The Influence of Central Insulin Signals on Stress Response in Mice

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ABSTRACT

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Stress is an anticipatory or actual disruption of homeostasis which triggers physiological responses to prepare for the threat or to reestablish the system's internal equilibrium. Other than physical stress (e.g. blood loss, infection, pain, and cold) and psychogenic stress (e.g. novel environment (rodent) and immobilization (rodent)), nutrient (metabolic) stress such as high fat diet and fasting can also trigger stress responses. Central to the activation and regulation of the stress response in mammals is the hypothalamus, where signals from the periphery and other regions of the brain are integrated and processed. Insulin and leptin are two important hormones that provide information about the energy status of the body to the hypothalamus. The hypothalamic insulin- and leptin-sensing circuits integrate these signals to coordinate metabolic outcomes such as food intake, energy expenditure, fuel partitioning, etc. Given the tight correlation between stress axis activity and nutrient status, it raises the possibility that hypothalamic insulin- and leptin- sensing circuits may also be involved in coordinating responses of the stress axis, particularly during stress pertaining changes in energy homeostasis. My thesis research focused on understanding the roles and the interactions of hypothalamic insulin and leptin signals in regulating and coordinating metabolic and hypothalamic-pituitary-adrenal (HPA) axis functions.

My first project involved understanding the role of hypothalamic insulin signals in hypothalamic *Leptin receptor* deficient animals ($L^{2.1}$ KO) in regulating

energy metabolism (Chapter 2). We observed an increase in body weight and adiposity in $D^{2.1}$ KO mice that lack both hypothalamic insulin receptor (InsR) and leptin receptor (LepRb) signals. These changes were accompanied by reduced energy expenditure, rather than an increase in food intake. Unexpectedly, there was a drastic loss in body temperature in $D^{2.1}$ KO during fasting that was significantly exacerbated by single-housing. These results suggest that interactions between hypothalamic insulin and leptin signals are important for regulating energy expenditure and body temperature. Furthermore, this study also highlights the influence of housing conditions on the evaluation of thermogenic defects in mice.

My second project involved the study of hypothalamic insulin signals in regulating stress-related functions by using the non-obese hypothalamic InsR-deficient mice ($I^{2.1}$ KO)(Chapter 3). We showed that $I^{2.1}$ KOs have elevated baseline hypothalamic *Avp* synthesis, increased activity of the HPA axis after restraint, as well as increased anxiety-like behaviors. This study demonstrated that hypothalamic InsR signals suppress the stress response to restraint, possibly by influencing AVP release to the median eminence and decreasing hypothalamic glucocorticoid receptor (GR) signals, and may also modulate anxiety-like behaviors.

My current research focuses are: to remove InsR signals by using more restrictedly expressed *Cre* lines in order to identify brain nuclei mediating insulin's effect on stress response and/or anxiety-like behaviors; to identify the hypothalamic AVP neuronal population affected by the loss of InsR and potential

extra-hypothalamic downstream targets of these neurons; to pinpoint the hypothalamic nuclei where GR signaling is altered in $I^{2.1}$ KO. By identifying the critical anatomical and functional components mediating insulin's effects, we hope to provide more understanding of the contribution of central insulin resistance to the development of HPA axis dsyregulation and anxiety disorders in humans.

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CHAPTER 1: BACKGROUND OVERVIEW

Hypothalamic Regulation of Physiological Functions

The hypothalamus is consisted of a highly diverse but specialized collection of interconnected neurons that allow the sensing and integration of peripheral and central signals to coordinate systemic responses, and maintain basic physiological functions and homeostasis. Early lesion studies in rodents revealed that the hypothalamus influence a wide ranges of essential body functions for survival, including appetite and feeding, stress response, maintenance of blood pressure, glucose metabolism, and body temperature regulation, etc. In this chapter, we will focus on discussing the role of hypothalamic circuits in regulating body temperature and stress response.

Hypothalamic Neural Circuits in Regulating Brown Adipose Tissue Thermogenesis

Central neural circuits coordinate thermogenic activities in peripheral organs to maintain body temperature in response to external (i.e. increased or decreased surrounding temperature) as well as internal stressors (i.e. fever, energy deficit). Maintenance of body temperature is important, because critical cellular functions are altered by deviations in body temperature. Thermogenesis is one of the major mechanisms for regulating and maintaining body temperature. There are two types of thermogenesis: non-shivering (adaptive) thermogenesis (mediated by brown adipose tissue (BAT) and skeletal muscle) and shivering-thermogenesis (mediated by skeletal muscle) (Lowell and Spiegelman 2000; Morrison and Nakamura 2011; Bal et al. 2012). Since relatively little is known about the central mechanism for regulating shivering

thermogenesis and shivering is usually engaged last for maintaining body temperature since the thermal threshold required for activation is lower than both cutaneous vasoconstriction and BAT thermogenesis (Morrison and Nakamura 2011), this discussion will focus on adaptive thermogenesis.

CNS circuits influence BAT thermogenesis by regulating sympathetic nerve activity (Morrison and Nakamura 2011). Sympathetic activity stimulates the expression of BAT *Uncoupling-protein-1*, which encodes a protein that generates heat by facilitating proton leakage across the electronic transport chain on the mitochondrial membranes (Morrison and Nakamura 2011). Temperature sensing occurs at several different levels: from cutaneous thermal receptors in the skin that respond to external temperature, to warm-sensing neurons in the POA which are activated in response to increased local temperature (Morrison and Nakamura 2011). Signals from cutaneous thermal receptors are transmitted via neurons in dorsal horn of the spinal cord (Craig 2002) to the lateral parabrachial neurons (Hylden et al. 1989; Li et al. 2006), which then projects to the POA (Nakamura and Morrison 2008; Morrison and Nakamura 2011)

Many hypothalamic nuclei, including include the POA, DMH, and PVH, are implicated in regulating BAT thermogenesis (Yoshida et al. 2009; Morrison and Nakamura 2011). However, the POA is believed to be the main hypothalamic site involved in sensorimotor integration of thermoregulation, since most thermoregulatory responses are dependent on the integrity of the POA (Morrison and Nakamura 2011). The POA is made up of several distinct populations of neurons that regulate thermogenesis separately. Warm-sensing neurons in the medial POA (mPOA) have been postulated to provide inhibitory signals to neurons in the DMH and rostral raphe

pallidus nucleus (rRPa), which normally act to promote BAT thermogenesis (Morrison and Nakamura 2011). The role of neurons in the median preoptic area (MnPOA), however, remains controversial. While Morrison et al. proposed that MnPOA activates thermogenesis by inhibiting neurons in mPOA that inhibit thermogenesis (Morrison and Nakamura 2011); Yoshida et al. suggested that neurons in MnPOA and dorsolateral preoptic area (DLPO) form parallel inhibitory pathways that modulate BAT thermogenesis (Yoshida et al. 2009). This inconsistency likely arose from the difference in injection sites and the proximity between the sites being investigated.

Downstream of the POA, thermogenesis-promoting DMH neurons project (possibly through caudal periaqueductal grey (cPAG)) to premotor neurons in the rostral ventromedial medulla, including the rRPa, which provide critical excitatory drive to thermal effectors (Morrison and Nakamura 2011). rRPa neurons provide inputs to preganglionic neurons in the IML that directly innervate BAT (Nakamura et al. 2004). It has been proposed that GABAergic neurons in the DMH that project to the PVH act to suppress BAT thermogenesis; however the exact functional and anatomical connections between PVH and rRPa are not known (Madden and Morrison 2009; Rezai-Zadeh and Münzberg 2013). The hypothalamus represents an important site for the integration of local and peripheral information to coordinate thermoregulatory events that maintain or alter body temperature in response to physiological needs. The central circuits regulating BAT thermogenesis are illustrated in Figure 1.1.

Figure 1.1

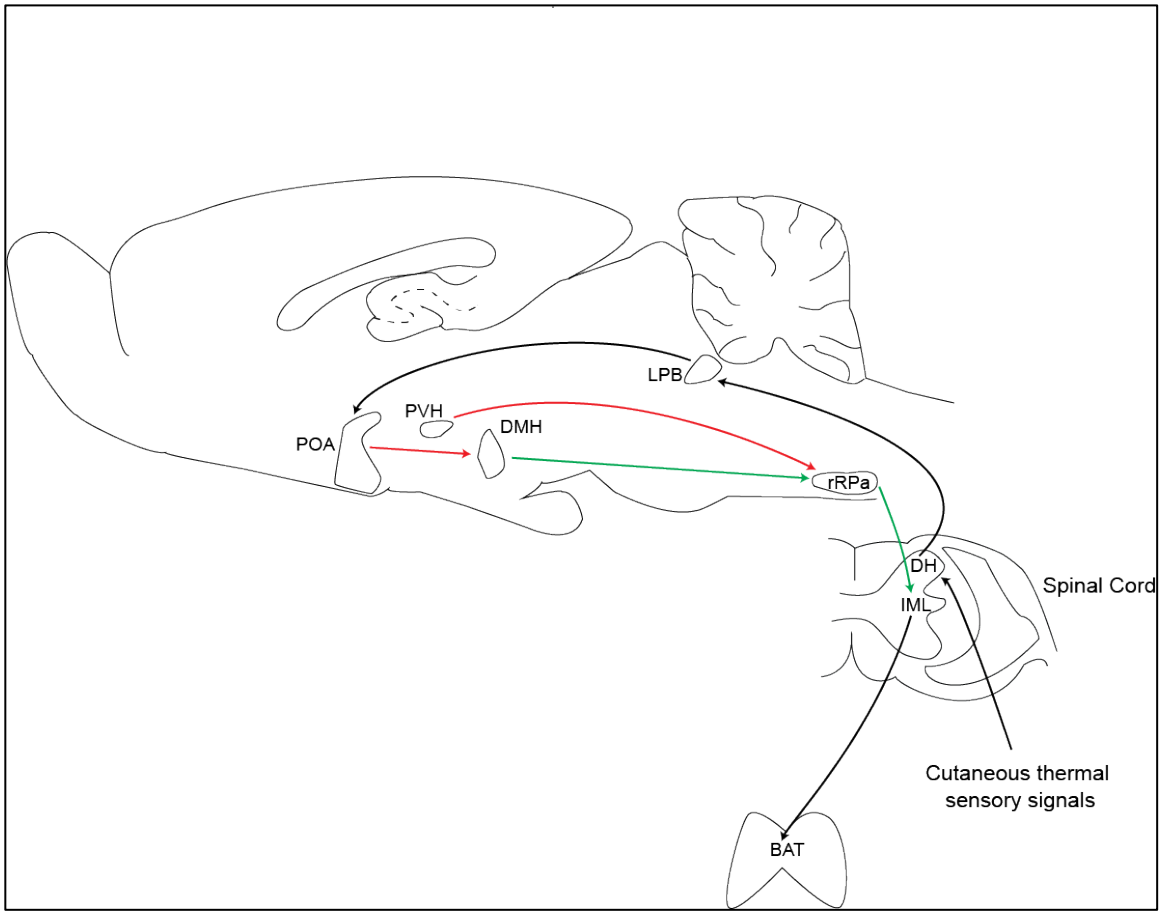

Figure 1.1: CNS Circuits Regulating BAT Thermogenesis
Cutaneous thermal sensory signals are transmitted to DH, which contains second-order thermal sensory neurons projecting to the LPB. POA neurons receiving thermosensory signals from the LPB send inhibitory outputs to the DMH. PVH also send inhibitory signals to the rRPa, which provide excitatory inputs to the IML of the spinal cord. Activation of the BAT sympathetic preganglionic neurons in the spinal IML increases BAT thermogenesis. (Excitatory projections are shown in green; Inhibitory, red) Abbreviations: DH (dorsal horn of the spinal cord); LPB (lateral parabrachial nucleus); POA (preoptic area); DMH (dorsomedial nucleus of the hypothalamus); NTS (nucleus of the solitary tract); PVH (paraventricular nucleus of the hypothalamus); RVM (rostral ventromedial medulla); rRPa (rostral raphe pallidus); IML (intermediolateral cell column); BAT (brown adipose tissue)

Hypothalamic Leptin and Insulin Signals in Regulating BAT Thermogenesis

Body temperature is tightly correlated with energy status, especially during energy deficiency, when it is important to reduce thermogenesis to preserve energy needed to maintain essential body functions for survival. Decreased serum leptin, indicative of insufficient energy stores, is associated with decreased thermogenesis. Thus, *Lep*$^{ob/ob}$ and *Lepr*$^{db/db}$ mice lacking leptin signaling exhibit lower body temperature at baseline, and are unable to maintain body temperature when subjected to an acute cold challenge (Trayhurn and James 1978; Bates et al. 2004). In addition, *Lepr*$^{db/db}$ mice dramatically reduce body temperature in response to fasting (Kinoshita et al. 2000). Leptin's effect on thermoregulation is believed to be partially mediated by hypothalamic neural circuits. In the CNS, *Leprb* expressing neurons in the POA and DMH/DHA (dorsal hypothalamic area) are labeled by pseudorabies virus (transsynaptic viral retrograde tracer) injected into the BAT (Rezai-Zadeh and Münzberg 2013), providing anatomical evidence to support a role of these neurons in mediating leptin's thermogenic effects. Supporting this idea, leptin injections into the DMH increase BAT sympathetic outflow (Enriori et al. 2011).

Similar to leptin, serum insulin levels reflect the energy status of an animal and have been shown to be involved in body temperature regulation and thermogenesis. STZ-treated rats cannot maintain body temperature in response to food restriction (Kinoshita et al. 2000). The effect of insulin on BAT thermogenesis is known to be partially mediated by hypothalamic insulin sensing circuits in the POA. Insulin signals directly inhibit warm-sensing neurons in the POA and increase sympathetic tone as well as BAT thermogenesis (Sanchez-

Alavez et al. 2010). These studies supported the idea that both hypothalamic insulin and leptin signals are involved in thermoregulation by influencing BAT thermogenesis.

Regulation of Stress Response (in Mammals) by Hypothalamic Circuits

Stress response promotes physiological adaptations that protect and prepare the animals for actual or anticipated threats to their well-being, and acts to maintain and restore physiological balance of the internal environment following the challenge. A wide variety of external and internal stimuli can elicit a stress response, including physical (blood pressure, pain, inflammation), psychological (smell, restraint), and nutritional (fasting, hypoglycemia) signals. There are two types of stress responses: the endocrine response and the autonomic response.

The endocrine stress response involves the stimulation of the adrenal gland through hormonal factors released from the hypothalamus and the pituitary, ultimately leading to an increase in the release of corticosterone (CORT) in rodents or cortisol in humans. Activation of the hypothalamic-pituitary-adrenal (HPA) axis elicits a slower-acting response than the autonomic stress response, as CORT levels take at least ten minutes to peak after exposure to a stressor (Ulrich-Lai and Herman 2009). The stress response initiated by the HPA axis involves the activation of neurons in the PVH, mainly corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) neurons, which secrete the respective peptide hormones into the hypophyseal-portal circulation in the median eminence. These hormones in turn stimulate corticotrophs in the anterior pituitary to release adrenocorticotropic hormone (ACTH), a 39-amino acid peptide derived from proopiomelanocortin (POMC), into the peripheral circulation. ACTH then

acts on the inner adrenal cortex (zona fasciculate) to increase synthesis and release of glucocorticoids (GC). The released GCs act both centrally and in the pituitary to provide negative feedback to the endocrine stress response (Figure 1.2).

Figure 1.2

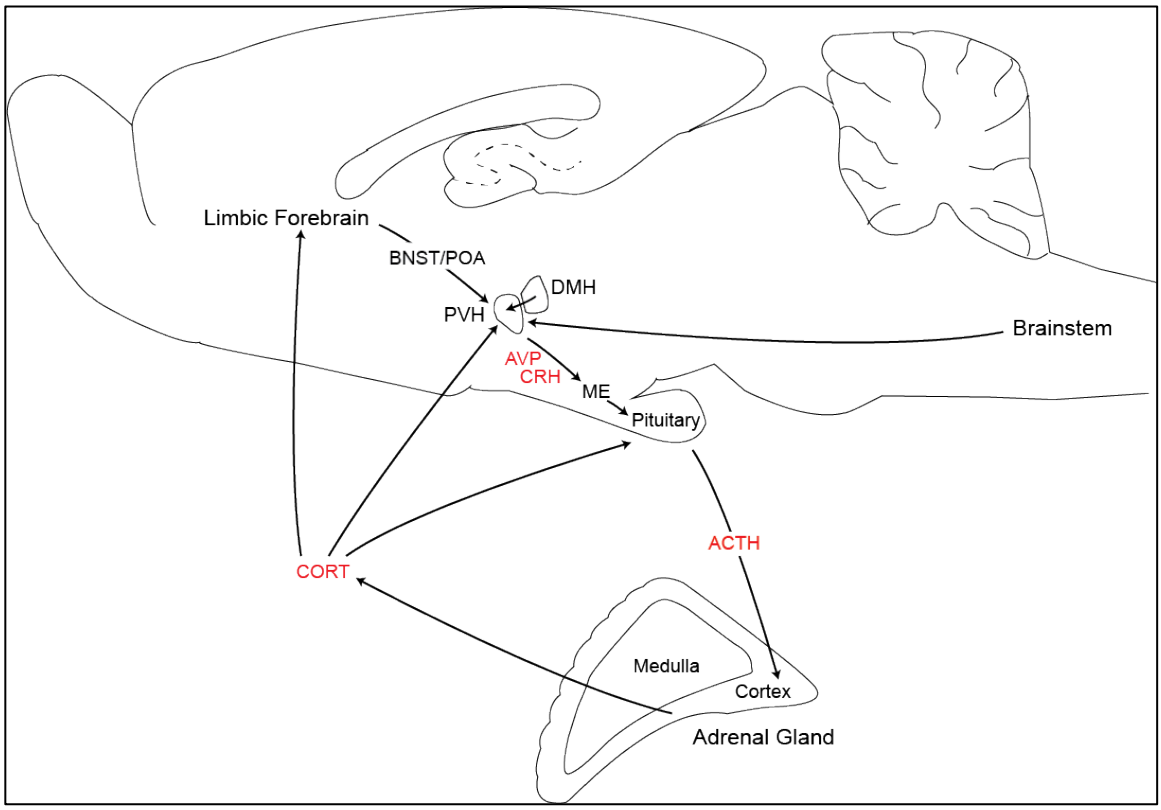

Figure 1.2: Endocrine Stress Response (Hypothalamic-Pituitary-Adrenal Axis). PVH integrates signals coming from the brainstem, BNST (which relays signals from the limbic forebrain), POA, and DMH to generate the appropriate stress response. Parvocellular PVH neurons release AVP and CRH into the ME and the hypophyseal-portal circulation. CRH and AVP signaling in the anterior pituitary stimulate the synthesis of ACTH and its release into the circulation. ACTH acts on cells in the adrenal cortex to stimulate GC production. Negative feedback actions of GCs are mediated through glucocorticoid receptors in the limbic forebrain, PVH and the pituitary. (Hormonal signals are indicated in red) Abbreviations: PVH (paraventricular nucleus of the hypothalamus); BNST (bed nucleus of the stria terminalis); POA (preoptic area); DMH (dorsomedial nucleus of the hypothalamus); AVP (arginine vasopressin); CRH (corticotropin-releasing hormone); ME (median eminence); ACTH (adrenocorticotropic hormone); CORT (corticosterone)

The autonomic stress response is critical for responses to acute physiological and homeostatic stressors. Major homeostatic imbalances, such as blood loss and pain, are sensed by peripheral organs, which send afferent signals to the brainstem. The nucleus of the solitary tract (NTS) in the brainstem relays this information to the rostral ventrolateral medulla, locus coeruleus, and paraventricular nucleus of the hypothalamus (PVH). The response to the stressor is then transmitted to the intermediolateral cell column of the spinal cord, which relays the information to peripheral organs. Direct innervation of the adrenal gland by sympathetic nerves that project from the spinal cord, allows for rapid transmission of stress-related stimuli through the release of catecholamines (ephinephrine/norephineprhine) from chromaffin cells in the adrenal medulla (Figure 1.3). The effects of catecholamines on target organs include a wide range of physiological adaptations that redirect the body's energy and resources to deal with the threat, such as increasing heart rate and blood pressure.

Figure 1.3

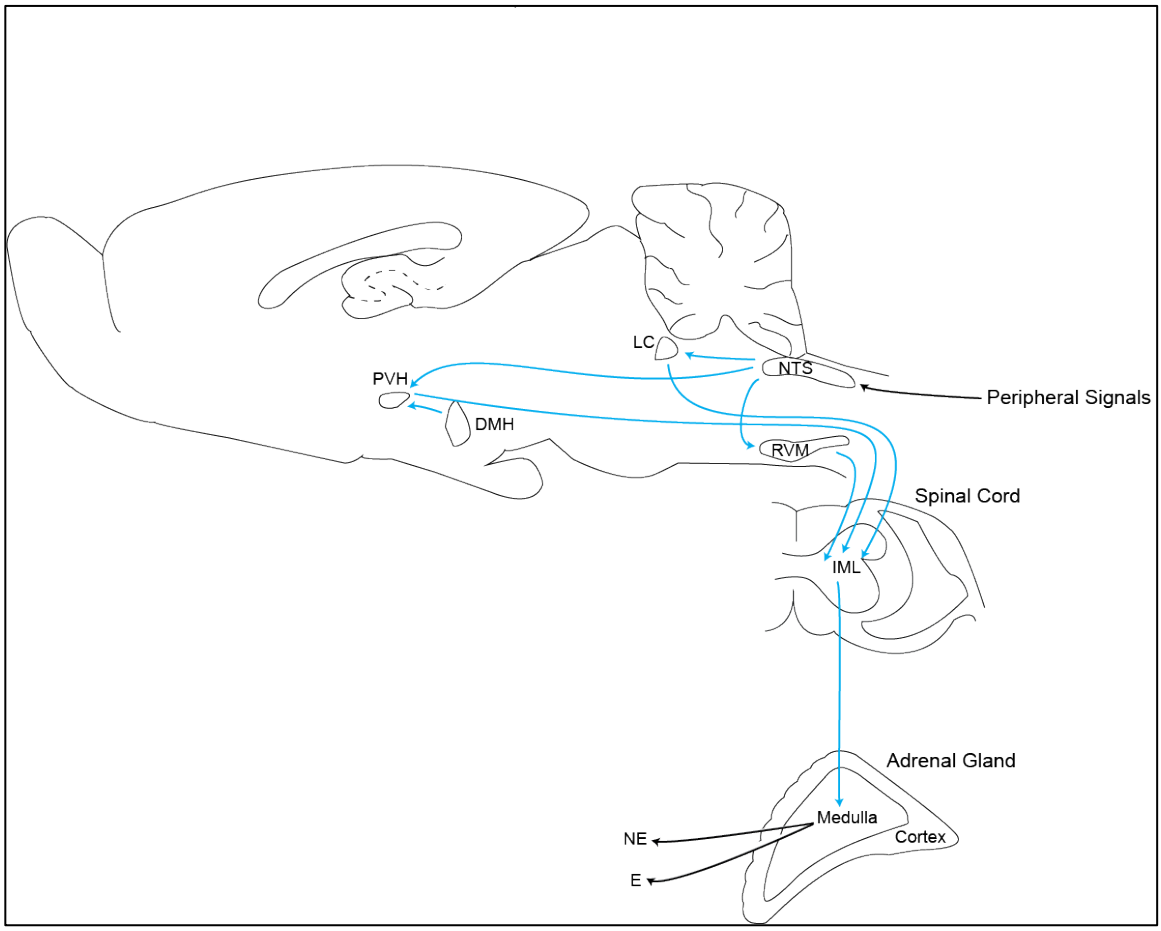

Figure 1.3: Autonomic Stress Response. After receiving afferent signals from peripheral sensory organs, the brainstem (NTS) relays this information to the PVH (which also receives input from DMH), LC and RVM, where pre-autonomic signals are believed to be initiated. The IML of the spinal cord relays these signals to the adrenal medulla through direct sympathetic innervations. Sympathetic activation of the adrenal medulla eventually leads to the release of NE and E. (Autonomic projections are shown in light blue) Abbreviations: NTS (nucleus of the solitary tract); PVH (paraventricular nucleus of the hypothalamus); DMH (dorsomedial nucleus of the hypothalamus); LC (locus coeruleus); RVM (rostral ventromedial medulla); IML (intermediolateral cell column); NE (Norepinephrine); E (Epinephrine)

Although the endocrine and the autonomic stress responses involved many different brain nuclei, there is a substantial degree of overlap between key components of the autonomic and endocrine stress responses in the hypothalamus. For example, neurons in the NTS, a critical node in autonomic circuits, can also modulate HPA axis activity through the release of neuropeptide Y (NPY), glucagon-like peptide 1, somatostatin and enkephalin to the PVH (Ulrich-Lai and Herman 2009). Conversely, pre-autonomic oxytocin (OXT) neurons that project from the PVH to the NTS influence sympathetic output to the adrenal glands (Buijs et al. 1999). The sympathetic nervous system also innervates the adrenal cortex and regulates corticosteroid release (Steckler et al. 2005). In the periphery, catecholamines can directly stimulate GC release from adrenocortical cells (Ehrhart-Bornstein 1998).

Other than central signals, peripheral hormones can also act on PVH neurons to influence the activity of the HPA axis. For example, elevated circulating angiotensin II stimulates HPA axis activity through projections from the subfornical organ to the PVH (Tanimura et al. 1998). In addition, sex steroids such as estrogen exerts a strong stimulatory influence on HPA axis responsiveness (Kudielka and Kirschbaum 2005) and estrogen receptor antagonist microinjected into the PVH inhibited CORT response to stress (Isgor et al. 2003). Since estrogen levels in females fluctuate dramatically during the estrus cycle, most studies on the stress axis and its components minimize the confounding influence of estrogen on the outcome of the experiments by analyzing males. Thus, the PVH represents an important site where information regarding both physiological and psychological stress is received and integrated in order to generate coordinated autonomic and endocrine responses.

Excitatory and Inhibitory Inputs to the PVH

Although stress stimuli activate various intra- and extra- hypothalamic regions in the brain, including the prefrontal cortex, amygdala, hippocampus, nucleus accumbens and hypothalamus, signals from these regions are processed and relayed to the PVH, where key regulators of the endocrine and autonomic stress response are located (Ulrich-Lai and Herman 2009).

The PVH integrates both inhibitory and excitatory inputs from various hypothalamic and extra-hypothalamic regions to coordinate the autonomic and endocrine responses. The dorsomedial hypothalamus (DMH), and medial preoptic area (mPOA) provide major hypothalamic inhibitory signals to the PVH. Stimulation of DMH neurons produces inhibitory GABAergic post-synaptic potentials in neurons in the PVH (Herman et al. 2003), and activation of ventral DMH, in particular, inhibits neuronal activity in the PVH (Ulrich-Lai and Herman 2009). Similarly, the mPOA sends GABAergic projections to the PVH, and lesions of the mPOA lead to an increase in stress-induced ACTH release (Herman et al. 2003). The limbic forebrain regions also provide indirect inhibitory inputs to the PVH mostly through GABAergic neurons in the bed nucleus of the stria terminalis and the hypothalamus (DMH, mPOA). These relays convert the excitatory glutamatergic outflow from the hippocampus and the prelimbic cortex into inhibitory signals to the PVN (Ulrich-Lai and Herman 2009).

On the other hand, excitatory inputs to the PVH from the hypothalamus, midbrain and brainstem act to increase HPA axis activity. Dorsal DMH likely contains neuronal populations that activate the endocrine stress response since inhibiting neurons in that region reduces ACTH releases and neuronal activation in PVH during psychogenic

stress (Cullinan et al. 1996; 2002). Serotonergic projections from the dorsal and the median raphe also increase HPA axis activity and stimulate ACTH and CORT secretion. The NTS is the major source of norepinephrine (NE) and epinephrine (E) (excitatory) inputs to the PVH, which promote CRH and ACTH release.

Neuronal Subpopulations that Regulate Stress Responses

The stress response can be regulated by several stress factors in the central nervous system, including CRH, AVP and Urocortins (UCN). In the PVH, two major neuronal populations, CRH and AVP neurons are involved in regulating the neuroendocrine arm of the stress axis by influencing ACTH release from the anterior pituitary. UCN peptides belong to the CRH family and also bind to CRH receptors; however, they have a widespread distribution in the CNS and potentially acts on multiple CNS sites to regulate stress responses.

Corticotrophin-releasing Hormone (CRH)

CRH neurons are not only found in the PVH, but also in the neocortex, central nucleus of amygdala, the bed nucleus of the stria terminalis, hippocampus, raphe nuclei, periaqueductal gray, olfactory bulb, several thalamic and brain stem nuclei, as well as the cerebellum (Bale and Vale 2004). However, CRH neurons in parvocellular PVH are known to be the critical components of the endocrine stress response, as their activation during stress stimulates ACTH release from the pituitary. In rats, the majority (~80%) of the CRH neurons in the PVH are located in the parvocellular region; while a smaller number (~15%) are found in the magnocellular region (where AVP and OXT predominate) and co-express *Avp* (Swanson et al. 1983). The organization of mouse PVH neurons is illustrated in Figure 1.4. When CRH neurons are activated, CRH is

released from the axonal terminals in the external zone of the median eminence to the hypophyseal-portal system, and finally to the pituitary, where it potently stimulates ACTH release and *Pomc* gene transcription (Autelitano et al. 1989) through binding to its receptor (CRH receptor 1, CRHR1) on corticotrophs. In the pituitary, *Crhr1* mRNA levels positively correlate with *Pomc* expression (Birnberg et al. 1983). During acute stress, there is a rapid release of CRH and an increase in *Crh* primary transcript within 5 to 30 minutes after stress (Luo et al. 1994), and CRH antagonists or antibodies can suppress the majority of ACTH release (Bale and Vale 2004). Not surprisingly, loss of CRHR1 function in mice leads to a failure of ACTH and CORT production during the diurnal peak (Smith et al. 1998), in response to stress (Bale and Vale 2004) or CRH stimulation (Muller et al. 2001); however, baseline ACTH and CORT levels are normal. These studies supported the idea that CRH is a critical player in the activation of the HPA axis.

Figure 1.4

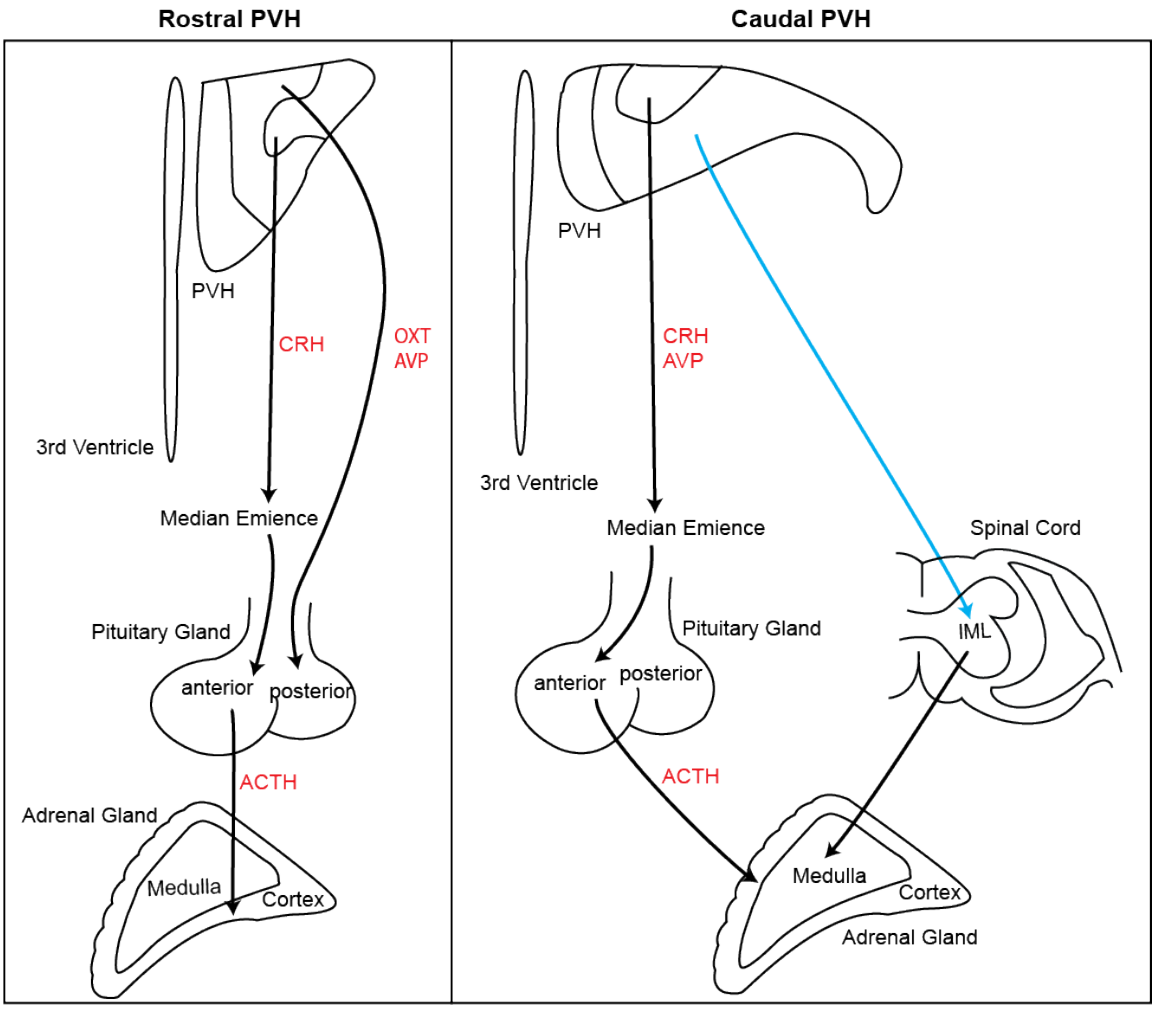

Figure 1.4: Neuroendocrine Organization of the Mouse PVH (Adapted from Biag 2012) In the rostral PVH (left panel), parvocellular neurons release CRH into the ME, and magnocellular AVP and OXT neurons release the respective peptides directly into the posterior pituitary. In the caudal PVH (right panel), parvocellular CRH and AVP release their peptides into the ME. Peptides released into the ME reach the anterior pituitary through the hypophyseal portal circulation and result in the release of ACTH, which stimulates cells in the adrenal cortex to release GCs. The autonomic division of the PVH sends direct projections to the IML, which innervates the adrenal medulla. (Autonomic projections are shown in light blue and hormonal signals in red) Abbreviations: PVH (paraventricular nucleus of the hypothalamus); CRH (corticotropin-releasing hormone); ME (median eminence); AVP (arginine vasopressin); OXT (oxytocin); ACTH (adrenocorticotropic hormone); GCs (glucocorticoids); IML (intermediolateral cell column); NE (norepinephrine); E (epinephrine); SNS (sympathetic nervous system)

There are two CRH receptors: CRHR1 and CRHR2. In the central nervous system (CNS), *Crhr1* is widely distributed and is found in the hypothalamus, cortex, and cerebellum; *Crhr2* is expressed in the hypothalamus (PVH), lateral septum, and the olfactory bulb. In the periphery, *Crhr1* is expressed in the adrenal cortex (Müller 2001). Conditional knockouts have been generated by various groups to study the functions of CRHR1 in the CNS without disrupting its function in the pituitary or adrenal glands. *Camk2a-Cre*-mediated recombination of a floxed *Crhr1* allele, which disrupts CRHR1 function in many forebrain neurons, did not affect basal HPA activity or response to restraint stress; however, both ACTH and CORT levels remained high after stress (Müller et al. 2003). These findings are consistent with a role for forebrain CRHR1 in providing negative feedback to the HPA axis, but do not support a role in the initiation of the stress response. It should be noted that *Crhr1* is highly expressed in the DMH and arcuate nucleus of the hypothalamus (ARH), and is induced in these regions in response to psychogenic stressors that stimulate CRH and AVP neurons in the PVH (Aguilera et al. 2004; P de Gortari 2012). However, little is known about the relative contribution of CRHR1 signaling in the PVH, DMH and ARH to the phenotype of the *Camk2a-Cre*-mediated knockout.

In contrast to CRHR1, stimulating pituitary CRHR2 *in vivo* and *in vitro* does not elicit the release of ACTH. As is the case for *Crhr1* knockouts, mice lacking *Crhr2* have normal baseline ACTH and CORT (Bale et al. 2000; Kishimoto 2000; Preil et al. 2001). Understanding the role of CRHR2 has been complicated by the discrepancies in the phenotypes of *Crhr2* null mutants in response to acute stress reported by various groups. Kishimoto et al. reported that *Crhr2* knockout mice have normal stressed levels

of ACTH and CORT at 10 minutes (Kishimoto 2000). However, Coste et al. and Bale et al. both reported that *Crhr2* knockout mice exhibit elevated CORT levels at 10 minutes, but not at 2 minutes after the initiation of the restraint test. Discrepancies between these findings are likely due to differences in experimental conditions. One of the factors that is known to significantly affect HPA axis reactivity is the housing situation of mice prior to experiment. Isolation of mice from weaning leads to heightened HPA axis response to restraint stress (Williams et al. 2009). The length of the single-housing period of the mice was only reported by one of the groups and it is not known whether the other two groups subjected their experimental mice to long-term isolation early on in life. If that is the case, this could potentially explain the discrepancy in the results of the restraint test on *Crhr2* knockout mice among the various groups. Despite the inconsistencies in the phenotypes reported for *Crhr2* null mutants, at least two of the groups have shown increased CORT levels during restraint. Interestingly, the Coste group also noticed an increase in CORT levels in these mutant 90 minutes after the restraint has stopped (Coste et al. 2000). These findings are consistent with the idea that central CRHR2 signaling provides negative feedback to activated HPA axis.

Arginine vasopressin (AVP)

AVP is primarily produced by the magnocellular neurons in the PVH and supraoptic nucleus (SON). These neurons project to the posterior pituitary and release the peptide into the circulation where they modulate fluid and electrolyte homeostasis (Wotjak et al. 2002). On the other hand, a small population of parvocellular neurons in the PVH releases AVP into the median eminence, where it influences anterior pituitary function through the hypophyseal-portal system (Antoni 1993; Tanoue et al. 2004). In

the pituitary, AVP appears to potentiate the effect of CRH on ACTH release, but does not appear to act directly. In response to psychological stress stimuli such as restraint, there is an increase in *Avp* expression in the parvocellular neurons (and not in the magnocellular neurons) of the PVH, as well as in AVP protein levels in the median eminence (Herman 1995; Ma et al. 1997a). Stress-induced increases in *Avp* transcription have been observed after 1-2 hours, and thus are delayed relative to the immediate increase in *Crh* transcription (which is observed within 5-30 minutes) (Ma et al. 1997a). Although AVP does not appear to stimulate *Pomc* transcription alone or in combination with CRH (Gillies et al. 1982; Abou-Samra et al. 1987), it markedly potentiates the stimulatory effect of CRH on ACTH release, partially via enhanced cAMP production (Gillies et al. 1982; Abou-Samra et al. 1987; Tanoue et al. 2004).

There are several AVP receptors: AVPR1A, AVPR1B, and AVPR2. *Avpr1b* is highly expressed on anterior pituitary corticotrophs, while both *Avpr1a* and *Avpr1b* are expressed in the CNS (AVPR1A: suprachiasmatic nucleus, PVH; AVPR1B: PVH, DMH, olfactory bulb, suprachiasmatic nucleus, SON) (Hurbin et al. 1998; Vaccari et al. 1998; Guillon et al. 2004). *Avpr1b* knockout mice are unable to mount a full ACTH response to most acute stressors (restraint, forced swim, novel environment), while the CORT response is largely intact in most cases, suggesting that there might be compensatory changes in the adrenal glands to maintain the stress response (Tanoue et al. 2004; Roper et al. 2011). Although AVP is capable of potentiating the stimulatory effect of CRH on ACTH releases, unlike CRH, AVP does not appear to be absolutely required for normal acute stress response. Other studies also suggested that hypothalamic AVP neurons might be involved in long-term regulation of the stress axis, particularly for

sustaining responsiveness to chronic stress (Aguilera et al. 2008).

Urocortin (UCN)

Urocortins are CRH-related neuropeptides that can also bind to CRH receptors. Three urocortins have been identified so far. While CRH is bound in a highly preferential manner by CRHR1, UCN1 binds with high affinity to both CRHR1 and CRHR2, and UCN2 and UCN3 bind selectively to CRHR2 (Lewis et al. 2001; Reyes et al. 2001; Ryabinin et al. 2012). The three urocortins are distributed differentially throughout the CNS. *Ucn1* is mainly expressed in the centrally-projecting Edinger-Westphal nucleus (Ryabinin et al. 2012). *Ucn2* mRNA is mainly found in the PVH magnocellular region, SON, and the ARH in the hypothalamus, as well as the locus coeruleus, the trigeminal, facial and hypoglossal motor nuclei and the meninges (Reyes et al. 2001). *Ucn3* mRNA is expressed in the hypothalamus (the median preoptic area, ventral premammillary nucleus, and the bed nucleus of stria terminalis), perifornical area, superior olivary nucleus, parabrachial nucleus, amygdala, and the brainstem (Lewis et al. 2001). Disparities in the distribution of these peptides have been reported in different strains of mice (Weitemier et al. 2005; Ryabinin et al. 2012).

To study the individual roles of UCN peptides in HPA axis function, various groups have generated mice lacking a single urocortin peptide. Multiple *Ucn1* null mutant models have been reported to exhibit normal baseline and restraint stress-induced HPA axis activity (Vetter et al. 2002; Wang et al. 2002; Zalutskaya et al. 2007; Ryabinin et al. 2012). In mice lacking either *Ucn2* or *Ucn3*, circadian rhythms of ACTH and CORT secretion are normal, as are stress-induced HPA axis responses (Chen et al. 2006; Deussing et al. 2010; Ryabinin et al. 2012). Consistent with results from the

knockout studies, *Ucn3* overexpressing mice also show normal baseline as well as restraint-induced CORT release (Neufeld-Cohen et al. 2012). Although *Ucn1/Ucn2* double knockout mice exhibit normal initiation of stress-induced response; they show a delay in recovery after stress (Neufeld-Cohen et al. 2010a; Ryabinin et al. 2012). Furthermore, mice lacking all three UCN peptides have normal baseline CORT levels, similar to what was found in *Crhr2* knockout mice. Yet, stress-induced CORT levels were not reported (Neufeld-Cohen et al. 2010b; Ryabinin et al. 2012).

Together, these studies are consistent with the idea that UCNs do not play a major role in regulating the baseline tone of the HPA axis or the initiation phase of the response to restraint stress. Similar to the *Crhr2* knockout mice, *Ucn1/Ucn2* double knockout mice show diminished ability to negatively regulate an activated HPA axis. This is consistent with the idea that the UCN/CRHR2 system plays a role in terminating the HPA axis response to stress. It is important to realize that since these knockout models involve the removal of gene function from birth, compensatory mechanisms could diminish the HPA axis phenotypes. Therefore, spatial- and temporal- specific genetic manipulations are needed to further elucidate the role of UCNs in regulating different stages (acute initiation/termination, chronic activation) of the HPA axis response.

Negative Feedback of HPA Axis Stress Response

The final product of the activated HPA axis, CORT, plays a variety of physiological functions in the body. It acts on peripheral tissues to modulate inflammatory responses and promotes stored energy mobilization through altering glucose and lipid metabolism, as well as potentiating sympathetic outputs (e.g.

vasoconstriction to maintain blood pressure) (Seematter et al. 2004). Another important function of CORT is to provide negative feedback to the HPA axis, which is largely mediated through the effects in the PVH and pituitary (Ulrich-Lai and Herman 2009). CORT can act through two types of receptors: mineralocorticoid receptors (MR), also known as type 1 glucocorticoid receptors and encoded by the gene *Nr3c2*; and glucocorticoid receptors (GR), also known as type 2 glucocorticoid receptors and encoded by the gene *Nr3c1*. MRs are mainly localized within the septum, hippocampus and the amygdala complex (Reul and de Kloet 1986; Rosenfeld et al. 1990; Gass et al. 2001; Kretz et al. 2001). They have a high affinity for glucocorticoids and are bound substantially even at the circadian nadir of corticosteroid secretion (Ulrich-Lai and Herman 2009). GRs are expressed in the pituitary and throughout the central nervous system, including the hypothalamus, corticolimbic system, brainstem and spinal cord (Reul and de Kloet 1985; Plotsky et al. 1993). They have a low affinity for CORT and thus are only activated by high CORT levels, such as during an acute stress response.

Effects of Glucocorticoids in the PVH

Exogenous CORT suppresses *Crh* and *Avp* transcription in the PVH as well as the secretion of CRH, while adrenalectomy increases *Crh* expression, consistent with a role for glucocorticoids in providing negative feedback to the HPA axis (Plotsky et al. 1993; Makino et al. 1994; Ma et al. 1997b; Kovács et al. 2000). Increased gene dosage of *Gr* leads to lower basal and restraint-induced CRH levels in the median eminence and plasma CORT levels (Reichardt et al. 2000). Conversely, *Gr* null mutants exhibit increased CRH and AVP levels in the median eminence (Kretz et al. 1999).

CRH neurons in the PVH express *Gr* (Coveñas et al. 1993) and CORT-mediated

suppression of *Crh* mRNA is specific to the PVH, as it doesn't affect *Crh* expression in central nucleus of the amygdala or SON (Itoi et al. 2004), raising the possibility that negative regulation of HPA axis by GC occurs at the level of CRH neurons in the PVH. Consistent with this idea, CORT microinjections into the PVH before restraint stress suppress the induction of *Crh* hnRNA in the PVH and *Pomc* hnRNA in the pituitary (Weiser et al. 2011). A PVH specific *Gr* knockout model was generated recently to examine the role of PVH GR signaling in HPA axis regulation. Mice heterozygous for *Gr* in the PVH (*Single-mined homolog 1* (*Sim1*)-*Cre Gr* knockouts) have increased CRH protein, as well as ACTH and CORT levels (Jeanneteau et al. 2012). Whereas CORT is reported to suppress *Avp* transcription as well, this likely occurs indirectly, as GR expression has not been detected on AVP neurons (Berghorn et al. 1995) and GC suppression of AVP gene expression in the hypothalamus requires neural synaptic transmission (Kuwahara et al. 2003).

In addition to providing negative feedback to the HPA axis during acute stress, GCs likely perform a similar role during diurnal fluctuations in HPA axis activity. There is a circadian rhythmicity in CORT levels as well as *Gr* expression, as measured by *in situ* hybridization, in the PVH. In rats, *Gr* expression is the lowest at the end of the light cycle and the highest during early light cycle, which is opposite to circulating CORT levels (highest during daytime in humans, nighttime in mice or rats) (Dallman 1993; Herman et al. 1993). Circadian fluctuations in *Gr* expression are thought to be caused by GC-mediated down-regulation of its own receptor (Dong et al. 1988; Rosewicz et al. 1988; Burnstein and Cidlowski 1989). This auto-regulation of *Gr* expression in the PVH potentially represents a powerful negative feedback mechanism mediated by GC to

reduce GR signaling when its levels are high.

Unlike manipulation of *Gr*, conditional loss function of limbic forebrain *Mr* does not lead to altered basal HPA activity or response to restraint (Berger et al. 2006). Transgenic mice with increased *Mr* in the forebrain also only lead to a mild suppression of the acute stress response in female, but not male, mice (Rozeboom et al. 2007). These data strongly support the idea that CRH neurons in the PVH are major targets of negative feedback through the GR signaling; signaling through forebrain MRs is less likely to be important in this respect.

Effects of Glucocorticoid Signaling in the Pituitary

Pituitary *Pomc* mRNA and plasma ACTH levels positively correlate with circulating GC levels (Birnberg et al. 1983; Watts 2005), as ACTH stimulates GC release from the adrenal cortex. On the other hand, GCs are capable of inhibiting both *Pomc* transcription and ACTH secretion in the pituitary (Drouin et al. 1987; Antoni 1996). Under stressed conditions, rats pretreated with GCs before stress show decreased capacity of the pituitary to secrete ACTH in response to stress (Plotsky et al. 1993; Schmidt et al. 2009). Since the administration of exogenous glucocorticoids (dexamethasone, DEX) can decrease *Crhr1* mRNA expression in anterior pituitary (Zhou et al. 1996), the negative feedback of the HPA axis at the level of pituitary could be mediated by the decrease in levels of CRHR1, through which CRH acts to induce ACTH release. Although *Pomc-Cre* (pituitary) *Gr* knockout adult mice have normal baseline and evening CORT levels, as well as normal activation of HPA stress response, they exhibit a delay in restoring normal baseline level of HPA axis activity after stress (Schmidt et al. 2009). These data are consistent with the idea that pituitary

GR signaling exerts an overall inhibitory tone on the HPA axis at both the level of the hypothalamus (CRH and AVP neurons) and the pituitary (corticotrophs).

Effects of Leptin on HPA Axis Activity

Leptin is an adipocyte-derived hormone in proportion to the amount of adipose tissue which acts in many hypothalamic nuclei to mediate its effect on energy homeostasis such as food intake, energy expenditure and glucose metabolism (Ring and Zeltser 2010). There is compelling evidence to support an additional role of hypothalamic leptin circuits in decreasing HPA axis activity. In addition to severe obesity, infertility, growth-retardation, defective thermoregulation, mice without leptin ($Lep^{ob/ob}$) or leptin receptor ($Lepr^{db/db}$) exhibit hypercorticosteronemia (Friedman and Halaas 1998). Chronic leptin replacement in $Lep^{ob/ob}$ mice suppresses the elevated CORT levels (Luque et al. 2007). Leptin can also partially suppress fasting-induced increase in CRH release, ACTH, as well as CORT levels in mice (Heiman et al. 1997; Ziotopoulou et al. 2000). Furthermore, rats pretreated with physiological levels of leptin exhibit suppressed ACTH and CORT response to restraint stress (Heiman et al. 1997). Consistent with the rodent data, humans that lack leptin also exhibit hyperactivity of the HPA axis (Ozata et al. 1999). Interestingly, these patients have normal basal ACTH and cortisol levels, but elevated hormonal levels at the diurnal peak.

In vitro and *in vivo* studies are consistent with the idea that leptin's effects on the HPA axis are likely to be centrally-mediated. Leptin can block CRH secretion from isolated rat hypothalamus and cannot directly inhibit ACTH secretion from rat primary pituitary cells (Heiman et al. 1997). Moreover, disrupting LepRb signaling throughout the CNS (Cohen et al. 2001) or in the hypothalamus (Ring and Zeltser 2010) is able to

recapitulate elevated CORT levels observed in *Lepr*$^{db/db}$ mice. Together these data support the idea that central leptin signaling plays a crucial role in suppressing the HPA axis during both metabolic and psychological stress.

Effects of Insulin on HPA Axis Activity

Central insulin has been shown to regulate food intake (Brown et al. 2006), as well as the metabolism of lipids and glucose in the periphery (Leona Plum 2006). Several studies are consistent with the idea that insulin and CORT signaling might be cross-repressive. Under baseline conditions, insulin is secreted with a diurnal rhythm that is opposite to that of CORT (Dallman et al. 1995). Furthermore, elevated glucocorticoid levels lead to systemic (liver, muscle, fat)(Meyuhas et al. 1976; Rizza et al. 1982; Pagano et al. 1983; Tappy et al. 1994; Lansang and Hustak 2011) and central insulin resistance (Piroli et al. 2007; Osmanovic et al. 2010). Diabetic animal models (Streptozotocin (STZ)-treated mice) exhibit hyperactivation of HPA axis, as evidenced by elevated ACTH and CORT levels, which is fully reversed by treatment with insulin (Chan et al. 2003). The insulin sensitizer, Rosiglitazone, can also suppress stress-induced CORT release (Ryan et al. 2012). Notably, HPA axis dysregulation is often observed in humans suffering from diabetes (Reynolds et al. 2010).

There is some evidence to support the idea that insulin's effects on HPA axis activity are, in part, mediated by signaling in the hypothalamus. Female mice with decreased FOXO1 (Forkhead box protein O1) signaling in the PVH (which has the effect of increasing insulin signals) show a strong trend toward decreased hypothalamic *Avp* expression. Dominant negative form of FOXO1 also directly decreases the activity of the *Avp* promoter (Redemann 2010). Together, these observations are consistent

with the hypothesis that central insulin signaling suppresses HPA axis activity through the regulation of *Avp* transcription and synthesis. If central insulin signaling normally functions to prevent over-activation of the HPA axis, it could provide a mechanistic basis for reported associations between diabetes and psychiatric disorders.

Regulation of HPA Axis by Hypothalamic Neuropeptides (NPY/AgRP/α-MSH)

NPY

In addition to the hormonal factors (CRH and AVP), other neuropeptides in the hypothalamus including neuropeptide Y (NPY), agouti-related peptide (AgRP), and alpha-melanocyte-stimulating hormone (α-MSH) can also modulate activity of the HPA axis. NPY is a 36 amino-acid neuropeptide that is widely expressed throughout the CNS, and about 1/3 of the NPY projections in the PVH originate from neurons in the ARH that co-express *AgRP* (Füzesi et al. 2007). The PVH is densely innervated by NPY neurons (Allen et al. 1983; Morris et al. 1989) and *Npy Y1* receptor is expressed on CRH neurons in the PVH (Dimitrov et al. 2007). Not only does the pattern of diurnal fluctuations in hypothalamic NPY levels is similar to that reported for plasma CORT levels (i.e. peaking at the end of the light period) (Jhanwar-Uniyal et al. 1990), elevated *Npy* expression in the ARH also coincides with increased plasma CORT levels in response to both psychological (i.e. restraint) and nutritional (i.e. fasting) stressors (White and Kershaw 1990; Makino et al. 2000; Sweerts et al. 2001; Makimura et al. 2003; Kas et al. 2005; Sato et al. 2005). Direct injection of NPY into the PVH *in vivo* leads to increased plasma ACTH and CORT levels (Wahlestedt et al. 1987; Haas and George 1989), and stimulates AVP release from hypothalamic cultures (Korbonits et al. 1999). Since pre-treatment with third ventricle infusion of NPY Y1 antagonist is able to

abolish the stimulating effect of NPY (Dimitrov et al. 2007) on CORT release in vivo, the effect of NPY on HPA axis is likely mediated by the NPY Y1 receptors. These studies are consistent with the idea that ARH NPY neurons stimulate HPA axis.

AgRP

In the CNS, *AgRP* co-expresses with *Npy* in the ARH (Füzesi et al. 2007). AgRP stimulates food intake by antagonizing melanocortin receptor 4 (MC4R) and therefore blocking the actions of α-MSH (Ollmann et al. 1997). Despite the opposite effect of α-MSH and AgRP in regulating food intake, intra-PVH injections of either AgRP or POMC similarly increases hypothalamic release of CRH and AVP, and stimulates ACTH release (Dhillo et al. 2002). However, since the effect of AgRP on the HPA axis is not influenced by the co-administration of α-MSH, it has been suggested to involve other independent receptors (Shimizu et al. 2008). While AgRP increases HPA axis activity, similar to *Npy*, it's mRNA expression is not down-regulated, but rather increased by GCs (Hagimoto et al. 2013).

α-MSH

Contrary to AgRP, α-MSH is an agonist of the MC3R and MC4R. It is derived from the 241-amino acids precursor polypeptide POMC by specific cleaving enzymes PC2 (Benjannet et al. 1991). Arcuate POMC neurons project to the MC4R in the PVH, where the major components of the HPA axis are located (Cone et al. 2001), and CRH neurons in the PVH also express MC4R (Lu et al. 2003). Consistent with the anatomical evidence, α-MSH increases AVP and CRH release from the hypothalamus and stimulates ACTH and CORT release in rats (Frijtag et al. 1998; Ludwig et al. 1998; Dhillo et al. 2002), and the effect on CORT release is blocked by a selective MC4R

antagonist (Lu et al. 2003). These studies are consistent with the idea that α-MSH acts directly on PVH CRH neurons to activate the HPA axis. Contrary to in the anterior pituitary, where GCs suppress POMC expression, *Pomc* and α-MSH and levels in the medial basal hypothalamus are increased by GCs (Wardlaw et al. 1998). Therefore, the hypothalamic melanocortin system (α-MSH and AgRP) appears to stimulate the HPA axis, but its components are not subjected to the negative regulation of glucocorticoid signaling.

Major components of the HPA axis such as CRH and AVP are subject to negative feedback regulation by GCs, yet GCs appear to stimulate arcuate *Npy* (White et al. 1994; Shimizu et al. 2008) and medial basal *AgRP* and *Pomc* (Savontaus et al. 2002). GC's effect on *Npy* and *Agrp* expression is possibly mediated by a direct mechanism, since ARH NPY/AgRP neurons also express *Gr* (Hisano et al. 1988). While these data are consistent with the hypothesis that ARH POMC and NPY/AgRP neurons promote stress responses, the role of these neurons in the regulation of the HPA axis during fasting (metabolic stress), and restraint (psychological stress) has not been examined. If ARH POMC and NPY/AgRP neurons stimulate the HPA axis, they might represent an additional source of negative feedback to the system in addition to GCs. Both insulin and leptin have been reported to inhibit the activity of NPY/AgRP neurons (Niswender et al. 2004), and thus actions of one or both of these signals could potentially provide negative feedback to the HPA axis. On the other hand, leptin activates while insulin suppresses POMC neuronal activity (Hill et al. 2010), suggesting that insulin, but not leptin provides negative regulation to the HPA axis through POMC neurons.

Effects of Central Leptin and Insulin Signals on Hypothalamic NPY/AgRP/POMC Neurons

If there is a need for GC-independent negative feedback to inhibit AgRP/NPY and POMC neuronal activity during stress, leptin and insulin might represent two possible candidates for this function, as they both hyperpolarize ARH NPY neurons (Yang et al. 2010) and insulin also inhibits POMC neuronal activity. In terms of the regulation of gene expression, both insulin and leptin decrease *Npy* and *AgRP*, and increase *Pomc* expression (Porte et al. 2002). It is interesting to note that insulin hyperpolarizes POMC neurons (usually associated with reduced peptide releases) but increases *Pomc* mRNA expression. This suggests a complex and yet unknown relationship between the regulation of gene expression and the electrophysiological effect of insulin in POMC neurons. Since both central insulin and leptin signals are important regulators of NPY/AgRP and POMC neuronal functions and activities, these neuronal populations may represent a way of integrating metabolic and stress-related signals to modulator HPA axis activity.

HPA Axis Dysregulation in Animal Models of Psychiatric Disorders

There are many types of psychiatric disorders, which include mood/affective disorders (e.g. anxiety, depression, bipolar disorder) and psychotic disorders (e.g. schizophrenia). HPA axis hyperactivity is commonly seen in patients with anxiety disorder, bipolar disorder, as well as depression (Keller et al. 2006; Jokinen and Nordström 2009; Zelena 2012). Furthermore, decreased HPA axis sensitivity to glucocorticoid suppression and increased sensitivity to CRH have been reported in patients with major depression and schizophrenia (Heuser et al. 1994; Lammers et al.

1995). Recently, an association has been reported between polymorphisms in *AVPR1B* and *CRHR1* genes and susceptibility of bipolar disorder patients to exhibit psychotic behaviors (Leszczyńska-Rodziewicz et al. 2012). These studies are consistent with the idea that there is a relationship between hyper-activation of the HPA axis and psychiatric disorders. The effects of genetic manipulations of distinct HPA axis components on anxiety-related behavioral phenotypes (as a surrogate for psychiatric disorders) in mouse models are summarized below.

CRH/UCN

Overexpression of CRH, the major PVH component of the HPA axis, leads to increased anxious behavior in mice (Keller et al. 2006; Dedic et al. 2012), while *Crhr1* null mice have reduced anxiety (Smith et al. 1998; Timpl et al. 1998). Forebrain specific *Crhr1* knockouts also exhibit a reduction in anxiety, suggesting that CRHR1 signaling in the forebrain increases anxiety-related behaviors. In contrast, CRHR1 signaling in midbrain dopaminergic neurons seems to be involved in suppressing pathways that increase anxiety as removing *Crhr1* from these neurons leads to increase anxiety (Müller et al. 2003; Refojo et al. 2011). Loss of the other CRH receptor, *Crhr2* has been reported to increase anxiety in some strains (Kishimoto 2000), but not others (Vetter et al. 2002; Wang et al. 2002; Ryabinin et al. 2012). Similar to some of the *Crhr2* knockout models, mice that lack all three ligands of CRHR2: UCN1, UCN2 and UCN3, show an increase in anxiety-like phenotypes (Neufeld-Cohen et al. 2010b). Although there have been some inconsistencies in findings from the various knockout models, most studies support a role for the UCN/CRHR2 system in suppressing anxiety-related behaviors (Chen et al. 2006; Deussing et al. 2010; Neufeld-Cohen et al. 2010b; Ryabinin et al.

2012). While signaling in the CRHR1 system can be anxiogenic (forebrain) or anxiolytic (midbrain), the CRHR2 system consistently seems to exert an anxiolytic effect. Nevertheless, due to the widespread distribution of the ligands (CRH, UCN1-3) and the receptors (CRHR1, CRHR2), conditional knockout models will be needed to parse out the roles of the CRH/CRHR1 and the UCN/CRHR2 systems in specific brain regions.

AVP

The other important component of the HPA axis, AVP, has also been implicated in the development of anxiety- and depressive-like symptoms in humans (Zelena 2012). Rats bred for high anxiety levels show an increase in *Avp* expression in the PVH (Wigger et al. 2004). Injection of AVP also leads to increase in anxiety in rats (Appenrodt et al. 1998). Consistently, *Avpr1a* knockout mice show a reduction in anxiety-like behaviors (Bielsky et al. 2004). Therefore, central AVP system is also likely to be involved activating pathways that increase anxiety-like behaviors in rodents.

NPY

In addition to CRH/UCN and AVP, the NPY system serves as another example of the intricate relationship between the HPA axis and behavioral responses. In addition to the previously discussed role of NPY in the HPA axis, central injections of NPY produce anxiolytic effects on animal models of anxiety. Similarly, intracerebroventricular injections of an NPY Y1 receptor agonist suppresses anxiety-like phenotypes in animal models (Britton et al. 1997). This effect has been shown to be partially mediated through NPY Y1 receptors in the amygdala where CRH elicits an opposite, anxiogenic effect (Thorsell 2010). Though often neglected in discussions of HPA axis regulation, NPY Y1 receptor signaling in the amygdala is likely a modulator of anxiety responses

during stressful events.

Glucocorticoid Receptor

Mice with conditional loss of glucocorticoid receptor function in the forebrain (*Alpha Calmodulin-dependent Protein Kinase II (Camk2a)-Cre*) or the whole-brain (*Nestin-Cre*) exhibit reduced anxiety-related behaviors (Gass et al. 2001; Boyle et al. 2005). It is important to note that the whole-brain *Gr* knockouts exhibit reduced anxiety in the face of elevated levels of CRH in the PVH (Tronche et al. 1999). The increase in CRH is consistent with the proposed role of GR in providing negative feedback to the HPA axis. As PVH neurons project to midbrain centers rich in dopaminergic neurons (Geerling et al. 2010) where CRHR1 signaling is reported to be anxiolytic (Refojo et al. 2011), it is possible that increased CRH signaling to the midbrain contributes to the reduction in anxiety in these mice.

In conclusion, there are compelling data to support a role for CRH/CRHR1, UCN/CRHR2, AVP/AVPR1A, and the GR systems in positively and negatively regulating anxiety-related behavioral phenotypes. Whereas AVP, GR and forebrain CRHR1 systems are generally anxiogenic, UCN/CRHR2 and midbrain dopaminergic CRHR1 systems appear to be anxiolytic. In addition to direct effects of stress hormones, interactions with other neuropeptide systems (e.g. NPY) appear to modulate both stress response and anxiety.

Insulin and Psychiatric Disorders

Diabetes has been associated with an increased likelihood of developing mental disorders, including anxiety, depression, and schizophrenia (Kruse et al. 2003; Kohen 2004; Lin et al. 2008). At the same time, it has been reported that schizophrenia is often

accompanied by metabolic dysregulation, including insulin resistance, impaired glucose tolerance and type 2 diabetes mellitus (Bushe and Holt 2004; Harris et al. 2012). Furthermore, in a recent publication, by using a computational approach (NETBAG+) to analyze schizophrenia-associated genetic variations, insulin signaling has been identified as one of the genetic networks that maybe functionally related to the etiology of schizophrenia (Gilman et al. 2012). The idea that deficits in central insulin signaling contribute to the pathology of mental disorders is also supported by studies using mouse models. Treatment with the neuronal insulin sensitizer, dicholine succinate (increase autophosphorylation of insulin receptors in neurons), reduces the anxiety scores of mice in various stress paradigms (Cline et al. 2012). Conversely, mice with deficits in neuronal Akt signaling, a downstream target of insulin signaling, also exhibit behavior associated with schizophrenia (Siuta et al. 2010).

Manipulations of the CRH, AVP, UCN or GR systems often lead to alterations in both HPA axis and anxiety levels in mice. These effects may be mediated via influences directly on the HPA axis or via limbic structures. Together with the observation that FOXO1 can directly regulate AVP expression (Redemann 2010), it raises the possibility that central, possibly hypothalamic, insulin resistance (la Monte et al. 2009; Zhao and Townsend 2009) occurring during diabetes could lead to dysregulation of HPA axis or/and increased anxiety through altered hypothalamic AVP system.

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CHAPTER 2: "Hypothalamic Leptin- and Insulin-sensing Circuits Coordinate to Regulate Core Body Temperature."

Introduction

Central injections of both leptin or insulin have been reported to increase energy expenditure and reduce food intake (Baskin et al. 1999; Kulkarni et al. 1999; Xu et al. 2008). Both hormones also activate similar signaling cascades including the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways to regulate energy intake (Schwartz and Zeltser 2013). While insulin does not stimulate signal transducer and activator of transcription 3 (STAT3) phosphorylation directly, it can potentiate leptin-induced STAT3 signaling (Carvalheira et al. 2001). Given the similar metabolic functions and receptor distribution of leptin and insulin in the hypothalamus (Schwartz and Zeltser 2013), it is therefore possible that interactions between hypothalamic insulin- and leptin-sensing circuits may provide a way to integrate information within the homeostatic circuits to maintain energy balance. Our lab has previously reported mice that lack hypothalamic leptin receptor (LepRb) signals ($L^{2.1}$ KO) exhibited a continuous increase in adiposity until 8 weeks of age, when stabilization of adiposity was observed. The increasing adiposity was accompanied by a progressive development of hyperinsulinemia, raising the possibility that increased central insulin signaling could act to limit further increase in the adiposity (Ring and Zeltser 2010). Since many of insulin's effects on energy metabolism are known to be mediated by hypothalamic circuits, we

embarked on a project that studied the contribution of hypothalamic insulin receptor (InsR) signals in $L^{2.1}$ KO to regulate energy homeostasis.

This chapter consists of a manuscript in preparation that describes the characterization of the metabolic phenotypes in *Nkx2.1-Cre* mediated conditional knockout animals that lack the majority of hypothalamic insulin ($I^{2.1}$ KO), leptin ($L^{2.1}$ KO) or both receptors ($D^{2.1}$ KO). The goal of the study is to provide some understanding into the nature of the interactions between hypothalamic InsR and LepRb signals in regulating body weight and energy metabolism.

Manuscript

(Author Contributions: Designed the experiments: CN Angie Chong, LM Zeltser. Analyzed data: CN Angie Chong. Performed experiments: CN Angie Chong, RA Greendyk (summer student, Figure 2.2C-D). Wrote the paper: CN Angie Chong, LM Zeltser.)

Hypothalamic Leptin- and Insulin-sensing Circuits Coordinate to Regulate Core Body Temperature.

Manuscript in preparation

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Abstract

Central injections of insulin and leptin activate similar intracellular pathways and evoke similar sets of physiological responses, raising the possibility that interactions between circuits sensing these signals help to coordinate physiological processes that maintain energy balance. Opportunities for cross-talk are particularly abundant in the hypothalamus, where networks of insulin- and leptin-sensing neurons are highly enriched and interconnected. To examine whether leptin and insulin signaling networks in the hypothalamus interact to regulate energy homeostasis, we used an *Nkx2.1-Cre* driver to generate conditional knockouts of leptin receptor ($L^{2.1}$ KO), insulin receptor ($I^{2.1}$ KO), and double knockouts of both receptors ($D^{2.1}$ KO). Consistent with previous reports, $L^{2.1}$ KO males were hyperphagic and severely obese, while $I^{2.1}$ KO males were similar to controls. Disruption of both signaling networks resulted in $D^{2.1}$ KO (males) with higher body weight and adiposity than $L^{2.1}$ KOs, without any

differences in food intake. Efforts to (further) characterize metabolic phenotypes using protocols that involved single housing were stymied by a significant mortality rate for $D^{2.1}$ KOs within several days, in part due to a refusal to eat. Whereas baseline (fed) core body temperature in $D^{2.1}$ KOs was similar to $L^{2.1}$ KOs under group-housing condition, overnight-fasting during single-housing condition resulted in a dramatic reduction of body temperature in $D^{2.1}$ KOs. Although also lacking hypothalamic InsR signals, $I^{2.1}$ KOs did not exhibit similar phenotype as $D^{2.1}$ KOs. This study highlighted the significance of the interactions between hypothalamic insulin- and leptin-sensing circuits in maintaining fasting body temperature, and the existence of multiple compensatory pathways in the central nervous system for thermoregulation (thermogenesis and social thermoregulatory behaviors) during energy deficit.

Introduction

The ability to accurately gauge and respond to energy requirements is essential for maintaining energy balance. Neuronal circuits in the brain and periphery sense and integrate nutrient, hormonal and neuronal signals of energy status to direct a coordinated series of physiological processes regulating energy intake and expenditure (Schwartz et al. 1996; Shimizu et al. 1997; Baskin et al. 1999; Porte et al. 2002; McMinn et al. 2004; Plum et al. 2005; Belgardt and Brüning 2010; Ring and Zeltser 2010; Moreno-Aliaga et al. 2011; Schwartz and Zeltser 2013). Beta cell-derived insulin and adipocyte-derived leptin relay signals of positive energy balance to neuronal populations distributed throughout the central nervous system (CNS) (Woods and Seeley, 2000). Patterns of fluctuations in plasma levels of these hormones are consistent with the idea that insulin provides short-term information about nutritional status, while leptin levels convey information about long-term energy stores. As obesity is associated with impaired sensitivity to both of these hormones, it is important to understand how these signals are transmitted and integrated under normal and pathophysiological conditions.

Similarities between receptor distribution, intracellular signaling cascades and physiological effects of central leptin and insulin led to the proposal that interactions between networks sensing these signals provide an important mechanism for integration within homeostatic circuits (Baskin et al. 1999; Kulkarni et al. 1999; Xu et al. 2008). Leptin and insulin act on overlapping populations of neurons that are widely distributed, but highly concentrated in

hypothalamic and brainstem nuclei that regulate energy homeostasis (Baskin et al. 1983; Corp et al. 1986; Werther et al. 1987; Elmquist et al. 1998; Ahren and Havel 1999; Chen and Farese 2002; Fulton et al. 2006; Hommel et al. 2006; Xu et al. 2008; Myers et al. 2009; Scott et al. 2009; Hayes et al. 2010; Könner and Bruning 2012). In the central nervous system, both hormones activate similar signaling cascades including the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways to regulate energy intake (Ahren and Havel 1999; Bruning et al. 2000; Porte et al. 2002; Niswender et al. 2004; Fulton et al. 2006; Hommel et al. 2006; Belgardt and Brüning 2010; Hayes et al. 2010; Ring and Zeltser 2010; Schwartz and Zeltser 2013). Whereas insulin does not appear to stimulate signal transducer and activator of transcription 3 (STAT3) phosphorylation directly, it can potentiate leptin-induced STAT3 signaling (Barr et al. 1997; Shimizu et al. 1997; Baskin et al. 1999; Schwartz et al. 2000; Carvalheira et al. 2001; Air et al. 2002; Niswender et al. 2004; Komori et al. 2005). While leptin and insulin signals may be integrated at the intracellular level in some types of neurons, there are also cases where leptin and insulin act on adjacent, but distinct neuronal subpopulations (Baskin et al. 1983; Corp et al. 1986; Werther et al. 1987; Menéndez and Atrens 1991; Elmquist et al. 1998; Ahren and Havel 1999; Myers et al. 2009; Scott et al. 2009; Sanchez-Alavez et al. 2010; Williams et al. 2010; Könner and Bruning 2012). Finally, central injections of both leptin and insulin have been reported to increase energy expenditure and reduce food intake (Barr et al. 1997; Shimizu et al. 1997; Baskin et al. 1999; Schwartz et al. 2000; Air et al. 2002; Niswender et al. 2004; Xu et al.

2008).

The hypothalamus is known to be one of the important sites of actions for insulin and leptin on energy metabolism and hypothesized site of interaction (Baskin et al. 1983; Corp et al. 1986; Werther et al. 1987; Schwartz et al. 1996; Shimizu et al. 1997; Elmquist et al. 1998; Ahren and Havel 1999; Baskin et al. 1999; McMinn et al. 2004; Plum et al. 2005; Fulton et al. 2006; Hommel et al. 2006; Myers et al. 2009; Scott et al. 2009; Belgardt and Brüning 2010; Hayes et al. 2010; Könner and Bruning 2012). In previous studies, we used *Nkx2.1-Cre*-mediated recombination of a floxed *Leptin receptor* (*Lepr*) allele to investigate the role of hypothalamic LepRb signaling in energy balance (Ahren and Havel 1999; Kulkarni et al. 1999; Porte et al. 2002; Niswender et al. 2004; Belgardt and Brüning 2010; Ring and Zeltser 2010; Schwartz and Zeltser 2013). This approach broadly disrupted LepRb signaling in the hypothalamus, as well as in the lung, posterior pituitary, thyroid and neurons derived from *Nkx2.1*-expressing domains in the medial ganglionic eminence (Barr et al. 1997; Shimizu et al. 1997; Baskin et al. 1999; Schwartz et al. 2000; Carvalheira et al. 2001; Air et al. 2002; Chen and Farese 2002; Niswender et al. 2004; Komori et al. 2005; Xu et al. 2008). However, it preserved signaling in midbrain and hindbrain nuclei that have been implicated in mediating some of leptin's effects on food intake and energy expenditure (Baskin et al. 1983; Corp et al. 1986; Werther et al. 1987; Menéndez and Atrens 1991; Elmquist et al. 1998; Ahren and Havel 1999; Bruning et al. 2000; Fulton et al. 2006; Hommel et al. 2006; Myers et al. 2009; Scott et al. 2009; Hayes et al. 2010; Sanchez-Alavez et al. 2010; Williams et al. 2010;

Könner and Bruning 2012). From birth to until 8 weeks of age, *Nkx2.1-Cre Lepr* knockout ($L^{2.1}$ KO) mice are indistinguishable from the global *Lepr* knockout ($Lepr^{db/db}$) and develop similar degrees of hyperphagia, adiposity, and insulin resistance. After 8 weeks of age, $Lepr^{db/db}$ progressively gain adiposity and are unable to adapt to a cold challenge throughout adulthood, $L^{2.1}$ KOs over 8 weeks of age maintain stable levels of body fat and develop adaptive thermogenesis (Ring and Zeltser 2010).

$L^{2.1}$ KO mice exhibit marked peripheral insulin resistance and hyperinsulinemia, raising the possibility that increased central insulin signaling could act to limit the severity of thermogenic-related deficits and adiposity. Consistent with this idea, hyperinsulinemic leptin-deficient $Lep^{ob/ob}$ mice have increased baseline levels of hypothalamic phosphorylated-protein kinase B (p-Akt) (Barr et al. 1997; Shimizu et al. 1997; Bruning et al. 2000; Schwartz et al. 2000; Air et al. 2002; Niswender et al. 2004; Komori et al. 2005), a downstream target of insulin receptor signaling. Insulin injections into the anterior or mediobasal hypothalamus have been reported to increase brown adipose tissue (BAT) thermogenesis and metabolic rate, respectively (Menéndez and Atrens 1991; Bruning et al. 2000; Sanchez-Alavez et al. 2010). In theory, stable levels of adiposity in $L^{2.1}$ KO could reflect the direct actions of insulin signals within and/or outside the hypothalamus or the potentiation of extrahypothalamic leptin signals. To begin to investigate the nature of interactions between leptin- and insulin-sensing brain networks, we compared metabolic and neuroendocrine phenotypes of mice with *Nkx2.1-Cre*-mediated disruption of InsR ($I^{2.1}$ KO), LepRb ($L^{2.1}$ KO),

or both receptors (double knockout, $D^{2.1}$ KO).

Methods

Generation of $I^{2.1}$, $L^{2.1}$ and $D^{2.1}$ KO mice

To generate mice with genetic deficiency of *Insr* and/or *Lepr* in the hypothalamus, the *Nkx2.1-Cre* driver line (C57BL/6J-Tg(Nkx2-1-cre)2Sand/J, provided by S. Anderson, Weill Cornell Medical College) (Xu et al. 2008; Zhang et al. 2011; Bartfai and Conti 2012) was crossed to mice homozygous for a floxed allele of *Lepr* (FVB.BKS(D)-*Lepr*^{db}/ChuaJ, provided by S. Chua, Albert Einstein College of Medicine) (McMinn et al. 2004; Ring and Zeltser 2010). The resulting F1 heterozygotes (*Nkx2.1*-Cre; *Lepr*$^{fl/+}$) were crossed to mice homozygous for a floxed allele of *Insr* (B6.129S4(FVB)-*Insr*^{tm1Khn}/J, provided by D. Acilli, Columbia University Medical Center) (Kulkarni et al. 1999; Saito et al. 2003; Streijger et al. 2009) to generate F2 *Nkx2.1-Cre*;*Lepr* $^{fl/+}$;*Insr* $^{fl/+}$ and *Lepr* $^{fl/+}$;*Insr* $^{fl/+}$ mice. F2 *Lepr* $^{fl/+}$;*Insr* $^{fl/+}$ mice were intercrossed to generate F3 *Lepr*$^{fl/fl}$;*Insr*$^{fl/fl}$ females, which were then crossed to F2 *Nkx2.1-Cre*; *Lepr* $^{fl/+}$;*Insr* $^{fl/+}$ males to generate our experimental animals: *Nkx2.1-Cre;Lepr*$^{fl/fl}$ ($L^{2.1}$ KO), *Nkx2.1-Cre;Insr*$^{fl/fl}$ ($I^{2.1}$ KO), *Nkx2.1-Cre;Lepr*$^{fl/fl}$;*Insr*$^{fl/fl}$ ($D^{2.1}$ KO), and *Lepr*$^{fl/fl}$;*Insr*$^{fl/fl}$ (control) in a Mendelian ratio. Due to the low probability of achieving the desired genotypes in the offspring, two crosses were generally required to generate one conditional knockout male. Mouse genotypes were assessed by PCR on genomic DNA from tail tips using the following primers:

Cre
5’ GCGGTCTGGCAGTAAAAACTATC 3’ (forward)
5’ GTGAAACAGCATTGCTGTCACTT 3’ (reverse)

Lepr
5’ GTCTGATTTGATAGATGGTCTT 3’ (forward)
5’ AGAATGAAAAAGTTGTTTTGGGA 3’ (forward)
5’ GGCTTGAGAACATGAACAC 3’ (reverse)

Insr
5’ TGCACCCCATGTCTGGGACCC 3’ (forward)
5’ GCCTCCTGAATAGCTGAGACC 3’ (reverse)

Animal husbandry

Mice were maintained in a temperature- and light-controlled environment (22°C ± 1°C; 12-hour light/12-hour dark cycle). Except where noted, mice were housed in sex-matched groups of 2–4 with at least 1 $L^{2.1}$KO, 1 $D^{2.1}$ KO, and 1 control animal per cage. Pregnant and nursing mice were housed no more than 1 dam per cage. Pups were weaned on postnatal day 21. Unless otherwise noted, mice had ad libitum access to chow (9% calories from fat, 5058 Mouse diet 20; Labdiet) and water until the time of sacrifice. For studies the involved single-housing, mice were singly-housed for 2-3 weeks before experiments were done. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at Columbia University Health Sciences Division.

Preservation of adipose tissue depots

At the time of sacrifice, animals were anesthetized with 2.5% Avertin, 0.02 ml/g i.p. before cervical dislocation. Brown adipose tissue (BAT) was weighed, dissected and preserved in Allprotect tissue reagent (Qiagen). White adipose tissue depots were fixed overnight and processed for paraffin embedding. Five micron-thick sections were stained with hematoxylin and eosin and 200X images

were acquired using (Nikon Eclipse 80i equipped with a Retiga EXi camera and X cite 120 fluorescent illumination system). Images were converted into a binary format and analyzed with ImageJ with an adapted analysis method (Chen and Farese 2002; Cannon and Nedergaard 2004). At least four images across each adipose tissue sample were acquired and a total of three to four animals from each group were analyzed.

Analysis of body composition

To determine body composition, mice were subjected to nuclear magnetic resonance imaging (the Minispec, Bruker).

Measurement of glucose, insulin, and leptin levels

All blood samples were collected between 10 am and noon. Fasting blood samples were taken following 14–16 hours of fasting with ad libitum access to drinking water. Whole blood for glucose levels was taken via tail nick and assayed using a glucometer with disposable test strips (Abbott). The upper limit of measurement was 500 mg/dl; any “HI” readings were recorded as 501 mg/dl. Serum for insulin and leptin assessment was collected via orbital sinus puncture of isoflurane-anesthetized animals. Blood was collected and clotted at room temperature for one hour then centrifuged; serum was decanted and stored at –20°C until used in leptin (Millipore) or insulin (Millipore) ELISA, per the manufacturer’s protocol.

Indirect calorimetry and food intake

Oxygen consumption and food intake were measured simultaneously using a 16-cage Indirect Calorimetry System combined with Feeding Monitor and

TSE ActiMot system (TSE-Systems). Oxygen consumption was measured using a paramagnetic O_2 sensor over a 24-hour period.

Measurement of food intake in group-housed animals

2-3 mice of the same genotype were placed in each cage with food hoppers filled with pre-weighed food. The amount of food remaining in the hopper was measured every 2-3 days. Averaged daily food intake was calculated based on the number of days between measurements and the number of mice in each cage.

Measurement of core body temperature

Core body temperature was measured by a rectal temperature probe thermometer (Thermoworks) between 10am to noon. Baseline measurements were obtained under ad libitum feeding conditions; fasted readings were recorded after an overnight fast of 12-16 hours. During short-term cold challenge, mice fasted overnight were subjected to cold by placing in a glass beaker submerged in ice for 75 minutes. Body temperature was then assessed every 15 minutes.

Real-Time Quantitative PCR

Total RNA was isolated from BAT that had been stored in Allprotect tissue reagent using the RNeasy Plus Universal kit (Qiagen). Reverse transcription was achieved using Transcriptor First Strand cDNA Synthesis kit (Roche). Quantitative PCR was done on a LightCycler 480 system (Roche) using the LightCycler 480 SYBR Green I Master. Expression levels for *Ucp1* (forward, 5' GTGAAGGTCAGAATGCAAGC 3'; reverse, 5' AGGGCCCCCTTCATGAGGTC

3') were normalized against *Beta actin* (forward, CGCCACCAGTTCGCCAT; reverse, CTTTGCACATGCCGGAGC) using the delta delta CT method.

Statistics

Data are presented as group mean ± SEM. Statistical comparisons were performed between sex- and age- matched groups using 2-tailed, unpaired Student's *t* test or 1-way ANOVA with Bonferroni post-hoc analysis. *P* value of 0.05 or less was considered to be statistically significant.

Results

Nkx2.1-Cre-mediated loss of InsR signaling exacerbates body weight and adiposity phenotypes of $L^{2.1}$ KOs

Consistent with findings from mice lacking neuronal InsR signaling (NIRKO mice) (Menéndez and Atrens 1991; Schwartz et al. 1992; Bruning et al. 2000; Leona Plum 2006), body weight and adiposity in $I^{2.1}$ KO males were not different from controls (Figure 2.1A, B). Body weight and adiposity were significantly higher in $L^{2.1}$ KO and $D^{2.1}$ KO males as compared to $I^{2.1}$ KO and controls starting from 6 weeks (Figure 2.1A, B). As we previously reported, adiposity in $L^{2.1}$ KO males stabilized after 8 weeks of age (Luquet et al. 2005; Ring and Zeltser 2010). In contrast, body weight and adiposity of $D^{2.1}$ KO started to diverge from $L^{2.1}$ KO at 10 weeks (2.1A, B). At week 14, the adiposity of $D^{2.1}$ KO males was significantly higher (7%) than $L^{2.1}$ KO males (Figure 2.1B).

Female $I^{2.1}$ KO mice weighed the same as the controls, but exhibited a mild increase (~2%) in adiposity starting from 10 weeks of age (Figure 2.1D, E), similar to observations in NIRKO females (Schwartz et al. 1992; Bruning et al. 2000). Both $L^{2.1}$ KO and $D^{2.1}$ KO females had significantly higher body weight and adiposity than $I^{2.1}$ KO and controls from 6 weeks of age (Figure 2.1D, E). Although $L^{2.1}$ KO and $D^{2.1}$ KO females had similar body weights throughout the study (Figure 2.1D), adiposity of the $D^{2.1}$ KO females started to diverge from $L^{2.1}$ KO from 9 weeks (Figure 2.1E); however, increased adiposity in $D^{2.1}$ KO females was not reflected in higher leptin levels at 12 weeks (Figure 2.1F).

As gonadal fat depots were reported to be preferentially affected in NIRKO females (Coyle et al. 2002), we performed histological analyses of inguinal and gonadal fat depots in $L^{2.1}$ KO and $D^{2.1}$ KO females. Gonadal adiposity in $D^{2.1}$ KO females was significantly increased as compared to $L^{2.1}$ KO females at 9 and 19 weeks (increased by 40% and 34%, respectively). This difference was associated with an increase in adipocyte size in gonadal, but not inguinal, fat pads (Figure 2.2A-D). This result supported the idea that the increase in gonadal fat pad weights in $D^{2.1}$ KO was at least partially due to increased adipocyte cell size (increased by 14% at 9 weeks and 24% at 19 weeks as compared with $L^{2.1}$ KO).

Nkx2.1-Cre-mediated loss of InsR signaling does not exacerbates impaired glucose homeostasis of $L^{2.1}$ KOs

Whereas $I^{2.1}$ KO males and females had the same blood glucose and serum insulin levels as controls, $L^{2.1}$ KO and $D^{2.1}$ KO males and females were hyperglycemic and hyperinsulinemic (Figure 2.3A-D). Despite the elevated adiposity in $D^{2.1}$ KO vs. $L^{2.1}$ KO males, fasting or fed blood glucose (Figure 2.3A), and serum insulin (Figure 2.3B) were similar in both groups. On the other hand, fed blood glucose was significantly higher (Figure 2.3C) and serum insulin trended higher (Figure 2.3D) in $D^{2.1}$ KO females as compared to $L^{2.1}$ KO at 12 weeks.

Energy expenditure is decreased in $D^{2.1}$ KO vs. $L^{2.1}$ KO, while food intake is similar

Next, we sought to ascertain whether increased energy intake and/or decreased energy expenditure contributes to the increased adiposity in $D^{2.1}$ KO vs. $L^{2.1}$ KO mice. To this end, males in both groups were singly-housed in their home cages with food hoppers or in metabolic cages at 8 weeks of age. In either type of cage environment, approximately 30% of singly-housed $D^{2.1}$ KO males died within 3-5 days, likely due to a dramatic suppression of food intake (light purple curve, Figure 2.4A). To overcome the high mortality rate of single-housing in $D^{2.1}$ KO males, intake from food hoppers was assessed under group-housing conditions, with two to three mice of the same genotype in a cage. $I^{2.1}$ KO males and females consumed the same amount of food as controls (Figure 2.4C). $L^{2.1}$ KO and $D^{2.1}$ KO males and females were hyperphagic at both 5 and 10 weeks of age as compared to controls, but did not differ from each other (Figure 2.4C). Oxygen consumption during the light and the dark cycles was decreased by approximately 16% in $D^{2.1}$ KO compared to $L^{2.1}$ KO males, although it did not reach significance (Figure 2.4B). Together, these observations are consistent with the idea that changes in energy expenditure, but not food intake, contribute to increased body weights and adiposity in $D^{2.1}$ KO mice.

***Nkx2.1-Cre*-mediated loss of InsR signals from $L^{2.1}$ KO increases fasting-induced loss of core body temperature**

As central leptin and insulin signaling have been implicated in regulating body temperature (Hill et al. 2010; Zhang et al. 2011; Bartfai and Conti 2012) and

we observed a trend toward decreased energy expenditure in $D^{2.1}$ KOs, we explored whether the deficits in thermoregulation might contribute to the high mortality rate of singly-housed $D^{2.1}$ KOs. To this end, we assessed the ability of $L^{2.1}$ KO and $D^{2.1}$ KO males to maintain body temperature in response to an overnight fast under group- and single-housing conditions. As we previously determined that $L^{2.1}$ KO mice acquire the capability to thermoregulate in response to short-term cold challenge by 6 weeks (Cuendet et al. 1975; Ring and Zeltser 2010), these studies were performed in males older than 8 weeks of age. When the mice were group-housed, fasting induced a larger decrease in core body temperature in $D^{2.1}$ KO than all other groups (Figure 2.5A). Furthermore, fasting-induced loss in core body temperature was significantly increased in $D^{2.1}$ KO by single-housing condition (Figure 2.5B: $D^{2.1}$ KO group -2.9±0.2°C vs. single -4.9±0.6°C). Some singly-housed $D^{2.1}$ KOs lost as much as 8-12°C, close to the approximately 7-10°C threshold associated with death (Saito et al. 2003; Streijger et al. 2009). Consistent with the increased loss of body temperature phenotype under fasted conditions, BAT *Ucp1* expression in fasted singly-housed $D^{2.1}$ KOs was 18% lower than $L^{2.1}$ KOs (Figure 2.5C). Single-housing was associated with increased loss in body temperature in response to fasting in $I^{2.1}$ KO and $L^{2.1}$ KOs, but it did not reach significance (Figure 2.5B: $I^{2.1}$ KO group -0.8±0.7°C vs. single -1.6±0.4°C; $L^{2.1}$ KO group -1.7±0.3°C vs. single -2.6±0.6°C).

Since body temperature has to be regulated in response to different types of stressors, we also looked at the ability of $L^{2.1}$ KO and $D^{2.1}$ KO to thermoregulate during a short-term acute cold challenge. Although body

temperature was dramatically decreased in singly-housed $D^{2.1}$ KOs in response to fasting at room temperature (Figures 2.5A, B), $D^{2.1}$ KOs (fasted overnight before subjected to the cold challenge) were able to maintain stable body temperature during the cold challenge (Figure 2.6). All groups (including the $D^{2.1}$ KOs, which had significantly lower body temperature at the beginning of the test) showed an increase in body temperature initially when subjected to the cold. The elevated body temperature of $D^{2.1}$ KO and $L^{2.1}$ KO were similar, but both groups had significantly lower temperature when compared with controls throughout the experiment.

Discussion

Increased adiposity in $D^{2.1}$ KO is due to decreased energy expenditure

As central injections of insulin can suppress food intake and increase energy expenditure (Antoni 1993; Wotjak et al. 1996), the initial goal of our study was to explore whether hypothalamic insulin signaling contributes to the stabilization of adiposity levels seen in $L^{2.1}$ KOs after 8 weeks of age (Redemann 2010). To this end, we used *Nkx2.1-Cre*-mediated recombination to disrupt signaling via InsR and/or LepRb. Consistent with observations in mice lacking InsR signaling throughout the CNS (Duan et al. 2007), all measured metabolic parameters in $I^{2.1}$ KO males were similar to controls, while older females exhibited mild increases in adiposity (Figure 2.1E). This failure to observe consequences of diminished InsR signals on parameters that are reportedly altered by central insulin injections (i.e. food intake, energy expenditure and lipogenesis), likely reflects the robust capacity for developmental compensation in circuits regulating energy balance (Kruse et al. 2003; Lin et al. 2008; Zelena 2012).

Despite the fact that we could not detect changes in any metabolic parameters in $I^{2.1}$ KO males, disruption of InsR signals in the $L^{2.1}$ KO genetic background resulted in further increases in body weight and adiposity (Figure 2.1B). Although central insulin can produce anorectic effects (Appenrodt et al. 1998), increased obesity of $D^{2.1}$ KO was not due to hyperphagia (Figure 2.4C), but decreased energy expenditure (Figure 2.4B). Preceding the changes in body weight and adiposity, oxygen consumption in $D^{2.1}$ KO males was 16% less than

in $L^{2.1}$ KO. This decreased in energy expenditure likely contributes to the increased weights and adiposity in older $D^{2.1}$ KO animals (Bielsky et al. 2004). However, due to the high mortality rate of singly-housed $D^{2.1}$ KO males in metabolic cages and the poor odds of generating a $D^{2.1}$ KO male (one in two litters), we did not generate sufficient numbers for the difference to reach significance. It is interesting to note that insulin and leptin have opposite effects on POMC neuronal activity and removal of InsR signals on POMC neurons in *Pomc-Cre Lepr* knockout mice resulted in a significant reduction, rather than an increase, in fat mass and body weight (Liebsch et al. 1996; Wigger et al. 2004). Together with our data, these observations support the idea that InsR signals via non-POMC hypothalamic neurons contribute to the stabilization of body weight and adiposity in adult $L^{2.1}$ KO mice.

High mortality rate of $D^{2.1}$ KOs under single housing conditions

The most striking observation in our study was that ~30% of $D^{2.1}$ KOs refused to consume food when singly-housed either in metabolic cages or in home cages with a food hopper (Figure 2.4A). It is unlikely that this aphagic response results from a failure to learn to eat from the food hopper, as all group-housed $D^{2.1}$ KOs consumed the same amount of food as $L^{2.1}$ KOs (Figure 2.4C). As severe stress can elicit anhedonia (Engelmann et al. 2004; Zhang et al. 2012; reviewed in Schwartz and Zeltser), it is possible that increased stress axis reactivity in $D^{2.1}$ KOs when confronted with a novel object (food hopper) in a novel environment (metabolic cage) contributes to the aphagic response. Consistent with this idea, we find that the response to fasting stress is elevated in

$D^{2.1}$ KOs as compared to $L^{2.1}$ KOs (Data are presented in Chapter 3). Aphagic $D^{2.1}$ KOs typically died within 3 days, sooner than has been reported for obese *Lepr*$^{ob/ob}$ solely due to starvation (Luppino et al. 2011). Death was likely hastened by the inability of singly-housed $D^{2.1}$ KOs to thermoregulate when fasted (Figures 2.5A-B).

Hypothalamic leptin and insulin signals interact to regulate body temperature in response to nutritional stress

Conserving energy by reducing body temperature promotes survival when food sources are limited. While leptin- and insulin-sensing neurons have been implicated in body temperature regulation (Sanchez-Alavez et al. 2010; Enriori et al. 2011), the degree to which these circuits interact is not known. Similar to *Lepr*$^{db/db}$ animals (McMinn et al. 2004), $L^{2.1}$ KOs exhibit a 1-2^{o}C decrease in baseline (fed) body temperature (Ring and Zeltser 2010) (Figure 2.5A). Disruption of LepRb signaling in AgRP and POMC neurons also leads to a similar decrease in baseline body temperature as *Lepr*$^{db/db}$ mice (van de Wall et al. 2008). Conversely, NPY deficiency (Erickson et al. 1996) and *Pomc* overexpression (Mizuno et al. 2003) in *Lep*$^{ob/ob}$ mice normalize the reduced baseline body temperature. Together, these data are consistent with the idea that loss of leptin signals in the hypothalamus, likely involving some neuronal populations in the arcuate nucleus of the hypothalamus (ARH), is sufficient to direct thermoregulatory circuits to conserve energy. However, lower body temperatures of $D^{2.1}$ KOs as compared to $L^{2.1}$ KOs supports the hypothesis that leptin- and insulin-sensing circuits interact to maintain body temperature. In

theory, increased circulating insulin levels in $L^{2.1}$ KOs could act to promote thermogenesis via actions on InsR in the hypothalamus.

In response to an overnight fast, group-housed $D^{2.1}$ KOs exhibited significantly decreased core body temperature as compared to all other experimental groups (Figure 2.5A). Nevertheless, there was a further decrease in body temperature when $D^{2.1}$ KOs were singly-housed (Figure 2.5B), suggesting that huddling might be masking the fasting-induced decreased thermogenesis in group-housed $D^{2.1}$ KO. As $L^{2.1}$ KOs are hyperinsulinemic even after an overnight fast (Figures 2.3B, D), it raises the possibility that hypothalamic InsR signals serve as important compensatory signals to maintain body temperature. The observation that core body temperature in fasted $I^{2.1}$ KOs did not differ from controls is consistent with the idea that hypothalamic leptin signals, which are not decreased after an overnight fast, can compensate for the loss of InsR signals under these conditions. The dramatic loss of core body temperature in $D^{2.1}$ KOs following an overnight fast supports the hypothesis that interactions between hypothalamic InsR and LepRb signals are critical to maintain body temperature under conditions of nutritional stress. Consistent with this idea, STZ-treated rats, which have dramatically reduced circulating leptin and insulin levels, also exhibit very low fasting body temperatures (Kinoshita et al. 2000).

While most analyses focus on the magnitude of fasting-induces decreases in body temperature, it should be noted that the capability to conserve energy in response to negative energy balance is maintained. The fact that fasted $I^{2.1}$ KOs, $L^{2.1}$ KOs and $D^{2.1}$ KOs all decrease their body temperature in response to an

overnight fast, strongly supports the idea that other signals are sufficient to provide this input. For example, ghrelin is induced by fasting (Toshinai et al. 2001) and is reported to suppress body temperature (Inoue et al. 2013). Redundancy in the signals that can direct thermoregulatory circuits to conserve energy likely reflects the importance of this adaptive strategy for survival.

Dramatic reduction in core body temperature in $D^{2.1}$ KO in response to fasting can be rescued by an acute cold challenge

Thermogenic adaption in response to environmental stressors is an important survival strategy for warm-blooded animals. Although $L^{2.1}$ KOs and $D^{2.1}$ KOs have significantly lower core body temperatures when fasted at room temperature, they dramatically increased body temperature to baseline (fed) levels within 15 minutes of an acute cold challenge (Figure 2.6). These observations are consistent with the idea that circuits engaged by the cold exposure are intact in $L^{2.1}$ KOs and $D^{2.1}$ KOs. Shivering-thermogenesis mediated through the skeletal muscles (Cannon and Nedergaard 2011) could promote thermogenesis. In addition, LepRb and/or InsR signals outside the domain affected by *Nkx2.1-Cre*-mediated recombination could act to promote cold-induced thermogenesis.

Phenotypes of mouse models with loss or gain of LepRb signaling in different brain regions can provide some insights into central circuits regulating the response to a cold challenge. The inability of $Lep^{ob/ob}$ and $Lepr^{db/db}$ to maintain body temperature in response to an acute challenge (Trayhurn and James 1978; McMinn et al. 2004) strongly implicates leptin signaling in this process.

Expressing the LepRb transgene in the CNS using rat synapsin I and/or neuron-specific enolase enhancer/promoter completely normalized the cold tolerance in *Lepr*$^{db/db}$ animals (Kowalski et al. 2001; de Luca et al. 2005), consistent with the idea that the critical site(s) of leptin action are in the brain. Importantly, *Nkx2.1-Cre*- ($L^{2.1}$ KO) and *Camk2a-Cre*- mediated removal of LepRb do not recapitulate the cold intolerance observed in *Lepr*$^{db/db}$ (McMinn et al. 2005; Ring and Zeltser 2010).

These results are consistent with the idea that leptin-sensing neurons mediating cold-induced thermogenesis likely reside outside of the domains affected by the *Nkx2.1-Cre* (active in the ARH, ventromedial hypothalamus, dorsomedial nucleus of the hypothalamus (DMH), paraventricular nucleus of the hypothalamus (PVH), preoptic area (POA), but not in the parabrachial nucleus (PB), nucleus of the solitary tract (NTS) and periaqeductal grey (PAG)) and *Camk2a-Cre* (active in the ARH, VMH, DMH, PVH, POA, NTS and PAG, but not in the PB) transgenes. LepRb-sensing neurons in the DMH and median preoptic area (MnPOA) have been implicated in regulating sympathetic outflow to promote BAT thermogenesis (Zhang et al. 2011). Since leptin signaling in both of these is disrupted in *Camk2a-Cre;Lepr*$^{flox/flox}$ and $L^{2.1}$ KO mice, they are probably not critical to maintain body temperature in response to an acute cold challenge.

Identification of leptin-sensing neurons that promote the response to cold stressors is an important area for future research. Retrochiasmatic area and NTS are two regions that contain LepRb neurons retrogradely labeled by pseudorabies virus injected into BAT (Zhang et al. 2011) or skeletal muscle (Babic et al. 2010). Since neither *Camk2a*-Cre (McMinn et al. 2005) nor *Nkx2.1-Cre* (Ring and Zeltser 2010) achieves recombination in these brain regions, these regions may contain the thermogenic leptin-sensing neurons that are responsible for the maintenance of body temperature during acute-cold challenge in $L^{2.1}$ KO and *Camk2a-Cre Lepr* KO animals. *Lepr* is also expressed in several other regions implicated in body temperature regulation (i.e. PB and PAG) (Scott et al. 2009; Shaun F Morrison 2012) that do not express *Camk2a*-Cre or *Nkx2.1-Cre* transgenes.

Conclusions

Our results raise the possibility that the hyperinsulinemia may be a compensatory response to the development of obesity. While neonatal loss of AgRP neurons in mice leads to developmental compensatory to maintain normal feeding (Luquet et al. 2005), dysfunctions developed during adulthood such as obesity may trigger different types of compensatory consequences to maintain energy balance. Although initially important for temporary alleviation of the metabolic dsyregulation, chronic activation of the compensatory system, however, may eventually lead to adverse outcomes.

The observation that $D^{2.1}$ KO mice exhibited significantly greater fasting-induced temperature loss than $L^{2.1}$ KO highlights the importance of the integration of nutrient-related signals (e.g. leptin and insulin) to the central thermoregulatory circuits in regulating body temperature in response to nutrient stress. The fact that $D^{2.1}$ KO can maintain their body temperature during acute cold challenge (when fasted) suggested that these mice can thermoregulate, but an appropriate thermoregulatory response were not triggered during fasting. And this phenotype was not secondary to obesity since $D^{2.1}$ KO and $L^{2.1}$ KO had similar adiposity when the study was conducted. These observations therefore raise the possibility that some comorbidities observed in obesity maybe independent of the increased fat mass and could be treated separately by targeting the specific pathway involved.

Figure 2.1

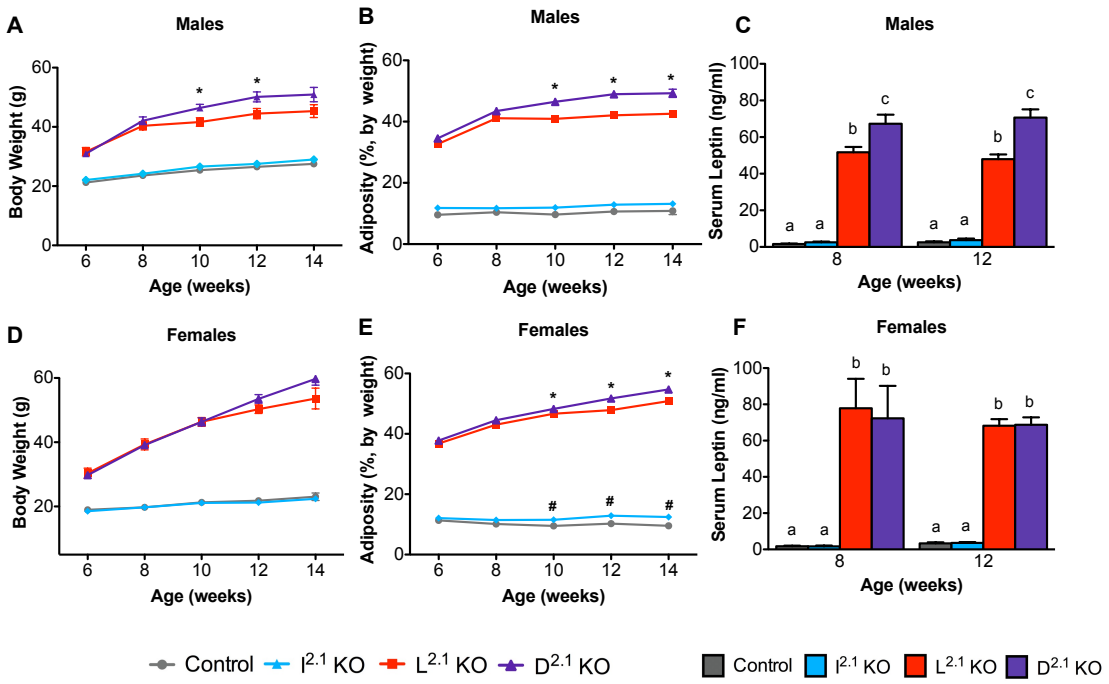

Figure 2.1: Nkx2.1-Cre-mediated disruption of InsR signals exacerbates obesity of $L^{2.1}$ KOs. (**A**) Body weight and (**B**) adiposity of male control, $I^{2.1}$ KO, $L^{2.1}$ KO, and $D^{2.1}$ KO mice; n ≥ 10 for all groups at all time points. (**C**) Serum leptin, as measured by ELISA, at 8 and 12 weeks of male control, $I^{2.1}$ KO, $L^{2.1}$ KO, and $D^{2.1}$ KO mice; n ≥ 4 for all groups at all time points. (**D**) Body weight and (**E**) adiposity of female control, $I^{2.1}$ KO, $L^{2.1}$ KO, and $D^{2.1}$ KO mice; n ≥ 9 for all groups at all time points for body weight. n ≥ 4 for all groups at all time points for adiposity. (**F**) Serum leptin at 8 and 12 weeks of female control, $I^{2.1}$ KO, $L^{2.1}$ KO, and $D^{2.1}$ KO mice; n ≥ 4 for all groups at all time points. All data are mean ± SEM. *P* values were calculated between age- and sex-matched groups. (**A-D**) $^{*}P < 0.05$ $L^{2.1}$ KO versus $D^{2.1}$ KO; $^{\#}P < 0.05$ $I^{2.1}$ KO versus controls. (**C, F**) Lowercase letters above bars denote statistically similar ($P > 0.05$) groups.

Figure 2.2

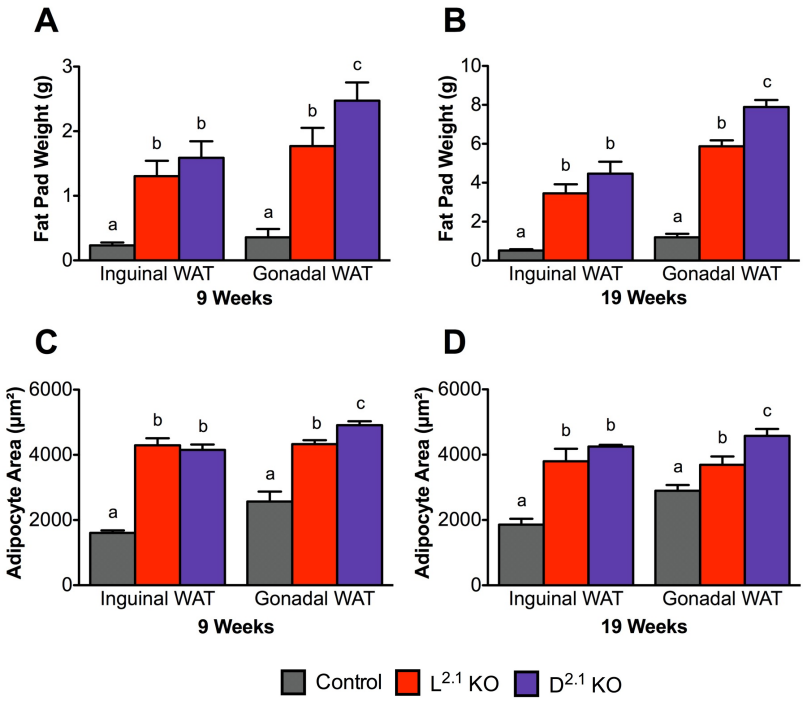

Figure 2.2: Preferential increase in gonadal adiposity and adipocyte size in $D^{2.1}$ KO females. (**A, B**) Gonadal and inguinal fat pad weights of control, $L^{2.1}$ KO, and $D^{2.1}$ KO females; (**A**) n ≥ 3 for all groups at 9 weeks of age and (**B**) n ≥ 7 for all groups at 19 weeks of age. (**C, D**) Average cross-sectional areas of gonadal and inguinal fat pad adipocytes, from histological sections, of control, $L^{2.1}$ KO, and $D^{2.1}$ KO females; (**C**) 9 weeks of age, n ≥ 3 for all groups; (**D**) 19 weeks of age, n ≥ 4 for all groups. All data are mean ± SEM. *P* values were calculated between age-matched groups. Lowercase letters above bars denote statistically similar ($P > 0.05$) groups.

Figure 2.3

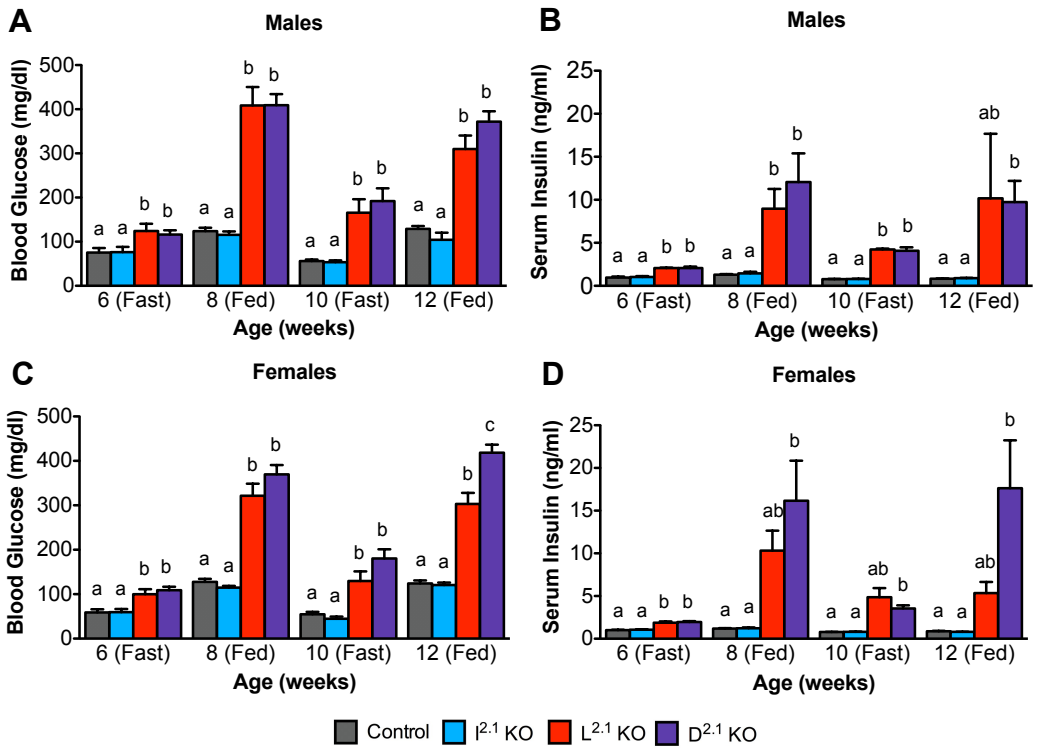

Figure 2.3: Disruption of hypothalamic InsR signals does not exacerbate glucose impairment in male $L^{2.1}$ KOs. (**A, C**) Whole-blood glucose at 6 (fasted), 8 (random-fed), 10 (fasted) and 12 (random-fed) weeks of age in control, $I^{2.1}$ KO, $L^{2.1}$ KO, and $D^{2.1}$ KO mice; (**A**) males, n ≥ 4 for all groups at all time points; (**B**) females, n ≥ 7 for all groups at all time points. (**B, D**) Serum insulin, as measured by ELISA, at 6 (fasted), 8 (random-fed), 10 (fasted) and 12 (random-fed) weeks of age in control, $I^{2.1}$ KO, $L^{2.1}$ KO, and $D^{2.1}$ KO males (**B**) and females (**D**); n ≥ 3 for all groups at all time points. All data are mean ± SEM. *P* values were calculated between age- and sex-matched groups. Lowercase letters above bars denote statistically similar ($P > 0.05$) groups.

Figure 2.4

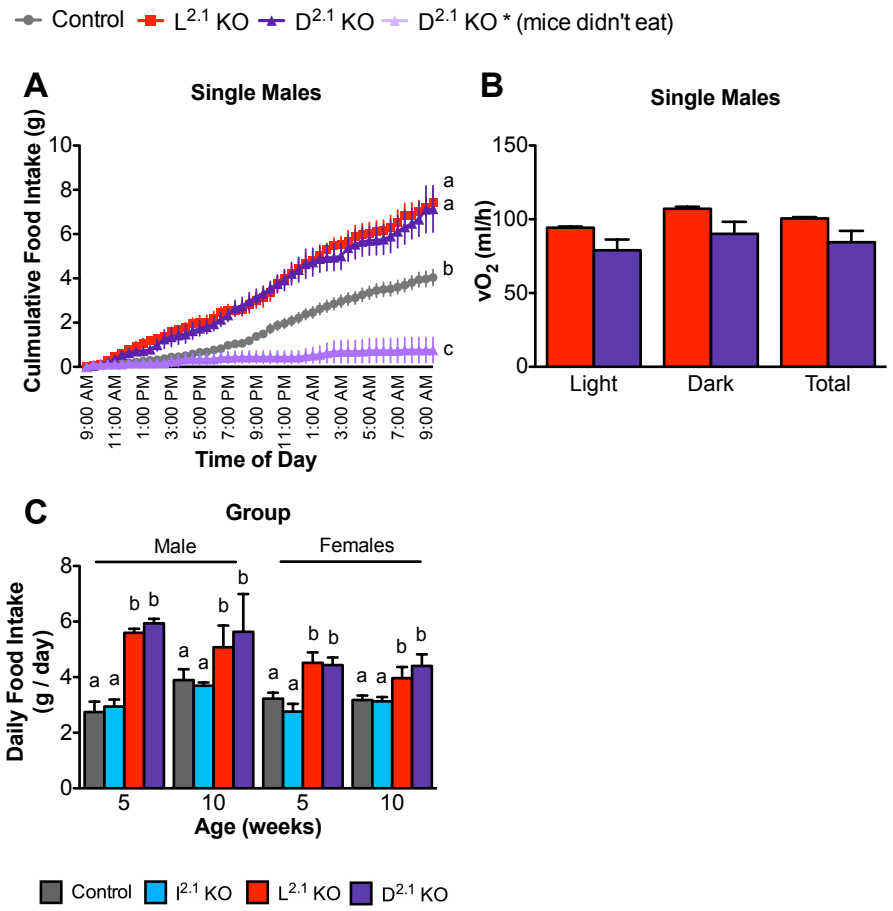

Figure 2.4: Increased adiposity in $D^{2.1}$ KOs is not due to effects on food intake. (**A**) Cumulative food intake, as measured in the metabolic cages of adult singly-housed male control, $L^{2.1}$ KO, and $D^{2.1}$ KO and $D^{2.1}$ KO* (mice refused to eat). (**B**) Daily oxygen consumption (vO_2), as measured in metabolic cages, of adult singly-housed male control, $L^{2.1}$ KO, $D^{2.1}$ KO and $D^{2.1}$ KO; n ≥ 5 for all groups. Data presented for light cycle, dark cycle or 24 hour total. (**C**) Averaged daily food intake, at 5 and 10 weeks of age, of group-housed male and female controls, $I^{2.1}$ KO, $L^{2.1}$ KO, and $D^{2.1}$ KO; n ≥ 3-4 for all groups at all time points**.** All data are mean ± SEM. *P* values were calculated between age- and sex-matched groups. Lowercase letters above bars denote statistically similar ($P > 0.05$) groups.

Figure 2.5

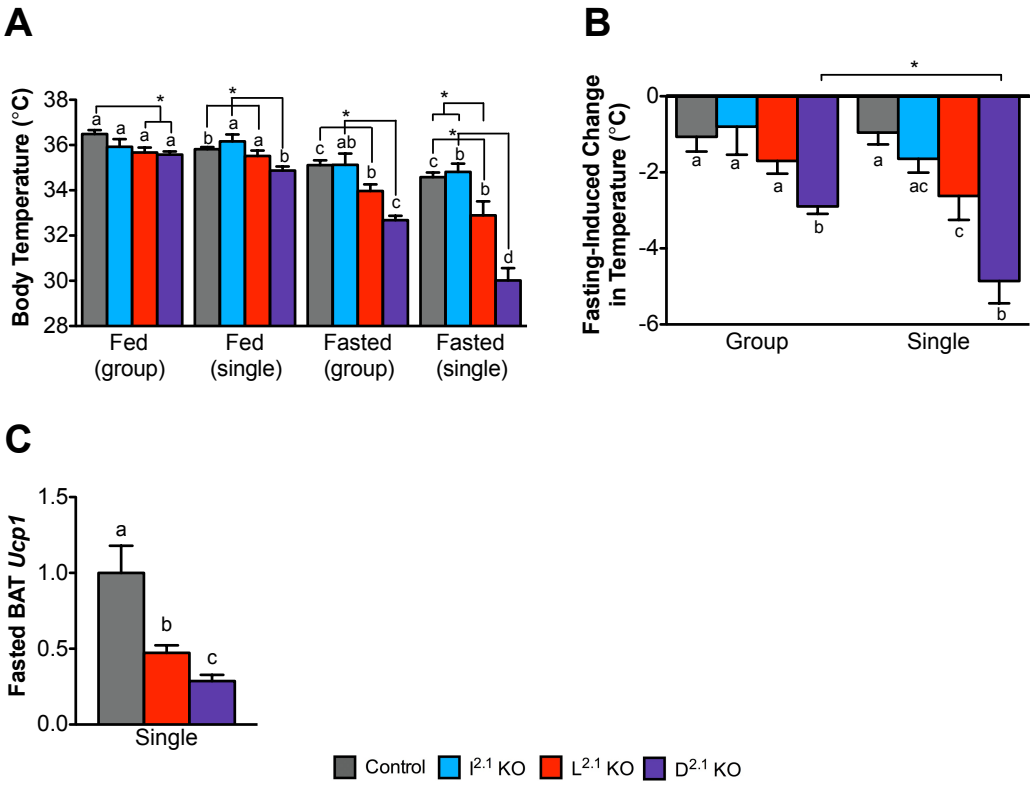

Figure 2.5: Disruption of hypothalamic InsR signals exacerbates fasting-induced loss of body temperature in $L^{2.1}$ KOs. (**A**) Core body temperature of group- and singly-housed, fed and fasted, control, $I^{2.1}$ KO, $L^{2.1}$ KO, and $D^{2.1}$ KO adult males; n ≥ 4 for all groups. (**B**) Change in core body temperature of group- and singly-housed control, $I^{2.1}$ KO, $L^{2.1}$ KO, and $D^{2.1}$ KO adult males in response to an overnight fast; n ≥ 4 for all groups. (**C**) BAT *Ucp1* mRNA expression, as measured by quantitative PCR, of fasted singly-housed control, $L^{2.1}$ KO, and $D^{2.1}$ KO adult males; n ≥ 4 for all groups. All data are mean ± SEM. (**A**) Lowercase letters above bars denote statistically similar ($P > 0.05$) body temperature of animals with the same genotype under different conditions (Fed-group, Fed-single, Fasted-group and Fasted-single). *P < 0.05 for statistical difference between different groups (control, $I^{2.1}$ KO, $L^{2.1}$ KO, and $D^{2.1}$ KO) under the same condition. (**B-C**) Lowercase letters above bars denote statistically similar ($P > 0.05$) groups under the same condition. *P < 0.05 for statistical difference of animals with the same genotype under fed versus fasted condition.

Figure 2.6

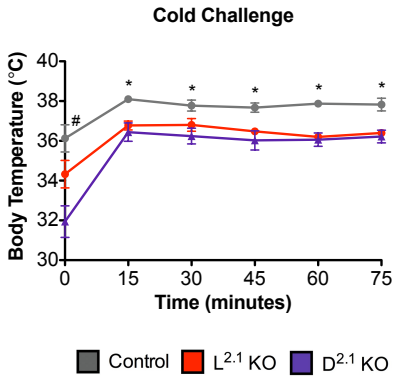

Figure 2.6: $L^{2.1}$ KO and $D^{2.1}$ KO males can mount a thermogenic response to an acute cold challenge. (**A**) Body temperature during short-term cold challenge of control, $L^{2.1}$ KO, and $D^{2.1}$ KO adult males; n ≥ 4 for all groups. All data are mean ± SEM. **P* < 0.05 $L^{2.1}$ KO and $D^{2.1}$ KO versus controls; $^{\#}P$ < 0.05 $D^{2.1}$ KO versus control.

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Author Contributions

Designed the experiments: CN Angie Chong, LM Zeltser. Analyzed data: CN Angie Chong. Performed experiments: CN Angie Chong, RA Greendyk (Figure 2.2C-D). Wrote the paper: CN Angie Chong, LM Zeltser.

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CHAPTER 3: "Hypothalamic Insulin-sensing Circuit Regulates Activity of the HPA Axis in Response to Stress."

Introduction

The higher prevalence of (hypothalamic-pituitary-adrenal) HPA axis dysregulation and mood disorders such as depression and anxiety in both type 1- and type 2-diabetes raise the possibility that decreased insulin signaling may contribute to the development of these disorders (Shaban et al. 2006; Bruehl et al. 2007; Li et al. 2008; Khuwaja et al. 2010; Torres et al. 2013). Intranasal insulin attenuated HPA axis activation (diminished saliva and plasma cortisol) in humans subjected to a psychological stressor (Bohringer et al. 2008; Zarkovic et al. 2008). In rodents, streptozotocin (STZ)-induced diabetes leads to increased circulating corticosterone (CORT) levels and *corticotropin-releasing hormone* (*Crh*) expression in the paraventricular nucleus of the hypothalamus (Tronche et al. 1999; Chan et al. 2001), suggesting hyperactivation of the HPA axis. These studies thus raised the possibility that central insulin signals may be involved in suppressing HPA axis activity. During the metabolic characterization of hypothalamic leptin receptor and insulin receptor double knockout ($D^{2.1}$ KO) mice in Chapter 2, we noted a high mortality rate when $D^{2.1}$ KO were single-housed. The search for the causes of death in $D^{2.1}$ KO led us to uncover an increase in activity of the HPA axis, suggesting that hypothalamic insulin-sensing circuit could provide negative regulation to the HPA axis. The following manuscript in preparation describes our effort to study the HPA axis function in the various

Nkx2.1-Cre mediated hypothalamic knockout animals ($I^{2.1}$ KO, $L^{2.1}$ KO, $D^{2.1}$ KO), as well as the neurobehavioral functions in $I^{2.1}$ KO mice.

Manuscript

(Author Contributions: Designed the experiments: CN Angie Chong, LM Zeltser, AS Hill (Figure 3.4). Analyzed data: CN Angie Chong, AS Hill (Figure 3.4). Performed experiments: CN Angie Chong, AS Hill (Figure 3.4). Contributed materials/analysis tools: LM Zeltser, AS Hill (Figure 3.4), R Hen (Figure 3.4). Wrote the paper: CN Angie Chong, LM Zeltser.)

Hypothalamic Insulin-sensing Circuit Regulates Activity of the HPA Axis in Response to Stress.

Manuscript in preparation

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Abstract

In our previous study using *Nkx2.1-Cre* mediated condition knockouts of leptin receptor ($L^{2.1}$ KO), insulin receptor ($I^{2.1}$ KO) and both receptors (double KO, $D^{2.1}$ KO), we noticed a high mortality rate in $D^{2.1}$ KOs, in part due to a refusal to eat, within a few days during single-housing studies in the metabolic cages, an environment with both social isolation and novelty stress. These types of stress have been shown to increase hypothalamic-pituitary-adrenal (HPA) axis sensitivity and induce anorexia in mice respectively. We examined the fasting-induced response in singly-housed $D^{2.1}$ KO mice and found that their corticosterone (CORT) levels were significantly elevated as compared to $L^{2.1}$ KO mice. Evaluation of $I^{2.1}$ KO mice, which had normal body weight and adiposity, also revealed an increase in stress-induced HPA axis activity. These observations in InsR-deficient $I^{2.1}$ KO and $D^{2.1}$ KO mice suggested that

hypothalamic InsR signals are involved in regulating HPA axis activity. In humans, diabetes and HPA axis dysregulation are often associated with psychiatric disorders such as depression and anxiety disorders, we therefore decided to further investigate the neuro-behavioral functions and found that $I^{2.1}$ KOs exhibited increased anxiety-like behaviors in open field, marble burying, and stress-induced hyperthermia tests. Our results provide evidence to support a role of hypothalamic insulin signals in suppressing HPA axis activity and some anxiety-like behaviors in mice.

Introduction

Insulin levels decrease during fasting, and upon feeding, its levels increase. This fluctuation in circulating insulin in relations to feeding status is opposite to that of corticosterone (CORT) levels, which is dramatically increased by fasting and reduced by refeeding (Poplawski et al. 2010). During fasting (a type of metabolic stress), the increased CORT and decreased insulin levels allow activation of metabolic pathways (e.g. hepatic gluconeogenesis) (Renga et al. 2012) that provide the substrates to meet the specific energetic demands of the body. Similarly, CORT released during psychological stress (e.g. restraint in rodents) increases glucose availability in preparation for a possible flight and fight response (Munck et al. 1984). Interestingly, insulin levels are also upregulated during restraint stress (Warne 2009), but the function of this increase is not known.

CORT release is mainly regulated by the hypothalamic-pituitary-adrenal (HPA) axis. Upon activation of the HPA axis by stress, corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are released from hypothalamic neurons into the anterior pituitary, where these hormones increase adrenocorticotropic hormone (ACTH) release. ACTH stimulates the adrenal cortex to secret CORT. Several studies suggested that insulin may play a role in suppressing HPA axis activity. Diabetic animal models (e.g. streptozotocin (STZ)-induced diabetic mice) exhibit hyper-activation of HPA axis with elevated ACTH and CORT levels; while insulin replacement restored the levels of these hormonal to normal (Chan et al. 2003; Engeland and Arnhold 2005). Similarly in

humans, patients suffering from diabetes mellitus often exhibit hypercortisolemia (Ferrini et al. 1999; Bruehl et al. 2007). Observations from both rodents and human studies are consistent with the idea that sufficient insulin signals are required for reducing HPA axis activity. However, as chronic stress and elevated circulating glucocorticoid levels can induce systemic insulin resistance and increase insulin levels (Zarkovic et al. 2008), it is still not clear whether elevated glucocorticoid levels promote the development of insulin resistance and diabetes or vice versa. In addition, both diabetes and HPA dysregulation are often associated with affective disorders including anxiety and depression (Bruehl et al. 2007; Kallen et al. 2008), suggesting that a common underlying pathway may contribute to the development of these disorders.

Preliminary studies on animals that lack hypothalamic leptin receptor (LepRb) and/or Insulin receptor (InsR) signals suggested that the interactions between these hypothalamic signals provide negative regulation of HPA axis activity. When comparing to mice that lack only hypothalamic LepRb signaling ($L^{2.1}$ KO), mice that lack both hypothalamic LepRb and InsR signals (Double KO, $D^{2.1}$ KO) exhibit heightened HPA axis response to fasting. This observation raises the possibility that hypothalamic insulin signals suppress HPA axis activation under some circumstances. Since Both $L^{2.1}$ KO and $D^{2.1}$ KO mice exhibited severe obesity, hyperphagia, hyperinsulinemia, hyperglycemia, and hypercorticosteronemia, the analysis of the HPA axis functions on top of the metabolic dysregulation was very challenge (for metabolic characterization of the different mutants used in this study, please refer to Chapter 2). Therefore, to

understand the role of hypothalamic insulin signals in modulating HPA axis function and neuro-behaviors, we generated $I^{2.1}$ KO mice that lack InsR signaling in the majority of the hypothalamus mice by crossing mice with floxed alleles of *Insr* to *Nkx2.1-Cre* driver animals. Since $I^{2.1}$ KO did not exhibit any major changes in body weight, adiposity and glucose metabolism, they present a better model for understand the role of hypothalamic InsR signals in regulating stress axis.

Methods

Generation of $I^{2.1}$, $L^{2.1}$ and $D^{2.1}$ KO mice

To generate conditional knockouts of *Insr* and/or *Lepr*, the *Nkx2.1-Cre* driver line (C57BL/6J-Tg(Nkx2-1-cre)2Sand/J, provided by S. Anderson, Weill Cornell Medical College) (Xu et al. 2008) was crossed to mice homozygous for a floxed allele of *Lepr* (FVB.BKS(D)-*Lepr^{db}*/ChuaJ, provided by S. Chua, Albert Einstein College of Medicine) (McMinn et al. 2004). The resulting F1 heterozygotes (*Nkx2.1*-Cre; *Lepr$^{fl/+}$*) were crossed to mice homozygous for a floxed allele of *Insr* (B6.129S4(FVB)-*Insr^{tm1Khn}*/J, provided by D. Acilli, Columbia University Medical Center) (Kulkarni et al. 1999) to generate F2 *Nkx2.1-Cre*;*Lepr $^{fl/+}$;Insr $^{fl/+}$* and *Lepr $^{fl/+}$;Insr $^{fl/+}$* mice. F2 *Lepr $^{fl/+}$;Insr $^{fl/+}$* mice were intercrossed to generate F3 *Lepr$^{fl/fl}$;Insr$^{fl/fl}$* females, which were then crossed to F2 *Nkx2.1-Cre*; *Lepr $^{fl/+}$;Insr $^{fl/+}$* males to generate our experimental animals: *Nkx2.1-Cre;Lepr$^{fl/fl}$* ($L^{2.1}$ KO), *Nkx2.1-Cre;Insr$^{fl/fl}$* ($I^{2.1}$ KO), *Nkx2.1-Cre;Lepr$^{fl/fl}$;Insr$^{fl/fl}$* ($D^{2.1}$ KO), and *Lepr$^{fl/fl}$;Insr$^{fl/fl}$* (control) in a Mendelian ratio. Due to the low probability of achieving the desired genotypes in the offspring, two crosses were generally required to generate one conditional knockout male. Mouse genotypes were assessed by PCR on genomic DNA from tail tips using the following primers:

Cre
5’ GCGGTCTGGCAGTAAAAACTATC 3’ (forward)
5’ GTGAAACAGCATTGCTGTCACTT 3’ (reverse)
Lepr
5’ GTCTGATTTGATAGATGGTCTT 3’ (forward)
5’ AGAATGAAAAAGTTGTTTTGGGA 3’ (forward)
5’ GGCTTGAGAACATGAACAC 3’ (reverse)
Insr
5’ TGCACCCCATGTCTGGGACCC 3’ (forward)
5’ GCCTCCTGAATAGCTGAGACC 3’ (reverse)

Animal husbandry

Mice were maintained in a temperature- and light-controlled environment (22°C ± 1°C; 12-hour light/12-hour dark cycle). Pups were weaned on postnatal day 21. Unless otherwise noted, mice had ad libitum access to chow (9% calories from fat, 5058 Mouse diet 20; Labdiet) and water until the time of sacrifice. In the single-housing experiment, mice were single-housed 2-3 weeks before experiments were done. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at Columbia University Health Sciences Division.

Preservation of hypothalamus and pituitary

At the time of sacrifice, animals were anesthetized with 2.5% Avertin, 0.02 ml/g i.p., before cervical dislocation. Hypothalamus (whole hypothalamus; paraventricular nucleus; median eminence) and pituitary were dissected out and frozen in liquid nitrogen immediately.

Measurement of serum corticosterone levels

Serum was collected from tail bleeds on minimally-stressed animals unless otherwise stated. Baseline (fed) blood samples were collected between 10 am and noon. Fasting blood samples were taken following 14–16 hours of fasting with ad libitum access to drinking water. Blood was collected and clotted at room temperature for one hour then centrifuged. Serum was decanted and stored at –20°C until used in CORT radioimmunoassay (MP Biomedicals) in the laboratory of S. Wardlaw (Columbia University, New York, New York, USA).

Quantitative RT-PCR

Total RNA was isolated from fresh frozen hypothalamus and pituitary using the RNeasy Plus Universal kit (Qiagen). Reverse transcription was achieved using Transcriptor First Strand cDNA Synthesis kit (Roche). Quantitative PCR was done on a LightCycler 480 System (Roche) using the LightCycler 480 SYBR Green I Master. Target genes were normalized against *Beta actin*.

Primers Sequence:

Beta actin (*β−actin*)
forward, 5' CGCCACCAGTTCGCCAT 3'
reverse, 5' CTTTGCACATGCCGGAGC 3'
Arginine vasopressin (*Avp*)
forward, 5' ACTACCTGCCCTCGCCCTGC 3'
reverse, 5' GCCACGCAGCTCTCGTCGCT 3'
Corticotropin-releasing hormone (*Crh*)
forward, 5' AGGAGGCATCCTGAGAGAAGT 3'
reverse, 5' CATGTTAGGGGCGCTCTC 3'
Propiomelanocortin (*Pomc*)
forward, 5' GCAACCTGCTGGCTTGCATCCG 3'
reverse, CCGAAGCGGTCCCAGCGGAA 3'
Glucocorticoid receptor (Gr)
forward, 5' ACTTCGCAGGCCGCTCAGTGTT 3'
reverse, 5' TGGTCCCGTTGCTGTGGAGGAGC 3'

Restraint Stress Response

Mice were placed in a 50-ml falcon tubes for 30 minutes. Tail blood was sampled at 0, 30 (end of restraint), and 60 minutes (after restraint) after the beginning of a restraint. Serum was collected as previously described.

Psychiatric Evaluations

Open Field Test (OF)

After one-hour acclimation to the testing room, mice were placed in the open field arena for 30 minutes. Open field tests were videotaped for scoring later. Total distance traveled, and ratio of time spent in the periphery and center of the field were recorded and used to evaluate the anxiety levels in rodents (Walsh and Cummins 1976).

Novelty Suppressed Feeding (NSF)

The novelty suppressed feeding test is a paradigm used to assess anxiety-like behavior in novel environment (Santarelli et al. 2003). In preparation for this test, animals were fasted overnight. During the test, animals were placed in a brightly lit box (30 cm x 40 cm) with bedding on the floor and an accessible food pellet tied down to a circle of Whatman paper in the center of the box. In this test, anxiety was measured based on the time it took an animal to enter the brightly lit center of the arena and to bite the food pellet.

Marble Burying Test (MB)

The marble-burying test was used for measuring anxiety- and obsessive-compulsive-disorder-like behaviors in rodents. Mice were placed in a cage filled with 4-5cm of wood chip bedding, and 12 marbles, evenly spaced, were placed on top of the bedding. The duration of the each test was 20 minutes. The number of marble buried in the bedding was recorded. Increased in marbles buried indicates increased anxiety-like behaviors (De Boer and Koolhaas 2003).

Stress-Induced Hyperthermia (SIH)

Stress-induced hyperthermia was used to measure the physiological change of body temperature (about 0.5-2^{o}C) after encountering stress. It has been used as a model to assess non-behavioral change in rodents due to anxiety. The stressors we used in this test were the handling and used of rectal probe to measure the core body temperature of the mice at baseline. Temperature was then measured again with a rectal temperature probe thermometer (Thermoworks) after 60 minutes (Bouwknecht et al. 2007).

Forced swimming Test (FST)

The forced swimming test was conducted with mice using a modification of established procedures that are used to evaluate antidepressant drugs. Swim sessions were conducted placing mice in individual beakers (46 cm tall x 32 cm in diameter) containing 23° - 25°C water 30 cm deep. Two swim sessions were conducted, always between 12:00 and 18:00 hours: an initial 6 min pretest followed 24 hours later by a 6 min test. Mice were continuously monitored while swimming. Swim sessions were videotaped for scoring later (Castagné et al. 2011).

Statistics

Data are presented as group mean ± SEM. Statistical comparisons were performed between sex-matched groups using 2-tailed, unpaired Student's *t* test or 1-way ANOVA with Bonferroni post-hoc analysis. *P* value of 0.05 or less was considered to be statistically significant.

Results

Loss of hypothalamic InsR signals does not affect baseline CORT levels

Since female sex hormones are known to influence activity of the HPA axis (Ferrini et al. 1999), we limited our analysis in this study to male mice. The lowest (baseline) levels of CORT are observed during the morning, at around 10am (a few hours after the beginning of the light cycle) (Engeland and Arnhold 2005), in mice. There was no difference in CORT levels between $I^{2.1}$ KO and control mice. While both $L^{2.1}$ KO and $D^{2.1}$ KO had significantly elevated baseline CORT as compared to $I^{2.1}$ KO and controls, they were not different from each other (Figure 3.1A). The loss of hypothalamic InsR signals on wild type or $L^{2.1}$ KO background did not affect baseline HPA axis activity. Since single-housing (social isolation) has been shown to alter stress response (Bartolomucci et al. 2003; Williams et al. 2009; Niwa et al. 2013), a separate group of mice were subjected single-housing prior to the assessment of HPA axis activity. Consistent with what was reported by other groups, single-housing did not influence baseline HPA axis activity (Figure 3.1A).

Increased HPA axis activity after restraint in $I^{2.1}$ KO mice

Restraint (immobilization) stress is one of the commonly used tests for evaluating sensitivity of HPA axis to psychogenic (non-physical) stress in rodents. Since changes in basal HPA axis activity dramatically influence the dynamics of CORT response to stress in rodents (Windle et al. 1998; Hotchkiss et al. 2004; Pankevich et al. 2010), we mainly made comparison between $I^{2.1}$ KO

and controls (exhibited similar baseline CORT levels), and $L^{2.1}$ KO and $D^{2.1}$ KO (Figure 3.1A, $L^{2.1}$ KO and $D^{2.1}$ KO had similarly elevated baseline CORT).

Control and $I^{2.1}$ KO mice subjected to a 30-minute restraint showed normal initiation of CORT response, but a further increase in CORT levels after the restraint as compared to controls (Figure 3.1B). Since mice carrying the *Cre* transgene alone did not show an overall change in response to restraint when compared to wild-type animals (data not shown), the altered stress response in $I^{2.1}$ KO was likely due to the loss of InsR signals mediated by *Nkx2.1*-expressing neurons. The initial CORT response to restraint in $L^{2.1}$ KO and $D^{2.1}$ KO was about 2-folds higher than both $I^{2.1}$ KO and the controls, but the response was not different between $L^{2.1}$ KO and $D^{2.1}$ KO (Figure 3.1B). Similar to $I^{2.1}$ KO, loss of hypothalamic InsR signals (from the $L^{2.1}$ KO background) led to an increase, though did not reach significance, in CORT after restraint in $D^{2.1}$ KO mice.

Single-housing did not influence baseline activity of the HPA axis (Figure 1A), but similarly increased the initial CORT response to restraint stress in all groups of animals (Figure 3.1C). This is consistent with other studies which detected increased stress response, but not baseline HPA axis activity, in singly-housed mice (Bartolomucci et al. 2003). Unlike during group-housing, we did not detect higher CORT levels in $I^{2.1}$ KO after restraint (as compared to controls) (Figure 3.1B,C).

Altered gene expression of HPA axis components in $I^{2.1}$ KO

To find the neuroendocrine changes that might contribute to the elevated HPA axis activity in $I^{2.1}$ KO mice, we evaluated gene expression and peptide levels of several major HPA axis components in the hypothalamus and pituitary. Hypothalamic gene expression and median eminence (ME) peptide levels were assessed at various time points: 0 (baseline), 30 (end of restraint), and 60 (30min after restraint) minutes. Although usually considered the major driver of HPA axis activity, hypothalamic *Crh* expression (Figure 3.2E) and CRH levels in the ME (Figure 3.2A) were not changed. On the contrary, both *Avp* mRNA expression (Figure 3.2F) and AVP levels in the ME (Figure 3.2B) were significantly increased by 53% and 18% respectively at baseline in $I^{2.1}$ KO. Furthermore, while ACTH levels in the pituitary remained the same (Figure 3.2C), pituitary *Pomc* expression was increased at 60 minutes (Figure 3.2G). These findings were consistent with the elevated CORT levels observed in the $I^{2.1}$ KO after restraint (Figure 3.1B). Since GR signaling in the hypothalamus and pituitary can negatively regulate the stress axis (Tasker and Herman 2011; Jeanneteau et al. 2012), we assessed *Gr* expression in both regions. We detected a 30% decrease in hypothalamic *glucocorticoid receptor* (*Gr*) expression at 30 minutes in $I^{2.1}$ KO (Figure 3.2D). *Gr* in the pituitary, however, was increased at 30 minutes (Figure 3.2H), suggesting a possible compensatory mechanism for counteracting the hypothalamic changes.

Increased fasting-induced corticosterone release in $D^{2.1}$ KO Mice

The observed elevated HPA axis activity in $I^{2.1}$ KO mice after restraint led us to further examine the fasting-induced CORT response in the mutants. Since CORT and insulin levels are reciprocally regulated by energy status (Poplawski et al. 2010) and STZ-induced diabetes leads to increased CORT and *Crh* expression in the paraventricular nucleus of the hypothalamus (PVH) (Chan et al. 2001), we explored the idea that hypothalamic insulin signals regulate fasting-induced CORT release. In group-housed environment, we detected a trended, but non-significant, increase in CORT levels in $I^{2.1}$ KO as compared to controls. Fasting CORT levels also trended higher in $D^{2.1}$ KO as compared to $L^{2.1}$ KO mice during group-housed. Interestingly, CORT levels in $D^{2.1}$ KOs were significantly higher than $L^{2.1}$ KO in single-housing condition (Figure 3.3A). Fasting hypothalamic expression of *Avp*, but not *Crh and Gr*, in group-housed $D^{2.1}$ KO and $L^{2.1}$ KO was significantly higher than both $I^{2.1}$ KO and controls (Figure 3.3B). Similarly to what was observed during restraint stress, single-housing environment significantly increased fasting-induced CORT response in $I^{2.1}$ KO and controls (Figure 3.3A), consistent with the idea that single-housing environment increases sensitivity of HPA axis to stress.

Increased anxiety-like behaviors in $I^{2.1}$ KO

Both HPA axis dysregulation and diabetes are often associated with psychiatric diseases such as anxiety and depression in humans (Bruehl et al. 2007; Kallen et al. 2008; Zelena 2012), and HPA axis hyperactivity is also consistently found in patients with anxiety disorders (Appenrodt et al. 1998;

Flandreau et al. 2012). In light of the observed increased HPA axis in $I^{2.1}$ KO mice, we performed various neuro-behavioral tests in order to evaluate the anxiety and depression levels in these mice.

Anxiety-Like Behaviors

Open field test (OF), novelty-suppressed feeding (NSF), marble burying (MB) and stress-induced hyperthermia (SIH) tests were used to evaluate anxiety-like behaviors in our mice. In OF, $I^{2.1}$ KO mice showed a significant decrease in locomotor activities after 20 minutes in the field (Figure 3.4A). However, no change in the number of entries to the center of the field was observed (Figure 3.4D). In MB, we did not detect any baseline changes. However, after stress, $I^{2.1}$ KO showed an increase in number of marble buried as compared to controls, suggesting an increase in anxiety-like behaviors (Figure 3.4B). Since $I^{2.1}$ KO mice showed a change in locomotor activities in OF, which sometimes complicates the interpretation of an anxiety phenotype in rodents, we decided to further assess the anxiety-like behaviors with tests that are less dependent on the levels of physical activities: novelty-suppressed feeding test (NSF) and stress-induced hyperthermia (SIH) (Bielsky et al. 2004; Vinkers et al. 2008). NSF assesses the anxiety levels of a fasted animal by the latency to feed in a novel environment, and increased latency indicates an increase in anxiety-levels. $I^{2.1}$ KO showed a trended non-significant increase in latency in feeding (Figure 3.4C). During stress-induced hyperthermia (SIH), an anxiety response that is mediated by the autonomic nervous system, and a larger induction of body temperature by the stressor indicates increased anxiety levels. $I^{2.1}$ KO exhibited higher body

temperature than the controls 60 minutes after the probe insertion stress (Figure 3.4E). Together, these results suggested there was an increase in anxiety-like behaviors in $I^{2.1}$ KO mice.

Depressive Behaviors

Forced swim test (FST) is a type of learned helplessness test used to assess depression levels of rodents (Castagné et al. 2011). Experimental animals were placed in a beaker with water, from which they could not escape. The level of depression was indicated by the time of the animals spent immobile, without attempting to escape or swim during the 6-minute test. We did not detect any change in $I^{2.1}$ KO during the test session (Figure 3.4F). $I^{2.1}$ KO mice did not seem to exhibit depressive-like behaviors.

Discussion

Interactions between hypothalamic InsR and LepRb signals regulate HPA axis activity in response to metabolic and psychogenic stress

Psychogenic Stress (Restraint)

In the current study, we showed that group-housed $I^{2.1}$ KO mice had increased HPA axis activity after restraint (Figure 3.1B), but not during fasting (Figure 3.3A). Comparing restraint CORT response in $L^{2.1}$ KO and $D^{2.1}$ KO also revealed a similar (trended) increase in HPA axis activity in $D^{2.1}$ KO mice (Figure 3.1B). $I^{2.1}$ KO mice also exhibited increased baseline AVP, but not CRH levels (Figure 3.2). These results raise the possibility that elevated AVP release may underlie the alteration in HPA axis activity of $I^{2.1}$ KOs as treatment of pituitary cells by AVP have been shown to decrease sensitivity to glucocorticoid negative feedback (Aguilera 1994).

Efforts to further understand the underlying cause of the increased HPA axis activity in $I^{2.1}$ KO by looking at expression levels of hypothalamic genes (*Crhr1, Crhr2, Avpr1a, Avpr1b, Gr, Ucn1, Ucn2, Ucn3*, data not shown) implicated in regulating stress response revealed that *Gr* expression was decreased in $I^{2.1}$ KO (Figure 3.2D). Since the hypothalamus has been shown to mediate the glucocorticoid negative feedback, (Nathan K Evanson 2010; Tasker and Herman 2011; Laryea et al. 2012), the decreased *Gr* (possibly GR signal) could provide an alternative explanation for the hyperactivity in HPA axis after restraint stress. Either the increased baseline AVP or decreased hypothalamic *Gr*

expression observed in $I^{2.1}$ KO is consistent with the idea that hypothalamic insulin signals are involved in the negative feedback of stress response.

The decrease in *Pomc* (baseline) and increase in *Gr* (30 minutes) expression in the pituitary (Figure 3.2G, H) likely represent compensatory responses to suppress the enhanced HPA axis activity due to the hypothalamic changes. This could provide a possible explanation for the relatively mild increase in CORT levels in $I^{2.1}$ KO despite the ~70% increase in pituitary *Pomc* expression (after restraint). Since glucocorticoids have been shown to suppress ACTH release from the pituitary (Dayanithi and Antoni 1989), the effect of increased pituitary *Pomc* synthesis on ACTH release could be partially corrected by the increased pituitary GR signals.

The elevated hypothalamic *Avp* expression in in $I^{2.1}$ KOs (Figure 3.2F) suggested that hypothalamic InsR signals may directly regulate transcription in AVP neurons; we do not know the exact site(s) of the AVP neurons being affected in $I^{2.1}$ KOs since they are found in multiple hypothalamic nuclei (suprachiasmatic nucleus (SCN), supraoptic nucleus (SON) and PVH). Given our observation that AVP levels in the median eminence were increased (Figure 3.2B), the involved AVP neuronal population likely resides in PVH or/and SON, since both populations release AVP to the median eminence (Antoni 1993; Wotjak et al. 1996). Supporting this idea, female mice with decreased forkhead box protein O1 (FOXO1) signaling in PVH (similar to the effect of increased InsR signals) exhibited a trended decrease in hypothalamic *Avp* expression, and FOXO1 was also shown to directly decrease promoter activity of the *Avp* gene

(Redemann 2010). Although further studies will be needed to determine if hypothalamic InsR signals directly regulate the functions of AVP neurons in the PVH as well as how the increased AVP levels may contribute to the decreased negative feedback, existing data (including ours) suggested that hypothalamic InsR signals on PVH AVP neurons may be important for down-regulating HPA axis activity after encountering a psychogenic (restraint) stress.

Metabolic Stress (Fasting)

An overnight fast resulted in a trended increase in serum CORT levels in group-housed $D^{2.1}$ KO as compared to $L^{2.1}$ KO (Figure 3.3A). Interestingly, during single-housing condition, $D^{2.1}$ KOs exhibited significantly higher fasting CORT levels than $L^{2.1}$ KOs (Figure 3.3A), suggesting that hypothalamic InsR signals are important in suppressing fasting-induced HPA axis in the absence of hypothalamic LepRb signals, since $I^{2.1}$ KO had similar CORT levels to the controls under the same condition.

Elevated fasting CORT levels in $L^{2.1}$ KO as compared to the control animals suggested that hypothalamic LepRb signal may be involved in regulating fasting-induced activity of HPA axis (Figure 3.3A). A significant elevation in fasting hypothalamic *Avp*, but not *Crh*, expression in $L^{2.1}$ KO raises the possibility that *Avp* synthesis is also regulated by hypothalamic LepRb signals. This observation is consistent with the elevated hypothalamic *Avp* expression reported in $Lep^{ob/ob}$ mice, which was suppressed by leptin treatment (Duan et al. 2007). These data raise the possibility that the suppressive effect of

hypothalamic LepRb signals on fasting-induced response might be mediated by AVP neurons in the hypothalamus.

Together, our data suggested that during restraint, hypothalamic insulin signal influences glucocorticoid negative feedback, and hypothalamic leptin signals seem to regulate baseline and the initial activation of the HPA axis. During fasting state, interactions between hypothalamic InsR and LepRb signals negatively regulate the HPA axis. And hypothalamic InsR signal alone seemed to be less important in suppressing this response, likely due to the low level of insulin signaling during fasting.

Increased hypothalamic AVP may underlie the increased anxiety-like behaviors in $I^{2.1}$ KO mice

Other than stimulating the HPA axis, AVP has also been shown to increase anxiety levels. In human, elevated AVP levels have been implicated in the development of anxiety- and depressive- like symptoms (Kruse et al. 2003; Lin et al. 2008; Zelena 2012); while injecting AVP into rats increases anxiety levels (Appenrodt et al. 1998). Consistently, *Avpr1a* knockout mice showed reduced anxiety-like behaviors (Bielsky et al. 2004). In the CNS, AVP neurons in the hypothalamus have been suggested to influence anxiety levels in rodents. Rat bred for higher anxiety levels have elevated *Avp* mRNA in the PVH, and rats experienced maternal separation also exhibited increase activation of hypothalamic AVP neurons and anxiety levels (Liebsch et al. 1996; Wigger et al. 2004; Zhang et al. 2012). Therefore, the elevated hypothalamic *Avp* expression could be responsible for the increased anxiety-like phenotypes observed in $I^{2.1}$

KO (Figure 3.4). Interestingly, the increase in anxiety-like behaviors in $I^{2.1}$ KO was only detected after an initial stressful event (in OF, novel environment; MB, saline injection; SIH, probe insertion), and at a later time-course. The timing when the anxiety-like behaviors was observed therefore raised the possibility that the anxiety-like phenotypes in $I^{2.1}$ KO might be secondary to the decreased negative feedback and elevated CORT levels as acute CORT treatment can induce increased anxiety-like behaviors (Mitra and Sapolsky 2008). However, the exactly relationship between the increased activity in HPA axis and behavioral changes in $I^{2.1}$ KO is not known.

Conclusions

In this study, we have provided evidence for a novel role of hypothalamic insulin signals in modulating the negative feedback of the HPA axis, as well as anxiety-like behaviors in mice. Nevertheless, the exact sites of InsR signals that are mediating these effects, as well as the AVP neuronal population that might contribute to the elevated HPA axis activity and increased anxiety-like behaviors are not known. Since *Nkx2.1-Cre* is not only expressed in the hypothalamus, but also in interneurons of the striatum and cerebral cortex, neurons of the globus pallidus and septum, as well as the amygdala, and some of these regions are implicated in the development of anxiety disorders, we cannot eliminate the possibility that the observed phenotypes were due to the loss of InsR signals in these extra-hypothalamic regions. Moreover, the exact mechanism of how hypothalamic insulin regulates AVP levels is also not known. One possible mechanism, as discussed earlier, is that insulin signaling in the PVH may directly regulate *Avp* expression through FOXO1-mediated transcriptional pathway.

In humans, since metabolic syndrome is often associated with anxiety and other psychiatric disorders (Luppino et al. 2011), understanding the contribution of central insulin resistance to these disorders might therefore allow the development of more effective treatments for psychiatric disorders in these patients, which often makes the management of metabolic syndrome more difficult. Although the study of $I^{2.1}$ KO suggested a possible link between central insulin resistance, stress axis dysfunction and anxiety disorders, further studies will be needed to understand the exact relationship between the loss of InsR

signaling in *Nkx2.1*- expressing neurons, and the alterations in HPA axis and behavioral outcomes.

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Author Contributions

Designed the experiments: CN Angie Chong, AS Hill. Analyzed data: CN Angie Chong, AS Hill. Performed experiments: CN Angie Chong, AS Hill. Contributed materials/analysis tools: LM Zeltser, AS Hill, R Hen. Wrote the paper: CN Angie Chong, LM Zeltser.

Figure 3.1

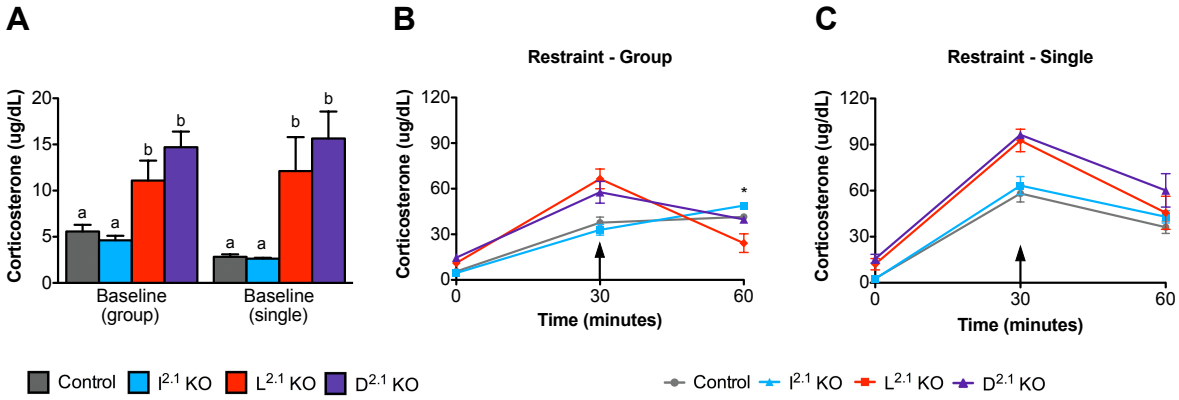

Figure 3.1: Loss of hypothalamic InsR signals resulted in increased CORT response to restraint. (**A**) Baseline (fed) serum CORT levels in adult group- and single-housed males; n ≥ 7 for all groups at all time points. (**B**) Serum CORT levels at baseline, at the end of a 30min restraint (indicated by arrow) and 30min after restraint in adult group-housed males; n ≥ 12 for $I^{2.1}$ KO and controls, n ≥ 3 for $L^{2.1}$ KO and $D^{2.1}$ KO, at all time points. (**C**) Serum CORT levels at baseline, at the end of a 30min restraint (indicated by arrow) and 30min after restraint in adult single-housed males; n ≥ 4 for all groups at all time points. All data are mean ± SEM of control, $I^{2.1}$ KO males, $L^{2.1}$ KO and $D^{2.1}$ KO. (**A**) Lowercase letters above bars denote statistically similar ($P > 0.05$) groups. (**B**) *$P < 0.05$ control versus $I^{2.1}$ KO

Figure 3.2

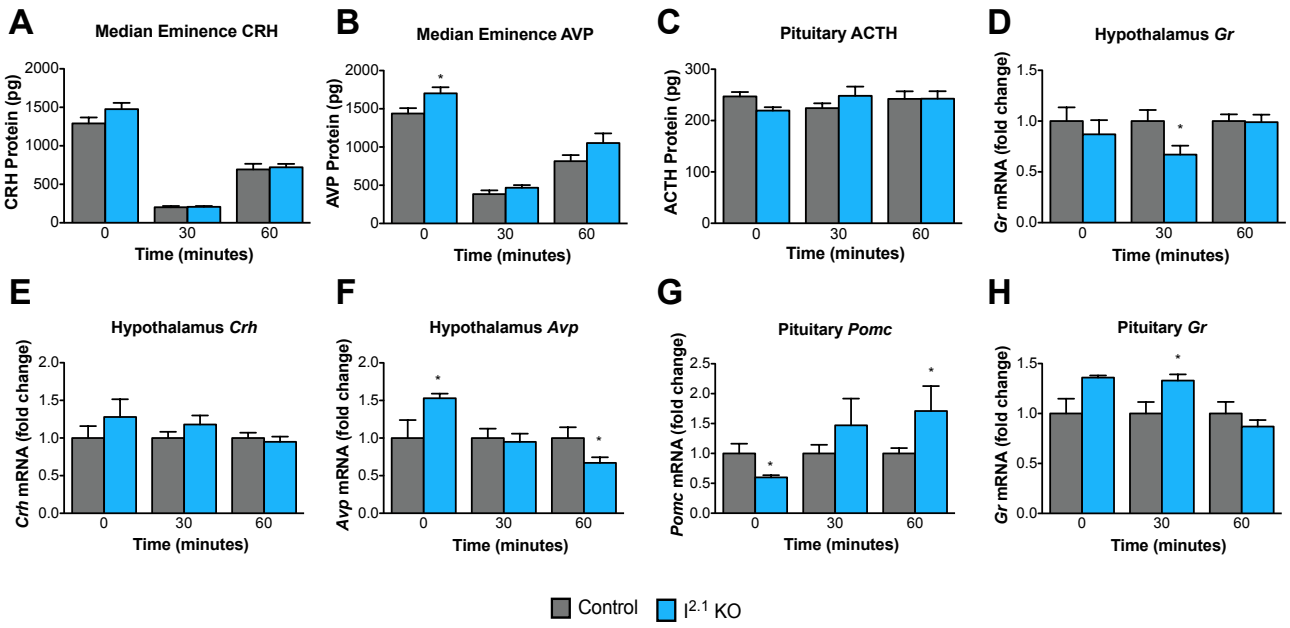

Figure 3.2: Altered gene expression of HPA axis components at baseline and during restraint test in $I^{2.1}$ KO. (**A**) CRH and (**B**) AVP protein levels in the median eminence; (**E**) *Crh* and (**F**) *Avp* mRNA expression levels in the hypothalamus at baseline, at the end of restraint (30min), and 30min post-restraint; n ≥ 5 for all groups at all time points. (**C**) ACTH protein levels and (**G**) *Pomc* mRNA in the pituitary;n ≥ 4 for all groups at all time points. (**D**) Hypothalamic, and (**H**) pituitary *Gr* mRNA; n ≥ 4 for all groups at all time points. All data are mean ± SEM of control and $I^{2.1}$ KO males. **P* < 0.05 control versus $I^{2.1}$ KO.

Figure 3.3

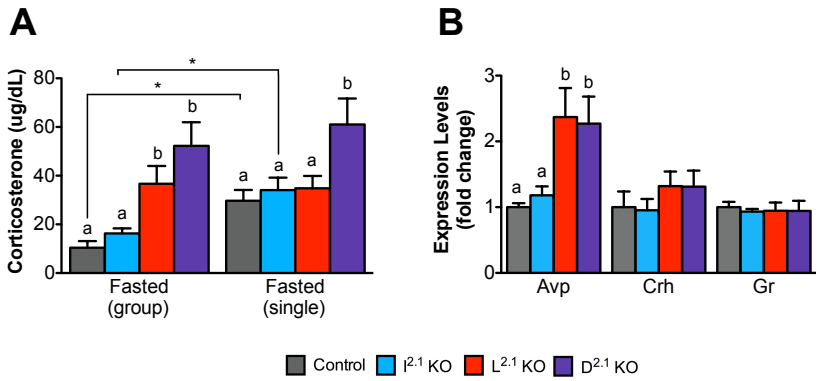

Figure 3.3: Loss of hypothalamic InsR signls resulted in increased CORT response to fasting in single-housed $D^{2.1}$ KO. (**A**) Fasting serum CORT levels in adult group- and single- housed males; n ≥ 4 for all groups at all time points. (**B**) *Avp*, *Crh* and *Gr* mRNA expression levels in the hypothalamus of fasted group-housed animals; n ≥ 3 for all groups at all time points. All data are mean ± SEM of control, $I^{2.1}$ KO males, $L^{2.1}$ KO and $D^{2.1}$ KO. Lowercase letters above bars denote statistically similar ($P > 0.05$) groups. *$P < 0.05$ for statistical difference between group- versus single-housed animals of the same genotype.

Figure 3.4

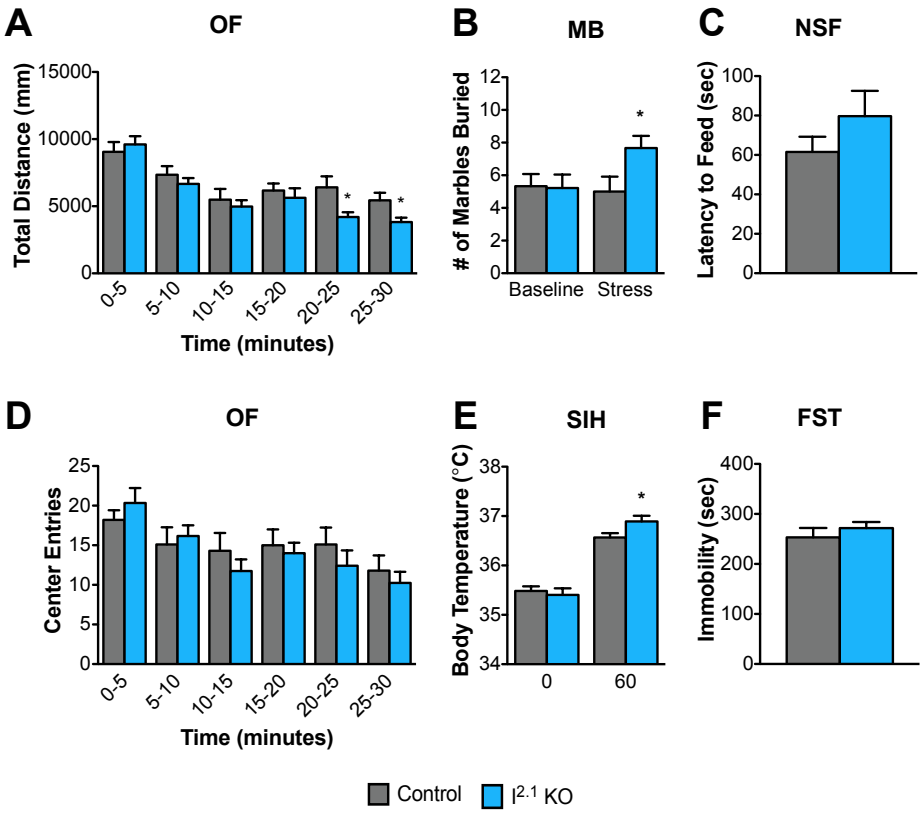

Figure 3.4: Defective behavioral responses in $I^{2.1}$ KO. (**A**) Total distance travelled and (**D**) number of entries to the center, in each 5-minute bin during the open field test (OF); n ≥ 9 for all groups. (**B**) Number of marbles buried during a 20min marble burying test (MB) at baseline and after stress; n ≥ 9 for all groups. (**C**) Latency to feed during novelty-supressed feeding (NSF) test; n ≥ 9 for all groups. (**E**) Stress-induced hyperthermia (SIH): body temperature at baseline and 60 min after stress; n ≥ 9 for all groups. (**F**) Time spent immoble during a 6min forced-swim test (FST); n ≥ 9 for all groups. All data are mean ± SEM of control and $I^{2.1}$ KO males. **P* < 0.05 control versus $I^{2.1}$ KO.

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CHAPTER 4: CONCLUDING REMARKS

Hypothalamic leptin receptor long isoform (LepRb) and insulin receptor (InsR) signals have been largely studied independently for their roles in regulating energy intake and expenditure, yet little is known about their interactions to modulate energy balance. Hypothalamic leptin signals can compensate for some of insulin's function in glucose metabolism in insulin-deficient mice (Fujikawa et al. 2013). On the other hand, it's not known if hypothalamic InsR signals can similarly compensate for some of leptin's functions. We therefore tested the hypothesis that hypothalamic InsR provides compensatory signals in $L^{2.1}$ KO (hypothalamic *Lepr* knockout) animals to regulate energy metabolism by using the $D^{2.1}$ KO model, which lacks LepRb and InsR signals in the majority of the hypothalamus.

From the study of the $D^{2.1}$ KO mice (Chapter 2), we have shown that the hypothalamic InsR signals in $L^{2.1}$ KO mice influence energy expenditure and adiposity, in part through effects on body temperature. The failure to observe similar phenotypes in $I^{2.1}$ KO (hypothalamic *Insr* knockout) mice is likely due to the compensatory actions of hypothalamic leptin signals to promote energy expenditure and thermoregulatory responses, while suppressing HPA axis activity. Strikingly, single-housing led to increased mortality rate and severe thermogenic dysregulation in $D^{2.1}$ KO, but not in $L^{2.1}$ KO mice, suggesting the interactions between environmental factors and the underlying genetic defects influence phenotypic outcomes in these mice.

In order to focus our study on the effects of loss of hypothalamic InsR on

the stress axis, we used $I^{2.1}$ KO mice as a model (Chapter 3). Unlike $D^{2.1}$ and $L^{2.1}$ KO mice, which are hypercorticosteronemic, $I^{2.1}$ KO mice do not exhibit major metabolic or HPA axis disturbances at baseline. Nevertheless, we found that the loss of hypothalamic InsR signals resulted in impaired negative feedback to stress response and some anxiety-like behaviors. Since the obese and insulin resistant state is associated with increased stress reactivity and anxiety disorders in humans, results from our studies support the idea that central insulin resistance might contribute to the observed HPA axis dysregulation and anxiety disorders in obesity.

Modification of phenotypic outcomes by the interactions between environmental and genetic factors

The dramatic effect of single-housing was first observed when $D^{2.1}$ KO animals were singly-housed for food intake or energy expenditure (in metabolic cages) studies. A significantly number of $D^{2.1}$ KO died in the middle of the study and the deaths were associated with a refusal to eat (Figure 2.4A). Since stress can induce anorexia in mice (Saegusa et al. 2011), the aphagic response in some $D^{2.1}$ KO during single-housing could be a result of increased stress response induced by the novel environment, which was then further exacerbated by the social isolation. Although being obese and hyperglycemic, $L^{2.1}$ KO did not exhibit similar mortality rates during the same studies. Therefore, we further examined why single-housing was causing significant mortality in the $D^{2.1}$ KOs.

Strikingly, single-housing brought out a dramatic body temperature phenotype in fasted $D^{2.1}$ KO, which was not observed in $L^{2.1}$ KO (Figure 2.5A),

consistent with the idea that hypothalamic InsR and LepRb interact to maintain body temperature in a state of negative energy balance via effects on brown adipose tissue (BAT) thermogenesis. InsR signals in the preoptic area (POA) inhibit the warm-sensing neurons, and activate the thermogenic pathways in BAT (York et al. 1985; Moriscot et al. 1993; Sanchez-Alavez et al. 2010). Similarly, LepRb signaling in the DMH mediates leptin's effect on BAT thermogenesis (Enriori et al. 2011). Theoretically, increased InsR signaling in the POA of hyperinsulinemic $L^{2.1}$ KOs could partially compensate for the loss of thermogenic hypothalamic LepRb signals. Thus, the loss of both hypothalamic leptin and insulin signals (as in $D^{2.1}$ KO) would severely impair BAT thermogenesis and the ability to thermoregulate.

The single-housing condition led to significantly greater fasting-induced loss in body temperature in $D^{2.1}$ KO, but not in $L^{2.1}$ KO, $I^{2.1}$ KO or the control mice (Figure 2.5A). Environmental factors, such as ambient temperature, are known to have a great impact on the thermoregulatory system in small rodents such as mice, which need to devote a large fraction of its energy metabolism to maintain body temperature (Shaun F Morrison 2012). Mechanisms to thermoregulate in mice range from the less energy costly mechanisms such as vasoconstriction, pilorection, and behavioral changes (e.g. changes in postures, huddling, etc.) to those that require more energy such as non-shivering adaptive and shivering thermogenesis. Mice housed at normal ambient temperature (~22^{o}C, most housing facilities) exhibit higher BAT *Ucp1* as compared with those at thermoneutrality (~30^{o}C) (Chen et al. 2003), indicating an increase in BAT

thermogenesis at sub-thermoneutral housing temperature.

As mentioned earlier, huddling is a social thermoregulatory behavior and increased huddling is observed in mice housed at lower ambient temperature (Canals and Bozinovic 2011). Higher BAT thermogenesis is also observed in singly-housed mice as compared to those that are group-housed at the same ambient temperature (Jennings et al. 1986). These studies support the idea that mice housed at either temperature below thermoneutrality and/or in isolation would require additional BAT thermogenic activity to maintaining body temperature. Therefore, the dramatic loss of fasting body temperature in singly-housed $D^{2.1}$ KO mice could be a result of the inability of these mice to provide the additional BAT thermogenesis (due to the lack of thermogenic hypothalamic LepRb and InsR signals). To minimize the impact of social isolation on physiological functions in rodents (Bartolomucci et al. 2003), we performed a subsequent study in which all mice were group-housed until the day when they were subjected to an overnight fast in single-housing. Their body temperature was then measured after the fast at the beginning of the light cycle. We allowed the mice to recover for a week, and repeated the same fasting experiment in group-housing condition. Consistent with what we reported in mice that were single-housed for two to three weeks (Figure 2.5A), in this experiment, fasting body temperature of $D^{2.1}$ KOs was significantly lower than $L^{2.1}$ KOs in both housing environments (Figure 4.1). Comparing body temperature of $D^{2.1}$ KOs and $L^{2.1}$ KOs in group- versus single-housing environment revealed a significantly lower temperature in single-housed $D^{2.1}$ KO (as compared to group-housed), but

not in $L^{2.1}$ KOs. These results therefore further supported the idea that in group-housed $D^{2.1}$ KO mice, huddling can partially compensate for the deficit in BAT thermogenesis associated with fasting.

The dramatic reduction in body temperature in singly-housed $D^{2.1}$ KOs was only observed during fasting (energy deficit) also raise the possibility that these mice were in a torpor state, a hypometabolic state generally defined as a regulated reduction in core body temperature below 31^{o}C (Gluck et al. 2006). However, since torpor in fasted mice has been reported to occur more frequently in group- than single- housed mice (Webb et al. 1982), and single-housing increased the proportion of $D^{2.1}$ KO mice exhibiting fasting body temperature lower than 31^{o}C (data not shown), the dramatically reduced body temperature in $D^{2.1}$ KO during fasting is unlikely to represent a true torpor state. So, the high mortality rate in single-housed $D^{2.1}$ KO might be a result of enhanced stress response, which was further exacerbate by a failure to thermoregulate, and eventually lead to death if the aphagia persist for several days.

These observations highlight the importance of thermoregulatory behaviors, in addition to BAT thermogenesis, for regulating body temperature. The consequences of housing animals below thermoneutrality for body weight and energy expenditure phenotypes have been the subject of several recent review (Speakman and Keijer 2012). For example, housing *Ucp1* knockout mice in thermoneutrality, but not at normal ambient temperature, increased susceptibility of the knockouts to high-fat-diet-induced obesity (Enerback et al. 1997; Feldmann et al. 2009). Our data highlight the need to consider differences

between single- and group-housing conditions as well, particularly in animals with impaired thermoregulatory circuits. Another observation that highlights the dramatic influence of housing environment on experimental outcomes is the increased CORT response to both restraint and fasting in $I^{2.1}$ KO and control mice when singly-housed (Figure 3.1, 3.3), which is consistent with what has been reported in wild type mice by Bartolomucci et al. (Bartolomucci et al. 2003). Nevertheless, increased fasting CORT response was not observed in singly-housed $D^{2.1}$ KO and $L^{2.1}$ KO (both lacking hypothalamic LepRb), suggesting a differential interaction between single-housing and this genetic mutation.

Interactions between single-housing (social isolation stress) and genetic factors have been reported in several different mouse models. Adolescent social isolation of animals with a putative dominant-negative DISC1 (disrupted in schizophrenia 1) under the expression control of a prion protein promoter exhibit behavioral phenotypes that were not seen in mice with either the genetic or the environmental component alone (Niwa et al. 2013). There was also an increase in CORT levels in the socially isolated mutants, and the effect of social isolation on the phenotypes was blocked by glucocorticoid receptor II antagonist (Pilkis and Granner 1992; Rebuffé-Scrive et al. 1992; Niwa et al. 2013), supporting the idea that social isolation causes dysregulation of the HPA axis. Together with our study, these results supported the idea that interactions between housing environment and certain genetic factors can bring out or mask a phenotype.

The influence of social isolation stress (a very commonly used housing condition) on the functions of HPA axis would be potentially problematic for many

studies focusing on energy metabolism since activation of HPA axis exerts systemic effects on multiple target organs including the liver, skeletal muscles, and adipose tissue (Pilkis and Granner 1992; Rebuffé-Scrive et al. 1992; Liu et al. 2010), etc. to influence body fat distribution, fuel metabolism and energy partitioning. And the observed phenotypes in an animal model may therefore be secondary to their differences in stress response to the housing condition.

Future Directions

Since the singly-housed fasted $D^{2.1}$ KO exhibit significantly lower body temperature and higher serum CORT levels (Figure 2.5A, 3.3A), and CORT can suppress BAT thermogenesis in both wildtype and *Lep*$^{ob/ob}$ animals (York et al. 1985; Moriscot et al. 1993; Nonogaki et al. 2007), it raises the possibility that the increased CORT might contribute to the reduction in fasting body temperature in $D^{2.1}$ KO mice. In order to investigate the effect of elevated CORT on BAT thermogenesis, we can perform adrenalectomy (ADX) on both $L^{2.1}$ KO and $D^{2.1}$ KO mice and then clamp their circulating glucocorticoid levels (by peripheral replacement) at a similar level. If the fasting temperature phenotype persists, elevated CORT levels in $D^{2.1}$ KO unlikely underlie the phenotype. If the phenotype is corrected, we could further explore the contribution of peripheral versus central GR signals in the dampening of thermogenesis in $D^{2.1}$ KO with central GR-II antagonist administration. Normalizing the fasting-induced decreases in body temperature in $D^{2.1}$ KO by ADX and peripheral glucocorticoid replacement, but not by central injections of GR-II antagonist alone, would be consistent with the idea that the increase in peripheral GR signals contributes to

the body temperature phenotype. Either if central or peripheral GR signaling is responsible for the decreased temperature, site- (specific brain nucleus) or tissue- (liver, white adipose tissue, brown adipose tissue, skeletal muscles) specific removal of GR signals by viral-mediated knockdown of *Gr* in $D^{2.1}$ KO can then be used to identify the site(s) of action of glucocorticoid that is mediating this effect.

Possible contributions of glucocorticoids to increased adiposity in $D^{2.1}$ KOs

While $D^{2.1}$ KO males have higher body weights and adiposity than $L^{2.1}$ KO starting from 8 weeks, $I^{2.1}$ KO mice are the same as the controls (Figure 2.1A, B). These observations support the idea that interactions between hypothalamic InsR and LepRb are involved in adiposity and body weight regulation. The increased adiposity in $D^{2.1}$ KO is not due to changes in food intake, but likely decreased energy expenditure, supported by a trended decrease in oxygen consumption (Figure 2.4B: ~16% lower than $L^{2.1}$ KO, $P \sim 0.09$). Our current n number in this study was 5 mice in each genotype. Given the high variability in oxygen consumption and mortality rate during the metabolic cages study in the $D^{2.1}$ KO mice, approximately 20 more mice in each genotype would be needed to obtain significance in our result and therefore this arm of the study was not further pursued.

Another factor that could contribute to the increased adiposity in $D^{2.1}$ KO is the increased circulating CORT levels. $D^{2.1}$ KO exhibit a 25% increase in baseline CORT (Figure 3.1A, although this difference is not statistically significant), and a significantly increase in fasting CORT response, as compared to singly-housed

$L^{2.1}$ KO (Figure 3.3A). Increased circulating glucocorticoids levels have been shown to promote increase adiposity. Either chronic stress or exogenous glucocorticoid treatments have been shown to increase adiposity, and gonadal fat distribution in rats (Rebuffé-Scrive et al. 1989; Makimura et al. 2000; Fujikawa et al. 2013); while adrenalectomy reduces obesity in *Lep*$^{ob/ob}$ mice (Makimura et al. 2000; Wigger et al. 2004). In human studies, women with increased visceral obesity have been shown to exhibit hyperactive HPA axis (York et al. 1985; Moriscot et al. 1993; Strack et al. 1995; Pasquali et al. 2002, 1996). These studies suggested that elevated glucocorticoids could underlie the preferential gain in gonadal, but not inguinal, adiposity in $D^{2.1}$ KO mice (Figure 2.1, 2.2).

Future Directions

We could investigate the extent to which elevated CORT levels underlie the increased adiposity in D2.1 KOs vs. $L^{2.1}$ KOs by adrenalectomy (ADX) and then replacing with defined amounts of glucocorticoids. By clamping circulating levels of glucocorticoids in both $D^{2.1}$ KO and $L^{2.1}$ KO mice from 8 weeks when their adiposity started to diverge, we could detect adult changes in adiposity that are not due to a difference in glucocorticoid levels. On the contrary, ADX and clamping the glucocorticoid levels early during post-weaning development at a similar level, and then varying the levels of replacement in $D^{2.1}$ KO (higher levels) and $L^{2.1}$ KO (lower levels) in adulthood will allow us to determine if differences in adult glucocorticoid levels are sufficient for the development of the increased adiposity in $D^{2.1}$ KO mice. Alternatively, we could also raise circulating glucocorticoid levels in $L^{2.1}$ KO by exogenous CORT administration to that of

similar to $D^{2.1}$ KO and study the effect on adiposity. If raising circulating corticosterone levels in $L^{2.1}$ KO resulted in an adiposity level similar to that of $D^{2.1}$ KOs, it would then suggested that increased glucocorticoid levels contributed to the difference in adiposity between $L^{2.1}$ KO and $D^{2.1}$ KO.

The role of central InsR signals in regulating stress response and anxiety

From our studies, we have shown that the loss of InsR signals in the majority of the hypothalamic neurons led to increased activity of HPA axis (in $D^{2.1}$ KO and $I^{2.1}$ KO) and a mild increase in anxiety-like behaviors (in $I^{2.1}$ KO). However, the exact relationship between the HPA axis dysregulation and anxiety-like behaviors in $I^{2.1}$ KO is not known. Given the data we collected so far, we came up with a few possible explanations for the observed phenotypes in $I^{2.1}$ KO mice: central insulin signals acting on different neuronal populations (circuits) to influence HPA axis activity and anxiety-like behaviors; insulin acts on hypothalamic neurons (maybe AVP neurons) that are common regulators for both the HPA axis activity (by projecting to the median eminence) and anxiety behaviors (by projecting to other brain regions); increased anxiety in $I^{2.1}$ KO is a result of increased central GR signaling (due to increased circulating CORT levels).

Since *Nkx2.1-Cre* is not only expressed in the hypothalamus, but also in interneurons of the striatum and cerebral cortex, neurons of the globus pallidus and septum, as well as the amygdala (Xu et al. 2008), and some of these regions are also implicated in the development of anxiety disorders (Appenrodt et al. 1998; Shepard et al. 2000), we cannot completely eliminate the possibility that

the observed phenotypes were due to the loss of InsR signaling in these extra-hypothalamic regions.

Future Studies

To study the role of InsR signals in specific sites of the central nervous system (CNS) in regulating the stress axis and anxiety, specific *Cre* drivers can be used to target neurons in various intra-hypothalamic and extra-hypothalamic brain regions. For example, *Neuromedin S* (*NMS)-Cre* can be used to target neurons in the SCN (Kinoshita et al. 2000; Chang 2010). Viral-mediated acute knockdown of *Insr* in intra-hypothalmaic (PVH, SON) and extra-hypothalamic (amygdala) regions can be used to study the contribution of InsR signal in each of these regions to phenotypes observed in $I^{2.1}$ KO mice. Acute knockdown would also prevent the development compensation for the loss of InsR signal on NKX2.1 neurons from birth and may produce a more dramatic phenotype.

Further studies will also be needed to identify the neuronal AVP that are regulated by InsR signals, as well as their downstream targets. In order to evaluate the contribution of SON and PVH AVP neurons to the increased hypothalamic baseline *Avp* expression levels, microdissection of each nucleus (instead of whole-hypothalamus) can be done to look at gene expression in these specific regions. Viral-mediated shRNA knockdown of *Avp* in either PVH or SON in $I^{2.1}$ KO mice would then allow us to distinguish the contribution of each AVP neuronal population to the elevated ME AVP levels. If ME AVP is normalized, we could also evaluate the stress response to show the direct involvement of elevated baseline AVP in the negative feedback.

Hypothalamic AVP has been proposed to increase anxiety-like behaviors in rodents. Rat bred for higher anxiety levels have elevated *Avp* mRNA in the PVH (Liebsch et al. 1996; Wigger et al. 2004); while intra-septal injections of AVPR1 antagonist reduced anxiety in rats (Liebsch et al. 1996; Toshinai et al. 2001; Sanchez-Alavez et al. 2010), and activation of AVP receptors in amygdala increased anxiety levels (Zhang et al. 2012; Inoue et al. 2013). Together, these studies raised that possibility that hypothalamic AVP neurons projecting to the septal region and/or amygdala might be involved in the modulation of anxiety behaviors. In order to evaluate the effect of increased hypothalamic *Avp* synthesis on the AVP release in the septal regions or amygdala, AVP levels in each region can be measured separately by microdissecting out each region after the viral-mediated knockdown of *Avp* in the hypothalamic nucleus. Examination of anxiety behaviors in these mice will further confirm a functional role of these AVP neurons in mediating the anxiety response in $I^{2.1}$ KO mice.

In order to understand the contribution of increased GR signal (due to increased CORT release) to the anxiety-like behaviors, ADX $I^{2.1}$ KO and control animals with similar level of glucocorticoid replacement can be subjected to the anxiety behavioral tests. If increased CORT contributed to the anxiety phenotypes, ADX $I^{2.1}$ KO would no longer exhibit elevated anxiety levels as comparing to the control animals. Furthermore, viral-mediated knockdown of *Gr* in specific brain nuclei in the limbic regions and hypothalamus can then be done to identify the CNS regions where GR signaling is mediating this effect.

HPA axis dysregulation and anxiety disorders in obesity

People who are obese have been shown to have an increase risk in having affective/mood disorders (including anxiety disorders) and increased HPA axis activity (Peeke and Chrousos 1995; Gariepy et al. 2009; Cannon and Nedergaard 2011). Results from our studies raise the possibility that decreased hypothalamic insulin signal could be involved in the development of HPA axis dysfunction and anxiety disorders in diabetes and obesity. Obese humans who are leptin-deficient or have mutated leptin receptor gene (with normal glucose tolerance) have normal cortisol levels (Montague et al. 1997; Clement et al. 1998; Kahn and Flier 2000), and obese individual who showed normal glucose tolerance also had lower cortisol levels that those who exhibited impaired glucose tolerance. Further supporting a role of insulin resistance in the development of anxiety disorders in obesity, treating human patients with abdominal obesity with insulin sensitizer, thiazolidinedione, for 12 weeks significantly reduced both anxiety and depression symptoms, without a significant change in body weight (Kemp et al. 2012). Together with our data, these studies suggested that reduced hypothalamic insulin signal might be central to the development of HPA axis dysregulation and anxiety disorders in obese humans.

Future Directions

To understand the contribution of central InsR signal in regulating HPA axis functions and anxiety-like behaviors in obese animal models, intracerebroventricular (icv) injections of insulin sensitizer e.g. dicohline succinate, could be used to increase central insulin signals in obese *Lep*$^{ob/ob}$

mice. We would then evaluate the anxiety scores and HPA axis activity in these animals before any changes in body weight or adiposity happen. The advantage of using *Lep*$^{ob/ob}$ mice is that the experimental outcomes will not be influenced by the effect of insulin on leptin levels. This model can therefore also be used to look at the interactions between central InsR and LepR signals in modulating anxiety levels by modulating central InsR and LepR signaling levels with icv injections of leptin and/or insulin. In combination with viral-mediated knockdown of *Insr* in specific brain regions, the site of action for insulin's effect can also be identified.

Conclusions

In the current study, we have shown that hypothalamic InsR signals have a weight- and adiposity- reducing effect in $L^{2.1}$ KO mice. More importantly, $D^{2.1}$ KO mice, but not $L^{2.1}$ KO mice, also displayed severe dysregulation in HPA axis and thermoregulatory response, particularly after overnight fasting in social isolation, providing evidence for a critical role of hypothalamic InsR and LepRb signals interaction in maintaining normal functions of the HPA and central thermoregulatory circuits. The loss of hypothalamic InsR alone led to decreased negative feedback in stress response and elevated anxiety-like behaviors, raising the possibility that hypersensitivity of HPA axis in obesity may be secondary to the underlying central insulin resistance.

There are some evidence from human studies that support our current findings that central insulin suppresses stress response (Bohringer et al. 2008). Obese patients with normal glucose tolerance also exhibited lower cortisol levels than those who are glucose intolerance. Therefore, it's possibly that hypothalamic insulin resistance is central to the altered response of HPA axis seen in obesity (Pasquali and Vicennati 2000; Pasquali et al. 2006). If this is true, however, the dramatically enhanced fasting CORT response in $D^{2.1}$ KO, but not $I^{2.1}$ KO, would suggest that either obesity or reduced hypothalamic LepR signals also contribute to development of HPA axis disorders.

Understanding the contribution of central insulin resistance (sites of action, and downstream targets) to the comorbidities in obesity, such as cardiovascular diseases, stroke and HPA axis hyperactivity etc., would potentially allow more

effective and specific treatments for these disorders by targeting the underlying pathways even if weight loss cannot be achieved.

Figure 4.1

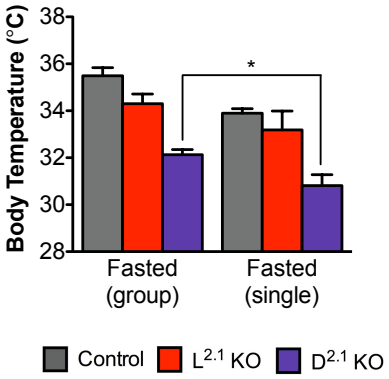

Figure 4.1: Fasting body temperature in group-housed $D^{2.1}$ KO was further reduced by single-housing. Fasting core body temperature of group- and singly-housed control, $L^{2.1}$ KO, and $D^{2.1}$ KO adult males; n ≥ 7 for all groups. All data are mean ± SEM. *P < 0.05 for statistical difference between group- versus single- housed $D^{2.1}$ KOs.

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APPENDIX A: Loss of function study to examine the role of LHX1 in NKX2.1 neurons in regulating energy balance and the reproductive axis

Overview

The arcuate nucleus of the hypothalamus (ARH) is located in the mediobasal hypothalamus, and adjacent to the third ventricle and the median eminence, where the blood-brain-barrier is compromised. Therefore, ARH neurons are structurally positioned to receive and integrate peripheral metabolic signals of energy status to modulate energy metabolism. POMC and AgRP/NPY neurons are two of the most studied leptin-sensing neurons in the ARH and have been shown to be important for mediating some of leptin's effect on energy balance (Luo et al. 2011; Berglund et al. 2012). Efforts in our lab to identify and study the role of non-POMC and non-AgRP/NPY leptin-sensing neurons in the ARH were started by a then graduate student - Stephanie Padilla. She performed genetic screens and used *in situ* hybridization to identify molecular markers (non-POMC and AgRP/NPY) in ARH leptin-sensing neurons.

Lhx1 is one of the markers that came out from the screen. It encodes LIM1 (Lim Homeobox 1), a transcription regulator that is essential for proper forebrain development (Shawlot et al. 1999). In the ARH, two functional markers are found in LHX1 neurons: Tyrosine hydroxylase (TH), an enzyme that catalyzes the synthesis of dopamine; and *cocaine- and amphetamine-regulated transcript* (*Cart*), which encodes a peptide that signals satiety and increases thermogenesis (True et al. 2013) (Figure A1). In order to study the functions of the ARH LHX1 neurons, we used mice that have a conditional loss of function of

Lhx1 in the hypothalamus using *Nkx2.1-Cre* (*Lhx1*$^{Nkx2.1}$ KO), which is expressed in the majority of hypothalamic neurons (Xu et al. 2008). *Lhx1*$^{Nkx2.1}$ KO mice were confirmed to have ~80% loss of LHX1-positive neurons in the ARH (Figure A2). We also detected a non-significant trend of decrease in TH- and *Cart*-positive neurons in the ARH of *Lhx1*$^{Nkx2.1}$ KO mice (data not shown).

Since some ARH LHX1 neurons are LepRb positive, and preliminary study showed that *Lhx1*$^{Nkx2.1}$ KO females exhibited reduced reproductive capacity (smaller litter size), we hypothesized that ARH LHX1 neurons may integrate reproductive behaviors and/or functions with metabolic status. We first evaluated different metabolic parameters in *Lhx1*$^{Nkx2.1}$ KO mice, included baseline and fast-induced feeding, body weight, body composition, length, glucose levels and glucose tolerance, and did not detect any difference (data not shown). High-fat-diet feeding of *Lhx1*$^{Nkx2.1}$ KO from weaning also did not bring out any metabolic differences (data not shown). Further study looking at functions of the reproductive axis in female *Lhx1*$^{Nkx2.1}$ KO mice including the onset of puberty (timing of vaginal opening, onset of estrus cycle), estrus cycle properties (length, cytology at various stages), gestational length, and latency to mate did not identify any changes. We also failed to detect a decrease in litter size found in earlier studies (data not shown).

Dopaminergic neurons in the ARH (part of the tuberinfundibular system) project and release dopamine to the median eminence and inhibit prolactin secretion from the pituitary (McCann et al. 1984). Prolactin is released in response to suckling stimulation during lactation and has been linked to

lactational hyperphagia (Woodside 2007), the increase in food intake in lactating mothers. Since a subset of ARH LHX1 neurons is TH-positive, we focused our study to the lactational period. We did not detect any baseline changes in prolactin levels during various stages of the estrus cycle (data not shown). During lactation, there was no change in body weight, body composition, food intake and glucose levels in *Lhx1*$^{Nkx2.1}$ KO females (data not shown). The energy and glucose metabolism during lactation did not seem to be altered by the loss of ARH *Lhx1* expression.

Since some LHX1 neurons express *Esr1* (*Estrogen receptor alpha*), a final study was performed to understand the relationship between hypothalamic LHX1 neurons and the anorexic effect of estrogen mediated by the hypothalamic circuits (Donohoe and Stevens 1982). We ovariectomized female *Lhx1*$^{Nkx2.1}$ KO mice and replaced estrogen by daily intraperitoneal injection of estradiol for one month and the study was continued for another month without estradiol injections. Throughout this study (with or without estradiol injections), food intake, body weight and adiposity were not different between *Lhx1*$^{Nkx2.1}$ KO mice and the controls (data not shown).

Overall, we did not find any metabolic or reproductive phenotypes in *Lhx1*$^{Nkx2.1}$ KO mice under various experimental conditions, and thus hypothalamic LHX1 neurons did not seem to be involved in regulating energy metabolism or reproductive functions. Therefore, this project was not pursued further. One possible explanation for the lack of phenotypes in *Lhx1*$^{Nkx2.1}$ KO mice could be due to the compensatory actions of the remaining ARH LHX1-postive neurons to

maintain normal functions. A large number of LHX1-postive neurons were detected in the region rostral (Suprachiasmatic nucleus anlage) to the primordial ARH from E13 to E15 (Figure A4). In adult *Lhx1*$^{Nkx2.1}$ KO mice, we detected only ~20% loss of LHX1-positive neurons in the suprachiasmatic nucleus (SCN) (Figure A3), where *Nkx2.1* expression is dramatically down-regulated by E13.5 (Shimogori et al. 2010; Morales-Delgado et al. 2011). Therefore, the transient activity of the *Nkx2.1-Cre* in the SCN anlage might not allow enough time for the recombination to occur. This supported the observation that LHX1 neurons in the ARH were not completely eliminated in *Lhx1*$^{Nkx2.1}$ KO mice and some of these neurons might originate from the SCN.

Figure A1

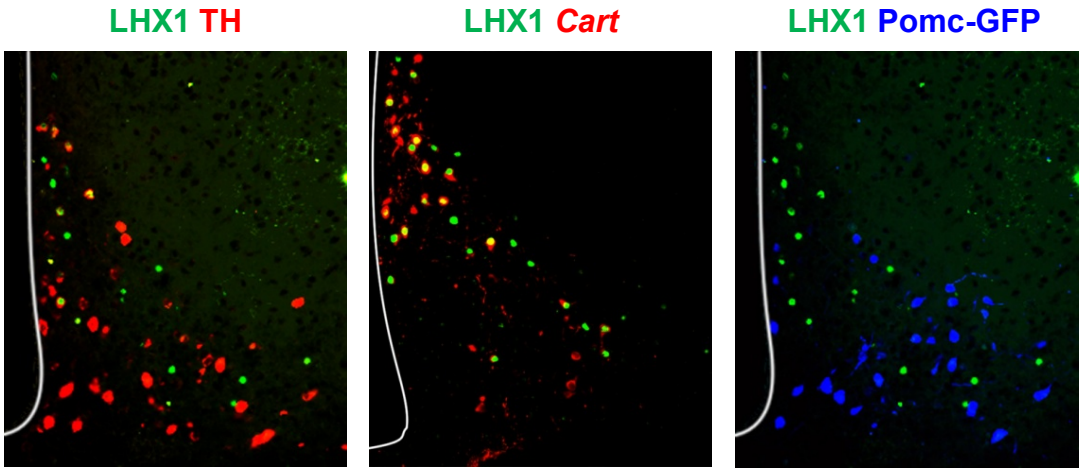

Figure A1: Functional markers of LHX1-positive neurons in the ARH. LHX1 colocalized with TH (left) and *Cart* (middle), but not Pomc-GFP (right) in the ARH. LHX1/TH neurons are mainly located in the dorsal ARH; LHX1/*Cart* neurons, dorsal-lateral ARH. (Staining was done by Stephanie Padilla)

Figure A2

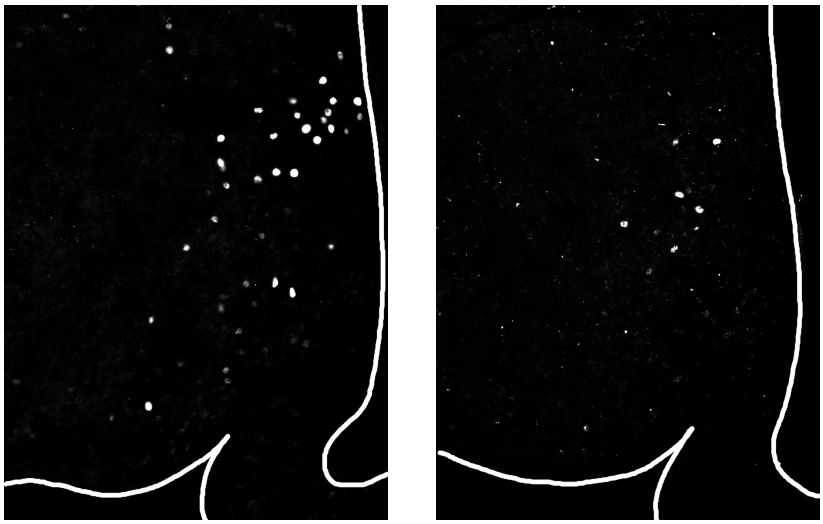

Figure A2: LHX1 positive neurons in ARH of control (left) and *Lhx1*$^{Nkx2.1}$ KO (right) adult animals. There was approximately an 80% reduction in LHX1 positive neurons in the ARH of the *Lhx1*$^{Nkx2.1}$ KO adult animals.

Figure A3

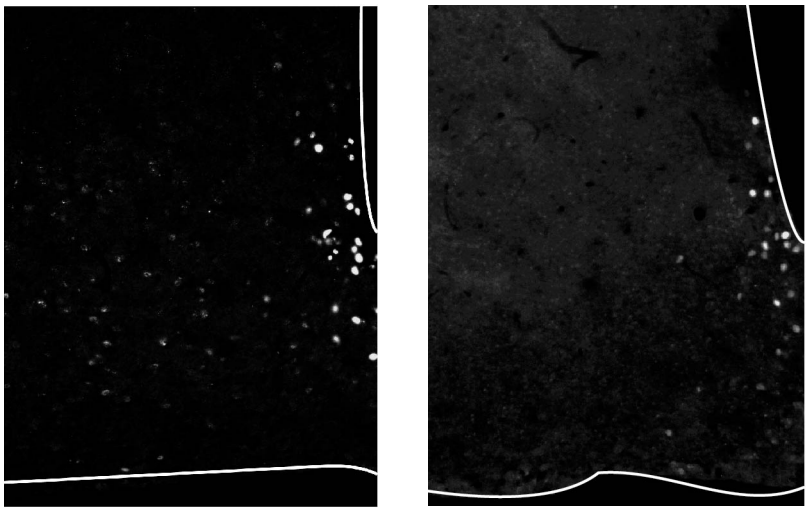

Figure A3: LHX1-positive neurons in the SCN of control (left) and *Lhx1*$^{Nkx2.1}$ KO (right) adult animals. There was about a 20% reduction in LHX1-positive neurons in the SCN of *Lhx1*$^{Nkx2.1}$ KO adult animals.

Figure A4

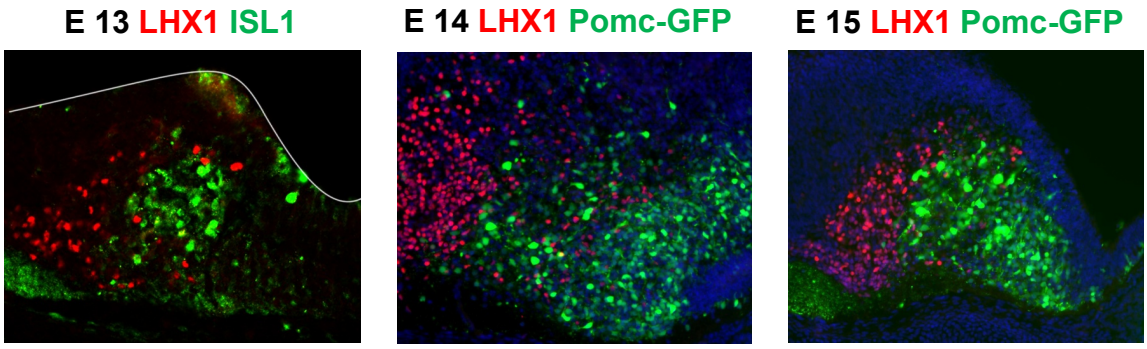

Figure A4: Sagittal sections of primordial ARH showing the distribution of LHX1-positive neurons. LHX1-positive neurons are also present in domain rostral to the ARH (anlage of the SCN) from E13 to E15. (Staining was done by Lori Zeltser)

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APPENDIX B: List of Abbreviations (in alphabetical order)

ACTH	Adrenocorticotropic hormone
ADX	Adrenalectomy
AGRP	Agouti-related peptide
AKT	Protein kinase B
ARH	Arcuate nucleus of the hypothalamus
AVP	Arginine vasopressin
AVPR1A	Arginine vasopressin receptor 1A
AVPR1B	Arginine vasopressin receptor 1B
BAT	Brown Adipose Tissue
BNST	Bed nucleus of the stria terminalis
CAMK2A	Calcium/Calmodulin-dependent Protein Kinase II alpha
CART	Cocaine- and amphetamine-regulated transcript
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
CORT	Corticosterone
CRHR1	Corticotrophin-releasing hormone receptor 1
CRHR2	Corticotrophin-releasing hormone receptor 2
DEX	Dexamethasone
DHA	Dorsal hypothalamic area
DLPO	Dorsolateral preoptic area
DMH	Dorsomedial hypothalamus nucleus
E	Epinephrine
FOXO1	Forkhead box protein O1
FST	Forced swim test
GR	Glucocorticoid receptor
GC	Glucocorticoid
HPA	Hypothalamic-pituitary-adrenal
ICV	Intracerebroventricular
IML	Intermediolateral cell column
INSR	Insulin receptor
LC	Locus coeruleus
LH	Lateral hypothalamus
LEPRB	Leptin receptor long isoform
LHX1	LIM homeobox 1
LPB	Lateral parabrachial nucleus
MAPK	Mitogen-activated protein kinase
MB	Marble burying
ME	Median eminence
mPOA	Medial preoptic area
MnPOA	Median preoptic area
MR	Mineralocorticoid receptors
NE	Norepinephrine
NPY	Neuropeptide Y
NSF	Novelty-suppressed feeding

NTS	Nucleus of the solitary tract
OF	Open field test
OXT	Oxytocin
PI3K	Phosphoinositide 3-kinase
PB	Parabrachial nucleus
PVH	Paraventricular nucleus of the hypothalamus
PAG	Periaqueductal grey
POMC	Proopiomelanocortin
RVM	Rostral ventromedial medulla
rRPa	Rostral raphe pallidus nucleus
SCN	Suprachiasmatic nucleus
SIH	Stress-induced hyperthermia
SIM1	Single-minded homolog 1
SON	Supraoptic nucleus
STAT3	Stimulate signal transducer and activator of transcription 3
STZ	Streptozotocin
TH	Tyrosine hydroxylase
UCN	Urocortin
VMH	Ventromedial hypothalamus