hrs before euthanasia.

(C) ISH with GAD1, Gsx1 and Hmx2 on the ARC of control and Isl1cko mice at E14.5 or E18.5. (D, E) The ARC of control and Isl1cko mice at E18.5 (D) or P21 (E) immunostained with anti-activated caspase 3 (Casp3) antibody. DAPI staining marks all nuclei in the section. Dotted yellow lines indicate the boundary of the ARC (E). (F) ISH with GAD1 performed on the ARC of control and Isl1cko mice at P21. Dotted yellow dotted lines indicate the boundary of the ARC.

4.4.5. Isl1 directly regulates the expression of AgRP in AgRP-neurons

Our results raise the possibility that Isl1 is critical to upregulate the expression of NPY, AgRP, POMC, GHRH and Sst during specification of arcuate neuronal subtypes in the developing hypothalamus. We have recently shown that Isl1 directly regulates cholinergic pathway genes, thereby determining a cholinergic neuronal identity of spinal motor neurons and forebrain cholinergic neurons (Cho et al. 2014). Likewise, it is possible that Isl1 directly controls the expression of key neuropeptides during ARC development. To test this possibility, we investigated the *AgRP* gene as a direct Isl1 target because we have previously identified critical gene regulatory regions in the *AgRP* gene, which drive gene expression specifically in AgRP-neurons (Lee et al. 2013). The AgRP promoter region was found to recruit two orexigenic transcription factors, a HD protein Bsx and a nuclear hormone receptor GR (Lee et al. 2013). Notably, the HD-binding motifs in the AgRP promoter, which have been shown to serve as Bsx-binding sites (Sakkou et al. 2007; Lee et al. 2013), are identical to the Isl1-binding consensus motif, which we defined by *in vitro* SELEX assays (i.e., CATTAG; (Lee et al. 2008)) (Fig. 20A). The CATTAG sequences in the distal HD response element (HD-RE) were completely conserved in multiple mammalian species (Fig. 20A). Similarly, the proximal HD-RE, located closer to the transcriptional initiation site, was also relatively well conserved (Fig. 20A). The evolutionarily conserved sequences of HD-REs in the AgRP promoter, along with the critical role of Isl1 in inducing AgRP expression (Fig. 17K and 18B), led us to hypothesize that Isl1 directly upregulates the *AgRP* gene by binding to the HD-REs in the AgRP

promoter. To test whether Isl1 directly binds to the HD-REs of *AgRP*, we performed electrophoresis mobility shift assays using ^{32}P-labeled DNA probe and in vitro translated Isl1 protein (Fig. 20B). Incubation of Isl1 with DNA probes containing each HD-RE led to the formation of Isl1/DNA complex (indicated as arrows in Fig. 20B) suggested that Isl1 binds both HD-REs in the AgRP promoter. To test the specificity of the interaction between Isl1 and HD-RE sequences, we competed the association between Isl1 and ^{32}P-labeled HD-RE probe with either unlabeled HD-RE oligonucleotides or unlabeled non-specific oligonucleotides. The formation of Isl1/HD-RE DNA complex was disrupted by HD-RE oligonucleotides, but not by non-specific oligonucleotides, further supporting the idea that Isl1 specifically interacts with each HD-RE in the *AgRP* gene. To further confirm the identity of Isl1/HD-RE DNA complex, we incubated the complex with either anti-Isl1 antibody or IgG and found that anti-Isl1 antibody, but not IgG, led to disappearance of the shifted bands. Thus, the electrophoresis mobility shift assays demonstrate that Isl1 directly binds to the *AgRP* promoter.

Given our previous finding that GR directly binds to the GRE in close proximity to the distal HD-RE, and activates *AgRP* transcription in response to peripheral cue glucocorticoids (Fig. 20A; Lee et al. 2013), we considered the possibility that Isl1 forms a transcriptional activating complex with GR and cooperates with GR in inducing the *AgRP* transcription in the hypothalamus. To test this possibility, we investigated the association between Isl1 and GR in HEK293 cells using co-immunoprecipitation assays. Isl1 interacted with GR in a GR ligand dexamethasone (Dex)-independent manner in cells (Fig. 20C),

supporting a possible cooperative action between Isl1 and GR. Next, to determine whether Isl1 binds to the *AgRP* gene in the hypothalamus and whether this recruitment requires glucocorticoids, we intraperitoneally injected P56 mice with either Dex or vehicle, collected the hypothalami from the mice 2 hrs after injection, performed chromatin immunoprecipitation (ChIP) assays with anti-Isl1 or anti-GR antibody and P56 mouse hypothalamus lysates, and did PCR analyses with a primer set encompassing the distal HD-RE and the GRE (Fig. 20A). This analysis revealed that Isl1 constitutively binds the HD-RE in the *AgRP* gene irrespective of glucocorticoid signaling, whereas GR is recruited to the GRE of the *AgRP* gene only in Dex-treated hypothalamus (Fig. 20D).

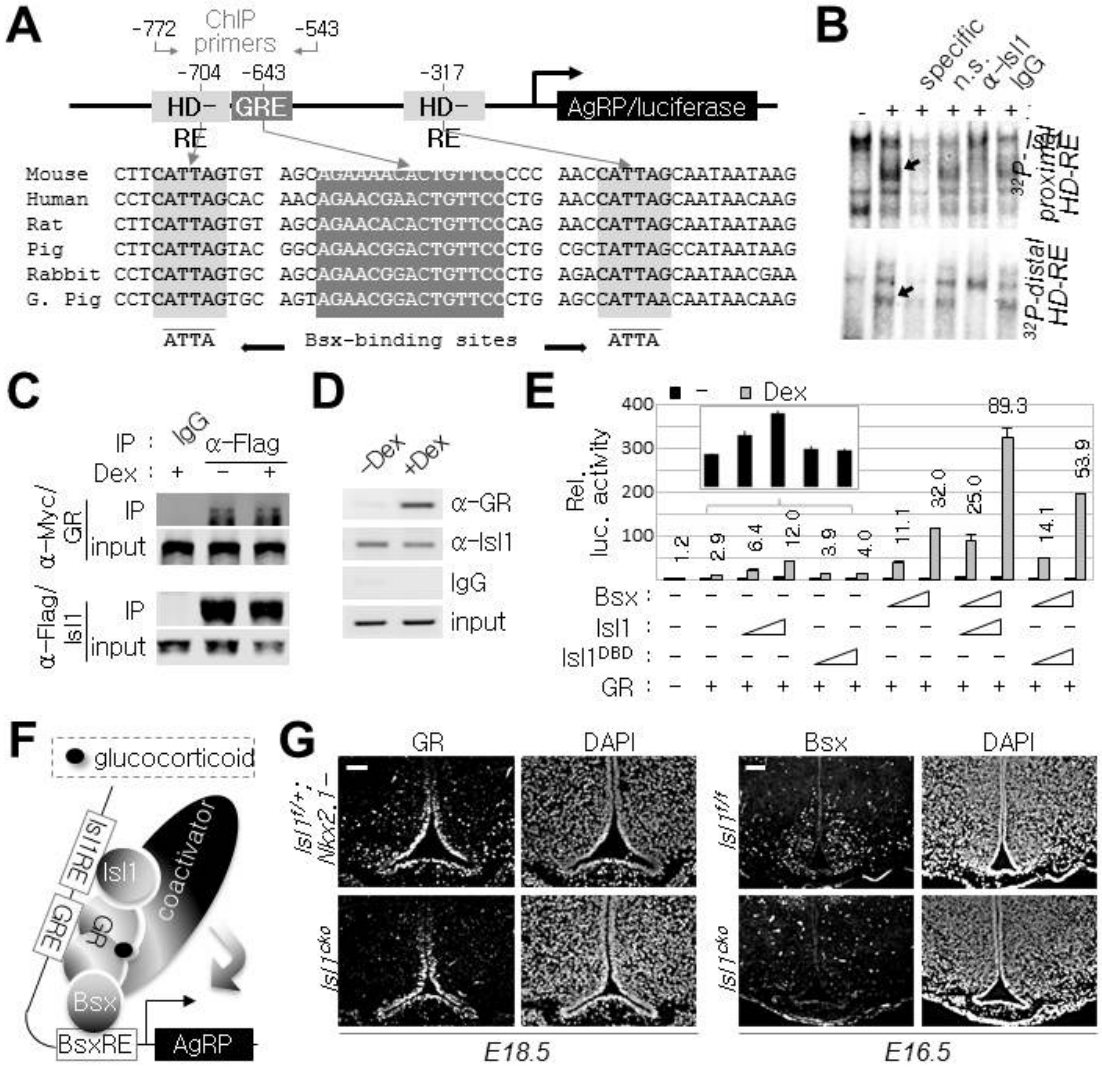
The co-recruitment of Isl1 and GR to the AgRP promoter prompted us to investigate whether Isl1 is capable of transactivating the AgRP promoter in combination with GR in response to the orexigenic glucocorticoid signaling. Given the presence of the two well-conserved HD-REs in the AgRP promoter and co-expression of Bsx1 with Isl1 and GR in the AgRP-neurons, we also asked whether Bsx participates in this transcriptional cooperation. To address these questions, we performed luciferase reporter assays with a reporter in which a 1kb region of the AgRP promoter is linked to a *luciferase* gene (Lee et al. 2013); Fig. 20A). Isl1 alone, but not a DNA-binding defective mutant form of Isl1, activates the reporter in a dose-dependent manner (Fig. 20E, inset), suggesting that Isl1 activates the *AgRP* gene transcription by directly binding to the gene. Interestingly, Isl1 strongly synergized with GR in the presence of Dex, while DNA-binding defective Isl1 was not as effective as wild type Isl1 in synergistically

activating the reporter (Fig. 20E). Bsx further enhanced the transactivation of the AgRP promoter by Isl1 and GR. Taken together, these results suggest a model in which Isl1, Bsx and GR form a ternary complex on the AgRP promoter by binding to the HD-REs and GRE, and that this Isl1/Bsx/GR ternary complex then synergistically stimulates the glucocorticoid-directed transactivation of the AgRP promoter, likely by facilitating recruitment of transcriptional coactivators to the *AgRP* gene (Fig. 20F).

Isl1 is important for the expression of orexigenic transcription factors GR and Bsx

Given the essential role of Isl1 in the fate specification of AgRP-neurons as well as in the transcription of the *AgRP* gene, we asked whether Isl1 is important to establish the expression of the two key orexigenic transcription factors GR and Bsx, which in turn cooperates with Isl1 to transactivate the *AgRP* gene during hypothalamus development. Interestingly, we found that the expression of both GR and Bsx was severely impaired in the ARC of *Isl1*^{cko} mice, while GR expression in the ependymal layer remained intact (Fig. 20G). These results indicate that Isl1 plays a crucial role in the expression of GR and Bsx in developing AgRP-neurons. Although it remains to be determined whether the *GR* and *Bsx* genes are direct or indirect targets of Isl1, our results demonstrate that Isl1 modulates the expression of multiple orexigenic genes (*GR*, *Bsx* and *AgRP*) in AgRP-neurons. These results also establish Isl1 as a key transcription factor that orchestrates not only AgRP-neuronal fate specification but also timely

induction of the orexigenic neuropeptide AgRP in response to an increased level of the peripheral orexigenic cue glucocorticoid.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-543
-704
-643
-317
HD-RE
GRE
HD-RE
AgRP/luciferase
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Human CCTCATTAGCAC AACAGAACGAACTGTTCCCTG AACCATTAGCAATAACAAG
Rat CTTCATTAGTGT AGCAGAACACACTGTTCCCAG AACCATTAGCAATAATAAG
Pig CTTCATTAGTAC GGCAGAACGGACTGTTCCCTG CGCTATTAGCCATAATAAG
Rabbit CCTCATTAGTGC AGCAGAACGGACTGTTCCCTG AGACATTAGCAATAACGAA
G. Pig CCTCATTAGTGC AGTAGAACGGACTGTTCCCTG AGCCATTAACAATAACAAG
ATTA
Bsx-binding sites
ATTA
B
specific
n.s.
α-Isl1
IgG
32P-proximal HD-RE
32P-distal HD-RE
C
IP : IgG α-Flag
Dex : + − +
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-Dex
+Dex
α-GR
α-Isl1
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Rel. luc. activity
400
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1.2
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32.0
25.0
89.3
14.1
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Bsx :
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Isl1DBD :
GR :
F
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Isl1RE
GRE
Isl1
GR
coactivator
Bsx
BsxRE
AgRP
G
GR
DAPI
Bsx
DAPI
Isl1f/+; Nkx2.1−
Isl1cko
Isl1f/f
Isl1cko
E18.5
E16.5

Figure 20. Direct regulation of AgRP expression by Isl1. (A)Schematic representation of the AgRP promoter as well as the evolutionary conservation of two HD-REs and GRE in mammals. (B) EMSA assays to test binding of Isl1 to both HD-REs. The specific bands (arrows) by 32P-probe/Isl1-complex were extinguished by non-labeled specific competitor or anti-Is1 antibody but not by non-specific competitor or IgG. (C) CoIP experiments with HEK293 cells transfected with the expression vectors for Myc-tagged GR and Flag-tagged Isl1 and treated with vehicle or 0.1 μM of Dex. (D) ChIP experiments with P56 wild type hypothalami using anti-GR and anti-Isl1 antibodies were performed following intraperitoneal injection of either vehicle or Dex (10mg/kg). (E) Luciferase reporter assay with AgRP-1kb:Luc reporter was performed in HEK293 cells transfected with expression vectors for Bsx, Isl1, Isl1DBD (Isl1-N230S) and GR as indicated. Isl1DBD is a DNA binding defective mutant form of Isl1. Inset shows the basal transactivation levels of the reporter in response to increasing amount of Isl1 or Isl1DBD. The numbers above each column indicate activation folds by Dex relative to the reporter activity without Bsx, Isl1, Isl1DBD and GR. (F) Model for a complex of Isl1-GR-Bsx to activate the transcription of AgRP by recruiting coactivators. (G) Immunohistochemistry with anti-GR and anti-Bsx was performed on E18.5 and E16.5 ARC. All bars indicate 100 μm.

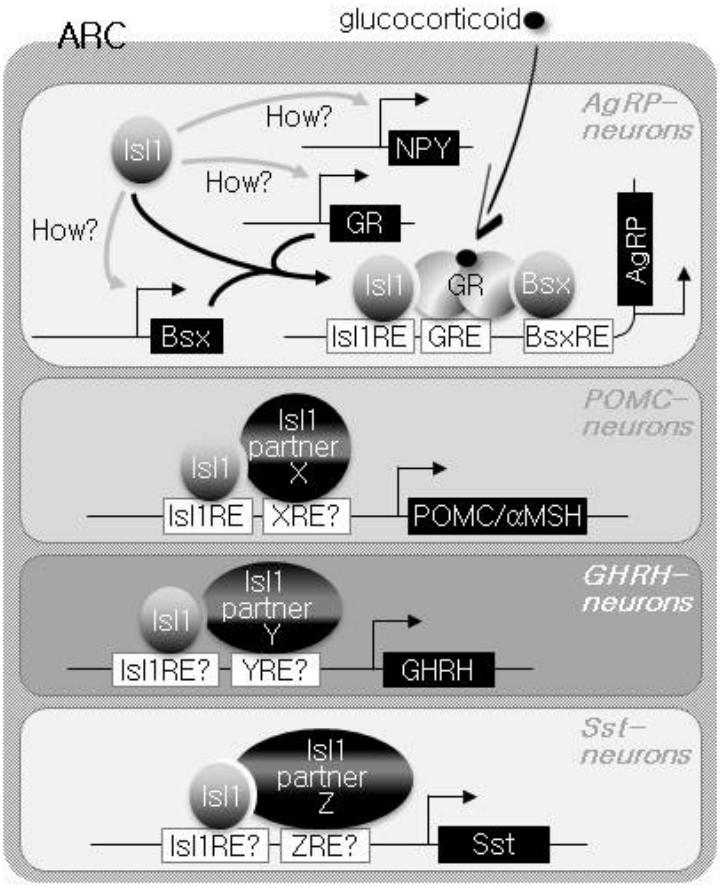

Figure 21. Model for Isl1 as a transcriptional regulator of key fate markers of multiple arcuate neurons during development. Isl1 forms a complex with Bsx and GR to induce the expression of AgRP Likewise, Isl1 may form one or more distinct complexes in each arcuate neuronal subpopulation to directly induce the expression of NPY, POMC/αMSH, GHRH and Sst, the central fate markers of the arcuate AgRP-, POMC-, GHRH- and Sst-neurons (see text).

4.5. Discussion

Our studies establish Isl1 as a central regulator of cell fate specification of arcuate neuronal cell types in the developing hypothalamus. Furthermore, we show that Isl1 directly controls at least one key neuropeptide gene (*AgRP*) by cooperating with two other orexigenic transcription factors (GR and Bsx), providing mechanistic insights into Isl1-directed gene networks in ARC development. In addition, our loss-of-function studies indicate that, in AgRP-neurons, Isl1 upregulates the expression of NPY, GR and Bsx. Given the cooperative actions of Isl1 with GR and Bsx, Isl1-dependent regulation of *GR* and *Bsx* is expected to amplify the positive regulatory action of Isl1 in AgRP expression (Fig. 21). Interestingly, while this manuscript was being prepared, Isl1 was reported to promote the expression of the anorexigenic neuropeptide POMC in the arucate POMC-neurons by directly binding to two related Isl1RE-containing enhancer elements in the *POMC* gene (Nasif et al. 2015). Combined with our $Isl1^{cKO}$ studies, this finding supports the idea that Isl1 specifies the identity of the arcuate POMC-neurons. In this regard, it is notable that both the *GHRH* and *Sst* genes possess Isl1-bound ChIPseq peaks in a ChIPseq dataset from embryonic stem cells (Mazzoni et al. 2013), pointing to the possibility that Isl1 directly binds to *GHRH* and *Sst* genes and induces their expression in developing arcuate GHRH- and Sst-neurons.

In this work, we demonstrated that Isl1 is required for the expression of key identity markers of several arcuate neuronal subpopulations; the orexigenic

neuropeptides AgRP and NPY of AgRP-neurons, the anorexigenic neuropeptide αMSH of POMC-neurons, and the growth stimulatory peptides, GHRH of GHRH-neurons and Sst of Sst-neurons. Our finding leads to the question of how a single transcription factor Isl1 directs the specification of distinct neuronal types. Notably, Isl1 has been shown to drive fate specification of many cell types during multiple tissue development, often by forming cell type-specific complexes with various partner transcription factors. For instance, Isl1 partners with the LIM-HD factor Lhx3 in specifying motor neurons in the spinal cord (Pfaff et al. 1996; Thaler et al. 2002), the LIM-HD factor Lhx8 in directing the fate of forebrain cholinergic neurons in the ventral telencephalon (Cho et al. 2014), the HD factor Phox2a in cranial motor neurons (Mazzoni et al. 2013), the basic helix-loop-helix (bHLH) transcription factor Beta2 in pancreas (Peng et al. 2005), the high-mobility group box factor Sox2 in epidermal Merkel cells (Perdigoto et al. 2014), and the POU-domain transcription factor Pou4f2 in retinal ganglion cells (Li et al. 2014). Given these results, it is probable that Isl1 forms distinct cell type-specific complexes in AgRP-, POMC-, GHRH-, and Sst-neurons in the ARC, which enable Isl1 to control distinct sets of target genes in each neuronal type in the developing hypothalamus (Fig. 21). Indeed, supporting this possibility, our study discovered a novel type of Isl1-complex consisting of Isl1, Bsx, and GR in AgRP-neurons that directly binds and triggers the expression of the *AgRP* gene (Fig. 20 and 21). In the future, it will be interesting to investigate whether Isl1 also forms one or more cell type-specific complexes with other partner transcription factors in regulating the expression of its target genes in AgRP-, POMC-, GHRH- and Sst-neurons (Fig. 21). In light of

this, it is noteworthy that the HD transcription factors Gsx1, Hmx2 and Hmx3 have been shown to be required for the expression of GHRH in the arcuate GHRH-neurons (Li et al. 1996; Wang et al. 2004). Gsx1 was also shown to directly bind to a Gsx-binding site in the GHRH promoter region (Mutsuga et al. 2001). Thus, it will be interesting to examine whether Isl1 cooperates with Gsx1 and Hmx2/3 in inducing the expression of GHRH in GHRH-neurons and whether this involves formation of an Isl1-containing complex with Gsx1 and/or Hmx2/3. It would also be interesting to investigate the mechanistic basis of Isl1-directed ARC neuronal fate specification by identifying a full range of direct target genes of Isl1 in distinct arcuate neurons using genome-wide approaches such as RNAseq and ChIPseq.

Deletion of *Isl1* in part of the developing hypothalamus, including the ARC, led to marked defects in linear growth and feeding behavior in *Isl1*^{cko} mice (Fig. 17). The loss of two growth-stimulating neuropeptides GHRH of GHRH-neurons and Sst of Sst-neurons likely contributes to the severe deficits in linear growth in our *Isl1*^{cko} mice. Notably, GHRH-null mice showed stunted growth but survived to adulthood (Alba and Salvatori 2004a). Thus, while the stunted growth of the *Isl1*^{cko} mice results from lack of both feeding and GHRH, their early death is likely caused by the defects in the hypothalamic feeding circuits and the resulting lack of feeding and starvation, rather than being an outcome of inadequate growth. The pathophysiological mechanism that underlies the strong feeding deficits of our *Isl1*^{cko} mice remains to be determined. Neonatal inactivation of *NPY* and/or *AgRP* does not affect food intake in mice (Qian et al. 2002). Similarly, neonatal ablation of AgRP-neurons displays minimal effects on feeding, whereas their

ablation in adults leads to rapid starvation and death (Bewick et al. 2005; Gropp et al. 2005; Luquet et al. 2005; Xu et al. 2005; Wu et al. 2009). These results led to a proposal that loss of AgRP expression or AgRP-neurons in neonatal stages triggers developmental compensation but such loss in adults is detrimental for survival. Of note, αMSH is a melanocortin-receptor agonist, while AgRP is an inverse agonist of melanocortin action (Biebermann et al. 2012; Myers and Olson 2012). Between the two predominant CNS melanocortin receptors, MC3R and MC4R, the latter expressed in the PVN and amygdala is known to primarily control food intake (Myers and Olson 2012). These results raise an interesting possibility that restoring AgRP-like activity, which antagonizes MC4R, might be a critical developmental compensatory component in AgRP- and AgRP/NPY-null mice (Qian et al. 2002). Our *Isl1*^{cko} mice may be defective in activating developmental compensation for loss of AgRP-like activity, thereby completely losing the antagonism function against MC4R. Alternatively, an additional MC4R-activating program might be inappropriately activated in our *Isl1*^{cko} mice despite the loss of αMSH expression. These additional mechanisms may involve Isl1 expression in Nkx2.1^{+} neurons in either the ARC and/or other nuclei in hypothalamic feeding circuits.

In summary, our results identify Isl1 as a key transcription factor that functions in cell fate determination of multiple arcuate neuronal subpopulations in the developing hypothalamus. Further studies of Isl1 in developing hypothalamus should provide important insights into the gene regulatory program directing the development of Isl1-expressing arcuate neurons.

Chapter 5

5. SUMMARY AND CONCLUSION

Over the last decade, a significant effort has been made to identify the neuronal pathway that controls the energy balance. My research has focused on both the development and the postnatal function of the hypothalamic arcuate nucleus (ARC) neurons that are crucial to regulating energy balance.

In chapter 2 and chapter 3 of this thesis, we identified the new regulatory mechanisms for the expression of AgRP and NPY, the orexigenic neuropeptides expressed in the ARC. First we showed that AgRP is a direct target of the glucocorticoid receptor (GR) in AgRP neurons by identifying a novel glucocorticoid response element (AgRP-GRE) in the AgRP promoter. AgRP-GRE has unique features that mediate the synergistic induction of AgRP gene expression by GR and Bsx in response to elevated glucocorticoid signals. In contrast, Bsx suppresses glucocorticoid-dependent activation of other downstream targets. Elevated blood glucocorticoid level upon food deprivation may lead to the unwanted activation of glucocorticoid target genes in AgRP neurons. Bsx, however, can help selectively activate the genes necessary for energy preservation and repress other glucocorticoid target genes that should not be activated. Our transgenic mouse line confirmed that AgRP-GRE drives fasting-dependent activation of target genes specifically in GR and Bsx double positive AgRP neurons, but not in other cell types expressing either GR or Bsx alone in the brain. By comparing the sequences of AgRP-GRE and other conventional

GREs, we were able to identify Per1 and Asb4 as two putative targets of GR and Bsx in AgRP neurons. More target genes of both GR and Bsx are expected to be identified using the unique sequence features of AgRP-GRE. Further characterization of these genes will provide critical insights into the molecular basis of the orexigenic function of glucocorticoid in the ARC.

We also uncovered that GR and NOR1, another nuclear hormone receptor, have a mutually antagonistic relationship to regulate AgRP and NPY expression. We characterized a novel molecular mechanism by which anorexigenic leptin and orexigenic glucocorticoid translate their signals to regulating the expression of appetite controlling neuropeptide. Leptin activates NOR1 transcription by activating the transcription factor CREB that binds to the promoter region of NOR1. The induced expression of NOR1 by leptin suppresses AgRP/NPY expression by inhibiting the action of GR. During fasting, glucocorticoid-bound GR antagonizes the action of CREB to reduce NOR1 expression, resulting the higher level of AgRP/NPY. Interestingly, NOR1 inhibits the expression of AgRP by interfering with the AgRP-GRE-binding activity of GR. Consistently Nor1-null mice showed higher levels of AgRP/NPY expression and a reduced response to leptin in decreasing AgRP/NPY expression as well as lowering body weight. It will be interesting to investigate if Nurr1 and Nur77, the other two members of NR4A subfamily, function similarly in this network in future studies.

Despite the extensive research on the function of ARC neurons in feeding regulation, the gene regulatory programs underlying their development remains poorly understood. We found that Isl1, a LIM-homeodomain transcription factor,

is expressed in several developing arcuate neurons including feeding-regulatory AgRP and POMC neurons, and growth-regulatory GHRH and SST neurons. Interestingly, deleting Isl1 in the developing hypothalamus results in the complete loss of the key marker genes of these neurons such as AgRP/NPY, αMSH, GHRH, and SST in the hypothalamic ARC. Among the genes lost in Isl1 conditional mutant mice, AgRP and NPY are orexigenic, POMC is anorexigenic, and both GHRH and SST are growth-stimulatory neuropeptides. As expected, the mutant mouse showed severe growth retardation. Surprisingly, however, Isl1 conditional mutant mice displayed profound anorexigenic phenotype. More studies are needed to show why the Isl1 mutants have such dramatic feeding deficits when both orexigenic and anorexigenic factors are missing. Another interesting future study will be to investigate the molecular mechanisms by which Isl1 regulates the expression of these important neuropeptides. I discovered that Isl1 directly binds to the AgRP promoter and activates its transcription in cooperation with GR and Bsx. But the identification of more Isl1 target genes and their Isl1 binding regions will be required to fully understand the regulatory mechanisms by which Isl1 controls the fate of multiple arcuate neuronal cell types.

My work provides novel insights into the molecular mechanisms that underlie the regulation of energy balance by revealing new gene regulatory networks in both development and postnatal feeding controlling paradigm. Furthermore, these studies could lead to the development of better tools to treat and prevent various metabolic and developmental disorders caused by hypothalamic neuronal dysfunction.

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