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Hypothalamic Substrates of Metabolic Imprinting

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Abstract

The mammalian brain develops according to intrinsic genetic programs that are influenced by a variety of environmental factors. Developing neural circuits take shape in two major environments: one *in utero* and a second during postnatal life. Although an abundance of epidemiological and experimental evidence indicates that nutritional variables during perinatal life have a lasting effect on metabolic phenotype, the underlying mechanisms remain unclear. Peripheral hormones are widely regarded as effective signals that reflect the state of peripheral environments and can directly influence the development of a variety of functional neural systems. Recent findings suggest that the adipocyte-derived hormone leptin may play an important role in directing formation of hypothalamic neural pathways that control body weight. The arcuate nucleus of the hypothalamus (ARH) is a key site for the regulatory actions of leptin in adults, and this same hormone is required for the normal development of ARH projections to other parts of the hypothalamus. In this review, the neurobiological role of leptin is considered within the context of hypothalamic development and the possibility that variations in both prenatal and postnatal nutritional environments may impact development of neural circuits that control energy metabolism through an indirect action on leptin secretion, or signaling, during key developmental critical periods.

Keywords

leptin; arcuate nucleus; paraventricular nucleus; development; obesity; glucose metabolism

Introduction

Effective management of bodily energy stores is essential to survival in any environment and is best served if the energy balance of the individual is optimized so as to promote efficient utilization of available metabolic fuels. Elaborate neuroendocrine mechanisms have evolved to facilitate coordination of energy balance in a variety of organisms that inhabit environments with variable food availability. Notable examples include mammals that hibernate during winter months and migratory birds that store energy in preparation for long periods of starvation during seasonal migrations [1,2]. In most parts of the world man has succeeded in maintaining a relatively steady source of food, so it would seem that the need for homeostatic mechanisms favoring storage of energy as fat is diminished. However, these mechanisms appear to remain intact and in modern environments, with widespread availability of energy dense food sources, may even be nonadaptive as evidenced by soaring rates of obesity and type 2 diabetes in juvenile populations [3,4]. Like all mammals, human infants are exposed to two major successive environments: one in utero and the other postnatal. Developmental mechanisms occurring in utero have the capacity to imprint a metabolic phenotype on the offspring that

promotes success in the postnatal environment. Both epidemiological and in vivo data from animal models suggests a variety of negative health outcomes ensue when these two environments are mismatched [5–7]. Thus, it is important to understand how homeostatic mechanisms established in utero predispose offspring to a favorable energy balance in its postnatal environment, and to identify the cellular and molecular events that specify metabolic phenotype.

Perinatal nutrition has been identified as a significant determining factor for predisposition to cardiovascular disease, obesity and type 2 diabetes. This relationship has been discussed extensively and in general the results of epidemiological studies indicate that fetal malnutrition can negatively impact the probability of developing metabolic disease [8–10]. Results of experiments in a variety of animal models also support a link between the perinatal nutritional environment and programming of energy balance "set points" [11–14]. Maternal obesity and high fat diets during gestation and lactation appear to promote obesity and insulin-resistance in offspring, as does maternal undernutrition during gestation [12,15–18]. The signals that mediate maternal aspects of metabolic disorders in offspring have not been identified, but hormones such as insulin and leptin, or nutrients such as glucose or fatty acids, may cross the placenta and influence fetal development. For example, patients with gestational diabetes display marked increases in leptin production and these elevations are out of proportion to their adiposity [19–21]. Maternal hyperglycemia is associated with fetal hyperglycemia, which in turn may cause elevations in fetal insulin levels leading to increased growth and adiposity of the offspring. Inadequate nutrition during key developmental periods may also promote obesity, perhaps by causing the offspring to become more metabolically efficient in anticipation of reduced availability of nutrients. Later, when provided with sufficient, or even excess, availability of calories these individuals may tend toward obesity and acquisition of insulin resistance [22]. Thus, an abundance of evidence suggests that alterations in the prenatal nutritional environment and subsequent postnatal growth can predispose an individual to obesity and diabetes. However, most studies have been focused on peripheral measures and outcomes. In this review, development of the hypothalamus will be examined within the context of developmental programming of metabolic phenotype.

Neurobiology of Energy Metabolism

Neural networks maintain homeostasis by coordinating endocrine signals with behavioral and autonomic function to ensure that behaviors and physiological responses remain in tune with environmental demands. The hypothalamus plays an essential role in this function by integrating endocrine, autonomic and somatomotor control mechanisms that coordinate a variety of neuroendocrine homeostatic processes [23,24]. The adaptive value of constructing homeostatic neural networks that are optimized for particular environments appears to be so important that physiological signals direct their development in a way that is largely independent of learning. Instead, this process is controlled by circulating hormones and other signals that reflect environmental conditions. Thus, a variety of homeostatic set points appear to be programmed during development in response to environmental constraints that include both prenatal and postnatal factors. Most functional neural systems acquire their unique properties during restricted developmental periods under the influence of intrinsic and environmental factors. Many learned behaviors are acquired early in life during times, termed critical periods, when experience has a lasting impact on subsequent behaviors. For example, avian song learning is only possible during a discrete critical period when the circuitry for auditory processing and vocal learning is being established [25]. Similarly, the accurate formation of ocular dominance columns in visual cortex depends on patterns of visual experience during a critical period of development when neurons in the optic tectum are finding their targets in the cerebral cortex [26]. This latter observation was, in fact, the first clear

evidence that the brain retains the effects of early experience well into adulthood through permanently altered wiring.

Neural pathways necessary for many instinctive behaviors and physiological functions are formed primarily during the perinatal period under the influence of what appear to be activity independent developmental mechanisms. The most memorable example of this phenomenon is the behavioral imprinting described by Konrad Lorenz in which early experience has a lasting effect on expression of certain behaviors [156]. The term metabolic imprinting refers to the biological impact of various events that occur early in life and exert long-term effects on metabolism. We understand very little about the molecular mechanisms underlying metabolic imprinting, but it is clear that when an individual is confronted with environmental conditions that differ markedly from those present during perinatal development, disaster can ensue. Both caloric restriction and overfeeding appear to cause lasting perturbations in energy balance, yet the underlying mechanisms remain unclear. One consistent observation, however, is that the timing of nutritional changes affects the outcome [27–29]. In humans, the development of hypothalamic feeding circuits occurs primarily in utero, under the direct influence of the maternal environment, while in rodents the majority of this development occurs during the first postnatal weeks, under the influence of preweaning nutritional cues.

Signals communicating environmental nutrient availability to the CNS

It is well established that many circulating hormones represent important environmental signals that act directly on the central nervous system to regulate its development and activity. In adults, hormones that regulate adiposity are secreted in the periphery and regulate the activity of brain circuits that control food intake. The best characterized hormonal signals of adiposity are insulin and leptin. Insulin is secreted by b-cells in the pancreas to promote energy storage. Increased circulating insulin is observed in response to nutrient repletion and in states of obesity. Insulin receptors are expressed centrally [30] and injections of small amounts of insulin into the brains of insulin-deficient animals eliminate their hyperphagia [31]. Deletion of the insulin receptor from the CNS results in obesity, further illustrating the importance of insulin action in the brain. Importantly, insulin levels are elevated and known to mediate compensatory responses such as macrosomia in the offspring of diabetic mothers [32]. Leptin is secreted by fat cells as a crucial signal of body energy stores and acts to blunt feeding behaviors and to permit energy expenditure through a variety of neural and endocrine outputs, such as alterations in thyroid hormone secretion or regulation of the autonomic nervous system. The functional “long” form of the leptin receptor (LRb) is highly expressed in regions of the hypothalamus involved in energy balance (see below for more details), and leptin acts directly on the CNS to mediate most of its action [33–35]. Data from a variety of sources suggests that leptin levels are regulated by perinatal nutrition, and that perinatal leptin levels influence the development of CNS feeding circuits. Thus, in contrast to micronutrients such as glucose, which fluctuates very little in the circulation, hormones such as leptin and insulin are well-suited for communication of environmental nutrient availability to the CNS during development. As noted above, there is an abundance of data from both rodent models and humans suggesting that nutritional status during early development programs the later metabolic fate of the organism. Insulin and leptin represent likely hormonal mediators of the environmental nutrient sensing apparatus that directs this programming. Indeed, insulin has a well-described role in mediating the macrosomic response to maternal diabetes [36–38], and several recent studies have defined a role for leptin in the developmental programming of hypothalamic feeding circuits.

Hypothalamic Neural Pathways Regulating Feeding and Energy Balance

The hypothalamus plays a critical role in the regulation of feeding and recent work has defined a core circuitry in the hypothalamus that appears to mediate many of the effects of leptin on

feeding and energy balance. For descriptive purposes, the hypothalamus can be divided into 3 longitudinal zones, yet each of these zones is functionally distinct. The periventricular zone contains most of the neurons that regulate hormone secretion from the anterior pituitary, and the medial zone contains nuclei that participate in neural networks that function as pattern generators controlling a variety of behaviors. The medial most part of the medial zone, and adjacent periventricular zone, contain most of the neurons that control autonomic outflow from the hypothalamus, while the lateral zone is involved primarily with arousal, behavioral motivation, and sensory integration (see ([39];[40];[41]). Each of these zones appears to play a distinct role in the regulation of body weight, and each zone is differentially innervated by descending cortical inputs [23,42] and ascending visceral sensory inputs [43]. The effectiveness of leptin in regulating energy stores is due to its direct access to hypothalamic neurons that control feeding behavior and other aspects of energy metabolism (see [33,34, 44–47] for reviews). Distinct subsets of hypothalamic neurons may respond to leptin by virtue of their location in a region where the blood-brain barrier is compromised, or through connections with circumventricular organs. The arcuate nucleus of the hypothalamus (ARH) fulfills both of these criteria since it resides above the median eminence and shares connections with regions such as the subfornical organ and vascular organ of the lamina terminalis.

The ARH has long been associated with obesity [48], expresses high levels of leptin receptors [49–51], and has high densities of neurons that express Fos protein in response to intravenous injections of leptin [52,53]. Moreover, recent genetic studies have demonstrated the importance of leptin receptor signaling, especially in the ARH [54–56]. The ARH lies in the periventricular zone of the hypothalamus, begins rostral to the infundibular recess, and ends just behind the median eminence. Two distinct cytoarchitectonic subdivisions of the ARH are generally recognized: a small-celled dorsomedial part and larger ventrolateral part that contains medium-sized neurons. The projections of the ARH have been studied in detail only recently [57] and the strongest inputs are to other sites implicated in the control of feeding such as the paraventricular and dorsomedial hypothalamic nuclei. These regions also show leptin induced increases in Fos expression, suggesting that the arcuate nucleus is a principal site of action for the central regulatory effects of leptin. In general, the projections of the ARH are largely confined to the periventricular zone, but notably avoid the suprachiasmatic nucleus, with the densest terminal fields found in many of the regions that supply strong afferents to the ARH (see below). ARH projections are largely intrahypothalamic with the greatest density of fibers and terminals restricted to the periventricular zone of the hypothalamus. Thus, ARH neurons provide dense inputs to the anteroventral periventricular nucleus, all parts of the periventricular nucleus, and to parvicellular parts of the paraventricular nucleus. The supraoptic nucleus also appears to receive a direct input. In addition to the periventricular nuclei, ascending projections appear to innervate the region surrounding the vascular organ of the lamina terminalis and they extend dorsally to terminate in the ventral part of the lateral septal nucleus. A significant projection appears to extend laterally through the DMH to end in the perifornical region, which has also been implicated in the neural control of feeding. Surprisingly, only a few projections extend from the ARH into the midbrain or medulla, but the periaqueductal gray, locus coeruleus and nucleus of the solitary tract, all appear to receive at least a few inputs.

The ARH contains two populations of neurons that play a particularly important role in distributing leptin signals centrally. Neuropeptide Y (NPY) and agouti-related peptide (AgRP) are coexpressed within a subpopulation of arcuate neurons, and a separate population of neurons in the ARH contains melanocortin peptides, such as alpha (αMSH), which are derived from proproopiomelanocortin (POMC). These anatomically distinct populations of ARH neurons provide overlapping projections to other key parts of the hypothalamus and appear to exert opposing regulatory functions: NPY and AgRP are orexigenic, while melanocortins are anorexigenic. Moreover, local circuits link these two populations of ARH neurons to provide an additional level of coordination and integration [58]. Insulin and the gut peptide ghrelin also

regulate the activity of ARH neurons [59–61], and circulating free fatty acids have been reported to influence ARH neurons [62], indicating that multiple peripheral signals related to energy balance may be integrated at the level of the ARH.

The paraventricular nucleus of the hypothalamus (PVH) is the most thoroughly characterized hypothalamic interface between the endocrine, autonomic and somatomotor systems that influence ingestive behavior and energy metabolism [34,44,63]. Of fundamental importance to the function of the PVH are its parvicellular neurons that regulate the pattern of ACTH and thyroid-stimulating hormone secretion into the hypophyseal-portal circulation. These neural pathways are, in turn, regulated by unique combinations of both neural and humoral afferent information. Other parts of the PVH contain neurons that innervate preganglionic parasympathetic and sympathetic neurons in the medulla and spinal cord, which provides a direct route for regulation of autonomic responses associated with energy homeostasis [63, 64]. Thus, the organization of PVH connections is well suited for integration of diverse signals that influence neuroendocrine control of energy metabolism. Lesions of the PVH produce obesity [65] and injections of the orexigenic peptide NPY directly into the region of the PVH increase feeding [66]. Intraventricular injections of leptin induce Fosimmunoreactivity in the dorsal and ventral parvicellular parts of the PVH [67], which provide inputs to autonomic neurons. However, this cellular activation may be due to transneuronal relay of leptin signals acting on the ARH and dorsomedial nucleus of the hypothalamus (see below).

Injections of retrograde tracers into the PVH label neurons in both the ARH and DMH that can be doubly labeled for Fos induced by intravenous injections of leptin, confirming that these cells may relay leptin signals to the PVH directly [68]. Lesions of the DMH cause changes in feeding activity [69];[70], and it contains a high density of neurons that express leptin receptors [71]. The ventral premammillary nucleus (PMv) also contains a high density of neurons that respond to leptin injections and it contains one of the highest densities of neurons that express leptin receptors [68]. The projections of the PMv are remarkable in that they largely overlap with those of the ARH, with equally dense inputs to the PVH and DMH, but the PMv innervates the ventrolateral (as opposed to dorsomedial) part of the VMH. The PMv is unique among hypothalamic nuclei in that it provides strong efferent projections to limbic regions such as the medial nucleus of the amygdala and ventral part of the lateral septal nucleus [72] Together, this anatomical evidence suggests that neurons responding to leptin in the ARH, DMH and PMv may converge onto neurons in the PVH. As in most parts of the CNS, amino acid neurotransmitters largely determine the activity of PVH neurons. Thus, the relative innervation of individual PVH neurons by excitatory glutamatergic neurons and inhibitory g-amino butyric acid (GABA) neurons establishes major aspects of neuronal response properties. PVH neurons receive both glutamatergic and GABAergic innervation from the ARH. The majority of NPY/AgRP neurons contain GABA, whereas POMC neurons appear to signal primarily via glutamate [73,74]. Therefore, synaptic rearrangements of terminals from these peptidergic neurons may also be reflected in synaptic densities of excitatory and inhibitory synapses on individual PVH neurons. Because of the functional compartmentalization of the PVH, changes in ratios of excitatory and inhibitory synapses within these compartments would have a profound effect on the functional output of the PVH.

The ventromedial nucleus of the hypothalamus (VMH) was one of the earliest sites in the hypothalamus thought to be involved in the regulation of body weight. The VMH consists of 3 distinct divisions, each with a distinct pattern of projections and inputs. The majority of neurons activated by peripheral leptin injections are localized to its dorsomedial part, yet these neurons do not appear to project to the ARH, DMH nor PVH, so these leptin sensitive neurons may represent a parallel set of pathways for transmitting leptin dependent information to other parts of the hypothalamus. The dorsomedial part of the VMH innervates several regions not innervated by the ARH such as the anterior hypothalamic nucleus, subaraventricular zone,

lateral parts of the bed nuclei of the stria terminalis, and dorsal parts of the periaqueductal gray. Each of these regions play important roles in expression of goal oriented behaviors so it is likely that leptin sensitive VMH neurons play a role in leptin's ability to regulate food intake. Consistent with this circuit architecture, virally mediated reexpression of leptin receptors in the VMH of a genetically engineered LRb null mouse has a much more significant impact on food intake than LRb rescue in the ARH [55]; [56]. Whether a similar molecular genetic manipulation of LRb expression in the DMH would also alter food intake and body weight has not been demonstrated, but would be consistent with the widespread projections of DMH neurons to a variety of hypothalamic nuclei involved in the regulation of energy metabolism [68];[75].

Although most regions of the periventricular zone of the hypothalamus do not provide significant inputs to the lateral hypothalamic area (LHA), the ARH is an exception and sends direct inputs to peptidergic neurons in the LHA that are thought to play an important role in the regulation of food intake [76].Two of the best characterized populations of LHA neurons, the orexin (a.k.a hypocretin) and melanin concentrating hormone (MCH) containing neurons, have partially overlapping distributions in the caudal half of the LHA [77], and both stimulate feeding [78]; [79]; [35]; [80]. Treatment of animals with exogenous leptin causes induction of Fos in orexin neurons [81], as well as increases in STAT3 phosphorylation in the lateral hypothalamus [82]. In addition, both orexin and MCH neurons have projections to components of the mesolimbic dopaminergic motivational circuitry, which has recently been shown to be directly regulated by leptin [83,84].

Afferent connections of the ARH have not been mapped in detail, but the strongest inputs appear to be from other parts of the periventricular zone of the hypothalamus, including the PVH, as well as from medial parts of the medial zone, such as the medial subdivision of the medial preoptic nucleus and PMv [72,85–88]. Although the VMH does not appear to provide many direct inputs to ARH neurons [89], a recent study used a sophisticated laser scanning photostimulation method to identify neurons in the ARH that appear to be synaptically activated by VMH neurons [90]. Interestingly, most of the active VMH neurons were located very near the border of the ARH and showed differential patterns of innervation to NPY and POMC containing neurons, suggesting that at least a few neurons in the VMH may influence the activity of NPY and POMC neurons differentially.

Extrahypothalamic projections of the ARH are sparse, but the ARH receives strong inputs from the principal nucleus of the BST and posterodorsal part of the medial nucleus of the amygdala [91–93], as well as from the ventral part of the lateral septal nucleus [94]. The ARH also receives substantial inputs from several brainstem sites thought to convey ascending visceral sensory information [39,95–97]. Regions such as the NTS contain neurons that respond to leptin, so these ascending pathways may represent additional routes for leptin-sensitive information to reach the PVH [98]. The long form of the leptin receptor, LRb, is expressed in abundance by ARH neurons [49–51]. As stated above, injections of leptin activate neurons in the ARH, but also acts on neurons in the DMH, VMH, PMv and PVH, as evidenced by induction of Fos immunoreactivity, SOCS3 mRNA and STAT3 phosphorylation. Although expression of Fos in hypothalamic neurons may represent direct activation by leptin, it may also be due to transynaptic neuronal activation. However, induction of STAT3 phosphorylation by leptin is lacking in db/db mice [99], so it is generally believed to be a reliable marker of LRb activation [82]. Accordingly, the distribution of neurons with detectable levels of phosphoSTAT3 is largely confined to the ARH, DMH, PMv, NTS and scattered groups of neurons in the LHA.

Development of leptin-sensitive circuits

The hypothalamus develops from the diencephalic vesicle below the hypothalamic sulcus and is discernable in the 5 vessicle stage embryo, which corresponds approximately to the 12th embryonic day (E12) in the rat [100]. Development of hypothalamic nuclei begins with neurogenesis in the neuroepithelium of the third ventricle, which involves generation of neuronal progenitor cells that ultimately produce postmitotic neurons. The postmitotic neurons leave the neuroepithelium and migrate to take up residence in various parts of the hypothalamus. In contrast to cortical structures, the hypothalamus follows an "outside-in" pattern of neurogenesis with more lateral regions forming earliest, followed by the more medial structures. Based on neuronal birth-dating studies in rats, the highest rate of neurogeneis in the lateral zone occurs between E12–E13, whereas the peak for the medial zone nuclei occurs at E14–E15, followed by the periventriclar zone which peaks at E16–E17.[100,101] Parvicellular neurosecretory neurons (e.g. neurons that express hypothalamic releasing factors such as CRH and TRH), located primarily in the periventricular zone, appear to be an exception to this rule and are largely generated between E12–E14. In addition, these neurons often show nuclei specific developmental gradients[102]. For example, neurosecretory neurons in the PVH display a dorsal to ventral gradient of neurogenesis, whereas neurons that express growth hormone releasing hormone in the ARH develop along a rostral to caudal gradient. Neurons located in key nuclei known to contain leptin-sensitive neurons follow the overall pattern for neurogenesis in the hypothalamus. Neurons located in the dorsal part of the VMH, or in the DMH, are born between E14–E17 with a peak at E15. Neurons in the ventrolateral part of the VMH tend to be born earlier (E12–E15 with a peak at E13), as are neurons of the PMv. The ARH shows an unusually long neurogenetic period. Some neurons are born as early as E12, which is unusual for a periventricular nucleus, but many are born as late as E17. However, this observation may be due to the common error of including the posterior part of the periventricular nucleus as part of the arcuate nucleus (see [24]). Only limited information is available about when particular cell types are born in the ARH. A double labeling study determined that most POMC neurons are born relatively early, between E12–13. The peak period of neurogenesis in hypothalamic nuclei may be slightly different for mice. For example, most neurons in the VMH and ARH of mice are born between E11–E14 [103]. In rats, the peak period of neurogenesis in the ARH is E15, as it is for the DMH and dorsomedial part of the VMH. Whether the leptin responsive neurons in each of these 3 cell groups are born at the same time is unknown, but it is curious that they share the same peak of neurogenisis. Equally interesting is determining when these neurons acquire sensitivity to leptin. In many developing neural systems neuronal fate is specified to some extent while the cells remain in the neuroepithelium [104]. Thus, a subpopulation of neurons may express leptin receptors shortly after they become post-mitotic, which may influence their neurobiological responses to leptin so as to impact migration and development of functional connections. The extent to which hormonal factors or neurotophins influence neurogenesis during development of leptin-sensitive hypothalamic circuits is unknown, but a recent report that CNTF can induce neurogenesis in the ARH of adult mice [105] suggests further study is warranted.

The development of neural projections between various parts of the hypothalamus has only recently been addressed with axonal labeling. Noradrenergic inputs from the brainstem to key parts of the hypothalamus such as the PVH and VMH are present at birth and continue to increase in density through the first 3 postnatal weeks [43]. Inputs specifically from the nucleus of the solitary tract do not appear to reach the PVH until postnatal day 6 (P6). The immaturity of visceral sensory innervation of the hypothalamus in neonates is reflected in the diminished densities of Fos labeling induced by visceral stimulation and suggests that hypothalamic neurons are less sensitive to viscerosensory signals than are adults [106].

Much of what we know about development of neural pathways in the hypothalamus has been inferred from patterns of immunohistochemically stained peptidergic axons (see [152,153, 154] [107] for reviews). The analysis of neuropeptide expression during development has indeed been informative, but the results must be considered within the context of state-dependent alterations in cellular levels of neuropeptide expression and cellular localization, which can influence interpretation. For example, NPY is transiently expressed in several hypothalamic nuclei during postnatal development, but the contribution of these NPY containing neurons to densities of NPY immunoreactive fibers in various regions has not been determined [108]. Moreover, aberrant expression of neuropeptides has been observed in various molecular genetic experiments, which may complicate interpretation of molecular events regulating development of connectivity [109]. Thus, it is not currently possible to determine if local changes in the density of neuropeptide immunoreactivity are due to alterations in the density of axon terminals, which reflect a true change in the organization of neural circuitry, or simply reflect alterations in neuropeptide synthesis and transport, or changes in local processing and release [110]. Nevertheless, changes in local levels of neuropeptides can be most informative about circuit regulation. This is especially true for AgRP, which in adults appears to be exclusively expressed in NPY neurons of the ARH [108,111]. Its utility as a marker in developing animals is more doubtful as significant densities of AgRP immunoreactive fibers have been observed in hypothalamic regions prior to their innervation by ARH neurons (Bouret S., personnal communication). Given the remarkable plasticity of peptidergic neurons during development, considerable effort will be required to establish patterns of neurotransmitter specific connectivity in the developing hypothalamus.

Development of ARH projections

It is remarkable how little information is available about development of hypothalamic pathways. Although a variety of axonal labeling methods have been available for studying development of neural connections, they have only recently been applied to the hypothalamus. An initial study used DiI to label the projections of the ARH in the mouse. The surprising finding is that the projections of this important neuroendocrine nucleus remain immature until well after birth, with the full pattern of projections not represented until the end of the second week of life [112]. Axons from ARH neurons extend rostally and remain largely confined to the periventricular zone and appear to innervate several key components of feeding circuitry during discrete temporal domains. The DMH is innervated relatively early (P6), followed by the anterior part of the periventricular nucleus, and then ascending fibers finally reach the PVH (P10). A subset of ARH projections arch laterally from the periventricular zone, through the DMH, and end in the LHA near the end of the second postnatal week. The overall pattern of ARH projections does not achieve a distribution resembling that of adult mice until nearly the end of the third postnatal week, and no evidence of regressive events has been reported [112]. Thus, the ARH does not appear to provide exuberant projectons to inappropriate targets that are then restricted through axon retraction later in development. Rather, the ARH axons appear to achieve their targets through a directed mechanism. This retarded development of ARH projections appears to be unusual among hypothalamic nuclei. Projections from the DMH to the PVH and LHA appear to be largely mature by P6, and VMH neurons provide widely distributed and robust projections to the LHA by P10 [112].

The neurochemical identity of ARH projections to regions involved in regulating feeding has not been established, but anterograde multiple labeling studies show that AgRP and POMC-derived peptides are expressed in ARH neurons that project to the DMH, PVH and LHA in both rats and mice [113][114]. AgRP-immunoreactive terminals are observed in neonatal rats in a developmental pattern that generally matches the development of neural projections from the ARH [108] suggesting that the ability of NPY-containing neurons to regulate the activity of neurons in the DMH and PVH matures along a similar time course. The sequencial

innervation of hypothalamic targets by ARH neurons suggests that the ability of leptin to regulate hypothalamic function may not be mature in neonatal mice. Indeed, leptin treatment has minimal effects on food intake in neonates [115]; [116]. In adult mice, peripheral injections of leptin cause marked increases in cfos expression, however, in neonatal mice, leptin does not induce Fos immunostaining in large numbers of neurons in the PVH and LHA until after innervation by the ARH has occurred [112]. Both the ARH and DMH contain neurons that express leptin receptors and a substantial number of these cells appear to be directly activated by leptin in neonatal mice. In contrast, most of the leptin dependent activation of neurons in the PVH seems to be transynaptic and dependent on maturation of ARH projections. Thus, developmental perturbations that disrupt development of ARH projections may contribute to leptin resistance at the level of the PVH.

Despite evidence from a variety of sources that neonatal nutrition and maternal factors have long term effects on obesity [28,29,117–119], we know relatively little about how the neonatal environment influences central mechanisms regulating food intake and energy balance. Although many of the CNS feeding circuits appear to develop *in utero* in humans and other primates [16], these circuits develop during the first postnatal weeks in rodents, facilitating the analysis of their development in rodent models. During gestational diabetes, or in overnourished neonatal rodents, there are significant perturbations in glucose homeostasis that may impact brain development. The increased availability of nutrients in these nutritional states increase circulating fetal/neonatal levels of insulin and leptin. The elevations in leptin that occur in these conditions can have lasting effects on body weight regulation later in life that correlate with changes in neuropeptide expression in the ARH (see [120]; [16] for reviews). Moreover, similar physiological perturbations are observed when insulin is applied directly to the region of the mediobasal hypothalamus during the critical period for development of projections from the ARH to the PVH [121]. Thus, it is clear that insulin acts on the brain to effect long term changes in body weight regulation. What is considerably less clear is how insulin signaling brings about changes in the organization and function of the hypothalamic circuitry that presumably underlie the observed physiological changes. Of note is the observation that ARH neurons of rats overfed during postnatal life display altered electrophysiological properties [11] suggesting direct involvement of ARH neurons in the physiological defects brought about by neonatal overfeeding and the accompanying hyperinsulinemia. Previous reports that insulin can exert neurotrophic effects on hypothalamic neurons [122] and can induce cellular differentiation of Y79 cells (originally derived from retinoblastoma cells) to acquire neuronal-like properties [123] provide additional support for the notion that insulin functions in hypothalamic development, and may provide a signaling mechanism for translating environmental perturbations into alterations in brain structure.

Developmental Neurobiology of Leptin

The discovery of leptin and its activity in regulating specific components of the central nervous system reaffirmed the primacy of the brain in regulating metabolism. An appreciation of the role played by the brain in developmental programming has been slower to emerge, but gained momentum as a result of recent studies of leptin neuroendocrinology in mice. A variety of observations in leptin deficient ob/ob mice suggest that leptin may influence brain development [124]; [125]. During the first week of life, body weights of Lep^{ob}/Lep^{ob} mice do not differ significantly from those of wild-type littermates, but they begin to diverge during the second week of life, and treatment of neonatal Lep^{ob}/Lep^{ob} mice with exogenous leptin does not alter milk intake or metabolic rates until after weaning [115]. The remarkable observation that there is a dramatic surge in circulating leptin levels during this period of apparent leptin insensitivity led Ahima and colleagues to suggest that leptin functions as a developmental cue for brain development [124]. That leptin-deficient Lep^{ob}/Lep^{ob} and LRb-deficient db/db mice have reduced brain weight and morphological defects supports this notion [126–131]. A more recent

report indicates that treatment of neonatal rats with leptin alters expression of hypothalamic neuropeptides in the ARH [116]. Both the PVH and ARH are known to play important roles in mediating central stress responses and leptin appears to influence glucocorticoid feedback on the hypothalamus in neonatal rodents [132], which may represent an indirect mechanism through which leptin influences brain development [133,134].

The existence of a discrete leptin surge between P4 and P16 in mice, which is independent of fat mass or food intake (unlike the situation in adults), raises the possibility that this adipocyte-derived hormone might regulate the establishment and patterning of ARH projections. The results of experiments carried out recently in leptin deficient (Lep^{ob}/ Lep^{ob}) mice provide compelling evidence that leptin promotes development of ARH projections through a direct action on the brain [135]. The density of projections from ARH neurons to other hypothalamic sites involved in control of food intake are severely disrupted in postnatal Lep^{ob}/Lep^{ob} mice and remain diminished throughout life [135]. Moreover, ARH projections appear to be uniformally impaired in Lep^{ob}/Lep^{ob} mice since labeling for ARH axons was reduced in all ARH targets, not just in the PVH, DMH and LHA. Both NPY/AgRP and POMC containing neurons appear to be affected, but it is unclear if they are reduced to the same extent, or are differentially altered so as to imbalance the impact of these two opposing neural systems. As with most important developmental factors, leptin appears to act primarily during a restricted postnatal critical period and this developmental window coincides with the naturally occurring surge in leptin. Treatment of adult Lep^{ob}/ Lep^{ob} mice with leptin did not restore ARH projections to the PVH, but daily injections of leptin between P4 and P12 rescued innervation of the PVH by ARH axons. The precise limits of this period of maximal sensitivity to the developmental actions of leptin are yet to be defined, and it remains possible that prenatal exposure to leptin may influence development of ARH projections. However, projections from the DMH to the PVH develop prior to the leptin surge and do not appear to be impaired in Lep^{ob}/ Lep^{ob} mice, despite abundant expression of LRb in DMH neurons [135]. Similarly, the integrity of a limbic-hypothalamic pathway that lacks significant leptin receptor expression was also normal indicating that at least during neonatal life, Lep^{ob}/Lep^{ob} mice do not have widespread defects in neural connectivity. The projection from the principal nucleus of the bed nuclei of the stria terminalis (BSTp) to the anteroventral periventricular nucleus of the hypothalamus (AVPV) is sexually dimorphic in rats and mice and is mature by P14 [136]. This pathway appears to be unaffected by leptin deficiency since the density of DiI labeled fibers in the AVPV was similar in Lep^{ob}/Lep^{ob} and wild type mice that had comparable implants of DiI in the BSTp. Taken together, these observations suggest that neonatal leptin deficiency does not lead to widespread disruption of hypothalamic circuitry, but may specifically affect development of the ARH. Hypothalamic circuits remain relatively plastic throughout life and a significant action that leptin shares with neurotrophins is the ability to cause synaptic rearrangements. Treatment of Lep^{ob}/ Lep^{ob} mice with leptin rapidly alters the ratio of glutamatergic and GABAergic synapses onto NPY and POMC neurons in the ARH, which may contribute to differential modulation of the activity of these neurons by leptin [137,138]. Collectively, the recent findings on leptin and brain development have renewed interest in the role of perinatal factors as major contributors to obesity.

The cellular mechanisms mediating the developmental actions of leptin on brain development are unknown. Direct application of leptin to organotypic explant cultures of the ARH in vitro cause a profound proliferation of neurites extending out from the explants suggesting a direct site of action [135]. This observation is consistent with the global impairment of ARH projections observed in Lep^{ob}/Lep^{ob} mice. While it remains possible that leptin may function in a target-dependent manner, perhaps by inducing release of diffusible guidance factors from sites such as the PVH or DMH, supportive evidence is lacking for this intriguing notion. Equally unexplored is the possible role of contact-mediated guidance factors that may guide and stabalize ARH axons as they project through the periventricular zone of the hypothalamus.

Based on an abundance of both pharmacological and molecular genetic studies in adult animals, a great deal has been learned about how leptin receptors signal in a variety of tissues (see [139] [140] [141] for reviews). Leptin receptors signal in neurons through the action of intracellular kinases. Binding of leptin to the LRb initiates signaling events mediated by Jak2 resulting in the phosphorylation and transcriptional activation of the latent transcription factor, signal transducer and activator of transcription-3 (STAT3). In addition to phosphorylation of STAT3, which appears to be the major mediator of leptin signaling in adult neurons, LRb activation promotes phosphorylation of the extracellular signaling kinase (ERK), as well as activation of PI3K signaling pathways. Leptin receptors are expressed in neonatal mice and are capable of inducing phosphorylation of STAT3, ERK and the PI3K effector Akt (S. Bouret and R. Simerly, unpublished observations). However, how these signals are linked to cellular mechanisms controlling axon outgrowth and targeting remains unknown. Leptin influences synaptic plasticity in the hippocampus. Exposure of hippocampal neurons in vitro to leptin increases synapse density and influences synaptic function [142]. Interestingly, leptin also alters growth cone morphology of cortical neurons in vitro, an event that appears to involve multiple convergent signaling pathways [143]. Convergent signaling between leptin receptors and other hormone signaling pathways should also be explored. For example, the neurobiological actions of leptin share certain similarities with those of estrogen (see [144] for review) and estrogen can alter leptin signaling events [145], as well as body weight [146].

Based on current information, it seems clear that perinatal nutrition has a profound effect on metabolic phenotype. An important priority in the coming years will be to identify signaling pathways for physiological signals that link nutritional status and developmental events impacting energy balance. A particularly promising avenue of research is to define epigenetic mechanisms that alter gene expression in response to changes in nutritional environments during critical developmental periods [147]. Central sensitivity to stress hormones appears to be permanantly altered by neonatal events through epigenetic regulation of glucocorticoid receptor gene expression [133];[148]. Changes in endocrine profiles permanently alter the structural organization and functional activity of the central nervous system represent an equally powerful mechanism for effecting long term changes in metabolic regulation in response to neonatal events. In addition to leptin, adrenal steroids may play a role in specifying the organization and sensitivity of a variety of neural pathways controlling energy balance [149] and possible interactions between the developmental actions of these hormone systems need to be defined. Finally, the impact of maternal nutrition and metabolic health on the physiology of pregnancy represents an especially important period of developmental sensitivity that may influence not only prenatal development, but may also affect the impact of postnatal nutrition on brain development. Even though we are just beginning to appreciate the importance of brain development in metabolic dysfunction, it is reasonable to expect that by gaining a better understanding of how developmental events influence the architecture of neural systems that regulate metabolism we may identify new therapeutic approaches for reducing obesity and type 2 diabetes in pediatric populations.

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