Mini-Symposium

Molecular Pathways Controlling Development of Thalamus and Hypothalamus: From Neural Specification to Circuit Formation

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The embryonic diencephalon gives rise to the vertebrate thalamus and hypothalamus, which play essential roles in sensory information processing and control of physiological homeostasis and behavior, respectively. In this review, we present new steps toward characterizing the molecular pathways that control development of these structures, based on findings in a variety of model organisms. We highlight advances in understanding how early regional patterning is orchestrated through the action of secreted signaling molecules such as Sonic hedgehog and fibroblast growth factors. We address the role of individual transcription factors in control of the regional identity and neural differentiation within the developing diencephalon, emphasizing the contribution of recent large-scale gene expression studies in providing an extensive catalog of candidate regulators of hypothalamic neural cell fate specification. Finally, we evaluate the molecular mechanisms involved in the experience-dependent development of both thalamo-cortical and hypothalamic neural circuitry.

Introduction

The developing vertebrate forebrain consists of two major parts: the telencephalon—which gives rise to cerebral cortex, striatum, amygdala, and associated structures—and the diencephalon. The diencephalon gives rise to two essential brain regions, the thalamus and hypothalamus (Fig. 1*A*). Though both structures are derived from a common region of the anterior neural tube, they serve very different physiological functions. The thalamus acts as a central integrator of sensory information, receiving afferents from receptors of all sensory modalities save olfaction, and serves as the sole path by which integrated sensory information reaches the cerebral cortex and gives rise to conscious perception. The hypothalamus, on the other hand, serves a more diverse range of functions. It is a central regulator of critical homeostatic physiological processes, including temperature regulation, food intake, and circadian rhythms, which it accomplishes in part by acting as the central regulator of the neuroendocrine system. It is also critical in the processing of many different emotional and social behaviors, including mating, fighting, and parental care. Finally, it plays a critical though still little-studied role in memory formation.

Both thalamus and hypothalamus are comprised of a highly diverse collection of cell groups and neuronal subtypes. This anatomical complexity, and the lack of well defined molecular markers that delineate particular cell types, has traditionally held back studies of these structures' development, particularly compared with other CNS regions such as the spinal cord, retina and cerebral cortex. With this in mind, these presentations aim to stimulate interaction between experts in this field and the broader neuroscience community, and encourage other researchers to enter this young and exciting field.

Patterning of developing thalamus and hypothalamus by secreted differentiation factors

Studies of early diencephalic patterning over the last 2 decades characterized a relatively small number of gene sets expressed in specific compartments of the developing diencephalon (Figdor and Stern, 1993; Puelles and Rubenstein, 1993). These gene sets were used to generate the prosomeric model, a representation of the hypothesized morphological boundaries of the developing diencephalon (Puelles and Rubenstein, 2003). The prosomeric

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model identifies three major compartments within the developing diencephalon (p1, p2 and p3, moving posterior to anterior) along the longitudinal axis. A hallmark of this model is the clear boundary that separates p2 and p3, termed the zona limitans intrathalamica (Zli), which includes the mid-diencephalic organizer (MDO) (Scholpp et al., 2009). This structure releases several secreted factors essential for patterning the developing thalamus, including Sonic hedgehog (Shh), and members of the Wnt and fibroblast growth factor (Fgf) family (Fig. 1*A*) (Bulfone et al., 1993).

Selective expression of hedgehog family genes in the MDO of vertebrate model organisms is observed in lamprey (Osorio et al., 2005), zebrafish (Macdonald et al., 1995), frog (Ruiz i Altaba, 1998), chick (Kitamura et al., 1997), and mouse (Kitamura et al., 1997). Experimental abrogation of Shh signaling in zebrafish, chick, and mouse results in loss of genetic fate determinants and cell identity in prethalamus and thalamus (Kiecker and Lumsden, 2004; Scholpp et al., 2006; Vue et al., 2009). Furthermore, ectopic activation of the Shh pathway by misexpression of a constitutively active Shh effector mutant (SmoM2) induces the expression of thalamic markers such as *Gbx2*, *Ngn2*, *Olig2*, and *Olig3* in the mouse pretectum (Vue et al., 2009). These studies showed that Shh induces the expression of two proneural genes within the developing sensory thalamus: a posterior-to-anterior wave of Ngn1 expression in the major caudal thalamus and a narrow stripe of *Ascl1* expression in rostral thalamic cells immediately adjacent to the MDO (Fig. 1*B*,*C*). Blockade of Shh signaling leads to both a substantial decrease in the size of the Ngn1-positive caudal thalamus and the absence of the Ascl1-positive rostral thalamus (Vue et al., 2009).

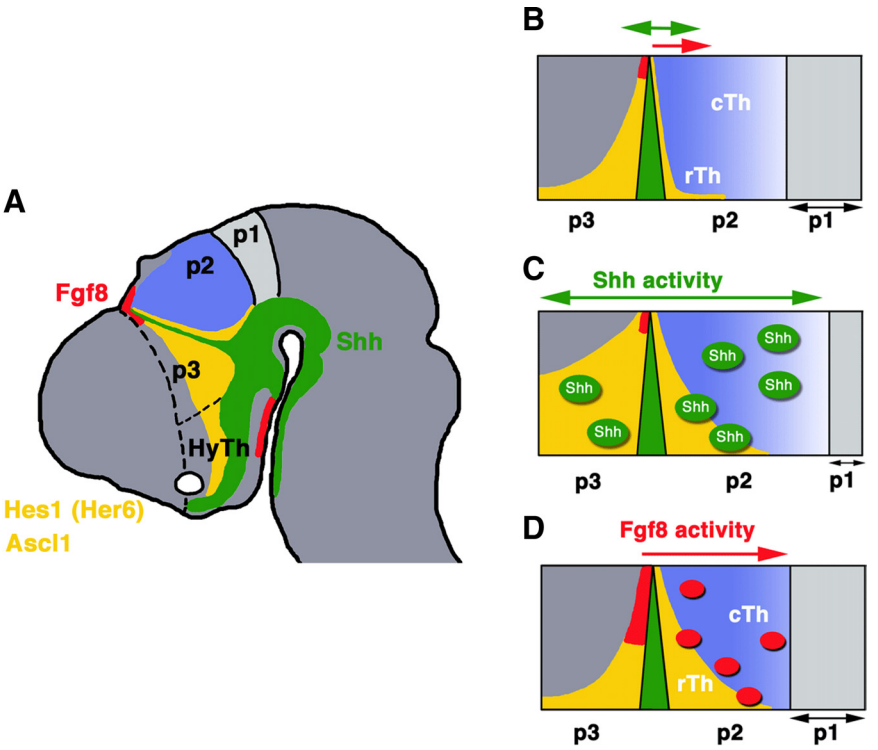

Figure 1. ***A***, Sagittal schematic of mouse embryonic brain at E10.5. The diencephalic neuraxis is divided in p1, p2, p3, and hypothalamus (posterior to anterior). Presumptive borders of the telencephalon and diencephalon, p3, and hypothalamus are shown by dashed lines. Shh expression domains in basal diencephalon and MDO/Zli are indicated in green, Fgf8-expressing region in dorsal diencephalon and hypothalamus are indicated in red, and Hes1 (Her6) and Ascl1 (acc1) are indicated in yellow. ***B***, Schema of detailed subdivision in the developing thalamus. Expression of Shh in MDO (green) divides p2 and p3, and expression of Fgf8 in dorsal diencephalon is detectable only in p3 (red). The p2 region can be further subdivided in two subregions: rostral thalamus [rTh (or the Rim)]; and caudal thalamus (cTh). Shh signaling is detectable both anterior and posterior to the MDO, whereas Fgf signaling is detectable only posterior to this region. ***C***, Overexpression of Shh expands the Hes1 and Ascl1 in both p2 and p3. ***D***, Overexpression of Fgf8 expands the Hes1 and Ascl1 only in p2.

How does Shh signaling selectively induce expression of these neurogenic basic helix-loop-helix genes in the two major divisions of the developing sensory thalamus? Studies in zebrafish have shown that the effect of Shh is controlled by the selector gene *Her6*, a homolog of the mouse *Hes1* gene (Fig. 1*A*). *Her6* is initially broadly expressed throughout thalamic neuroepithelium but is later progressively lost from the caudal thalamus. This creates a dynamic posterior-to-anterior "neurogenetic gradient" of *Ngn1* expression in the thalamus. Subsequently, the remaining anterior *Her6* induces *Ascl1* expression in a stripe of rostral thalamic cells. This zonation of proneural gene expression is followed by posterior-to-anterior differentiation of glutamatergic relay neurons from the *Ngn1*-positive precursors in the caudal relay thalamus and of GABAergic inhibitory neurons from the *Ascl1*-expressing precursors in the reticular nucleus of the rostral thalamus. Therefore, the dynamic expression of *Her6* defines neurotransmitter fates within the thalamus (Scholpp et al., 2009), and release of Shh determines the time point at which proneural gene expression, and subsequent neuronal differentiation, initiates within the developing thalamus.

In parallel to the Shh pathway, Fgf8 expression anterior to the MDO controls the anterior and posterior identity of thalamic nuclei (Kataoka and Shimogori, 2008). Using *in utero* electroporation to overexpress or inhibit endogenous Fgf8 in the mouse MDO, differentiation of two distinct progenitor populations in p2, marked respectively by *Ngn2* and *Ascl1* expression, were found to be regulated by Fgf signaling in a complementary manner (Fig. 1*D*). Furthermore, increased FGF activity shifts thalamic sensory nuclei posteriorly in postnatal mouse thalamus, which suggests that Fgf8 originating anterior to the MDO controls pattern formation in the region posterior to the MDO. However, the precise molecular mechanism by which Fgf8 controls diencephalic pattern and how Shh and FGF8 interact to correctly pattern the nascent thalamus has not yet been established.

Classical signaling factors are also expressed in spatially restricted fashions in the developing hypothalamus and play an essential role in its development and patterning (Fig. 1*A*). A Wnt activity gradient, from posterior to anterior, appears to govern the early acquisition of ventral hypothalamic identity: zebrafish embryos mutant for *Axin1*, an intracellular negative regulator of Wnt pathway activity, show a reduction of prospective ventral hypothalamic tissue and concomitant expansion of floor plate. Similarly, a reduction in Wnt signaling in the chick promotes ventral hypothalamic fate at the expense of floor plate. Wnt signaling also governs regionalization of the nascent hypothalamus, directing the emergence of posterior (mamillary) hypothalamic fates (Kapsimali et al., 2004; Manning et al., 2006). Although most Wnt ligands and components are restricted to the posterior

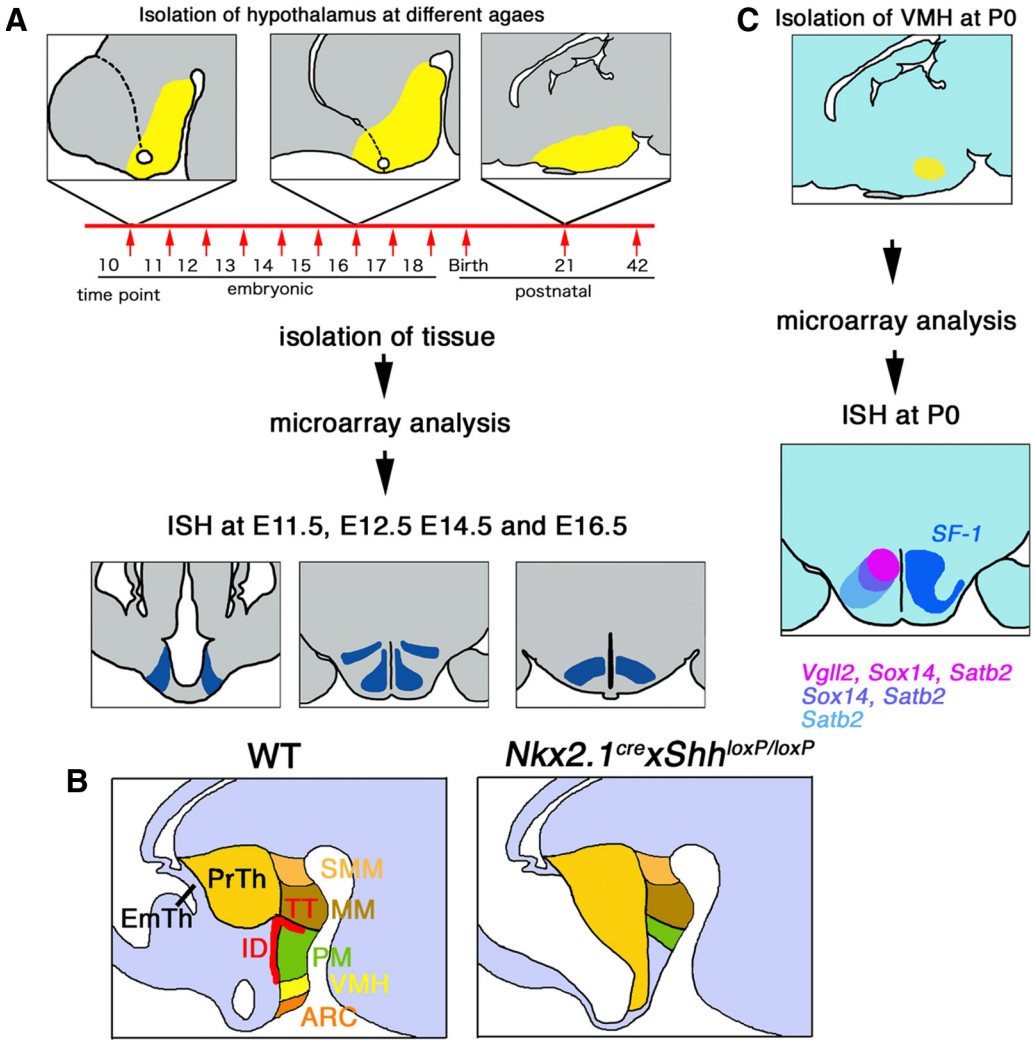

Figure 2. ***A***, Combined use of microarray-based expression profiling and large-scale *in situ* hybridization for the construction of the genomic atlas of mouse hypothalamic development. ***B***, Developmental phenotype of deletion of *Shh* from hypothalamic basal plate determined using regional markers identified in ***A***. PrTh, prethalamus (yellow, anterior; and green, posterior), EmTh, eminentia thalami; SMM, supramamillary nucleus; MM, mamillary nucleus; PM, premamillary nucleus; TT, tuberomamillary terminal. ***C***, Schematic for expression profiling of developing VMH in neonatal mice. Subdomains of VMH expressing different combinations of transcripts are indicated.

hypothalamus throughout development, *Shh* and multiple different bone morphogenetic proteins (BMPs) show dynamic spatio-temporal profiles. Expression of *Shh* and Shh signaling components is negatively regulated by BMP signaling (Manning et al., 2006; Ohyama et al., 2008). Studies in the limb bud show that cross talk between the BMP and Shh pathways is critical for integrated growth, pattern, and differentiation (Bénazet et al., 2009), and raises the possibility that a similar cross talk between BMP and Shh signaling regulates proliferation and spatial patterning of hypothalamic neural progenitors in a concerted manner. In support of this hypothesis, Shh is critical for the proliferation and patterning of anterior hypothalamic neural progenitors (Dale et al., 1997; Manning et al., 2006; Szabó et al., 2009; Shimogori et al., 2010), whereas BMP7 signaling regulates the proliferation and spatial identity of posteroventral hypothalamic progenitors. Forced maintenance of Shh and Shh signaling components abolishes posterior identities, defined through expression of the transcription factor *Emx2* (Manning et al., 2006). The BMP-mediated downregulation of Shh expression leads to expression of Fgf family members *Fgf8* and *Fgf10*, showing strong expression in the ventral hypothalamic floor following downregulation of Shh expression. Current studies are addressing the potential role of FGF signaling from the hypothalamic floor, demonstrating that it regulates the proliferation of progenitors in the mid (tuberal)-hypothalamus (Manning et al., 2006). Intriguingly, the spatially restricted expression of FGF and Shh along the hypothalamic dorsoventral axis not only persists throughout embryogenesis but is maintained into adulthood, suggesting that their activities might control progenitor cell proliferation in the adult hypothalamus.

Transcriptional control of cell identity in developing hypothalamus: from molecular atlas construction to candidate gene analysis

Secreted differentiation factors such as those described above confer identity on neural subtypes within the developing nervous system by inducing expression of regional and cell type-specific transcription factors. However, two major obstacles to the systematic analysis of hypothalamic development have been the lack of well defined molecular markers for identifying individual cell types, along with a dearth of candidate transcription factors that might direct specification of hypothalamic neuronal subtypes. To

overcome these obstacles, several large-scale efforts have been undertaken to comprehensively profile changes in gene expression both globally in the developing hypothalamus and in individual developing hypothalamic nuclei. In one such study, aimed at globally profiling genes expressed in developing mouse hypothalamus, microarray analysis of whole hypothalamic neuroepithelium at 12 different developmental time points was conducted. This was then followed by single-color and two-color *in situ* hybridization of >1000 transcripts in developing hypothalamus that showed dynamic expression by microarray analysis (Fig. 2*A*). From this analysis, multiple markers that stably label each major hypothalamic nucleus over the entire course of neurogenesis were identified (Shimogori et al., 2010). This detailed analysis not only identified hundreds of previously uncharacterized region and cell subtype-specific molecular markers but also revealed several previously uncharacterized domains within the developing hypothalamus. One notable novel compartment, termed the intra-hypothalamic diagonal (ID), consists of a zone of *Arx*-positive, *Gad67*-positive cells that separates the anteriodorsal and posterioventral portions of the hypothalamus This region expresses multiple Lhx family genes along its anterior–posterior axis and may give rise to the bulk of hypothalamic interneurons (Fig. 2*B*). Using this comprehensive set of region-specific hypothalamic markers, it became possible to directly analyze the effects of removal of Shh from the basal plate domain of the developing hypothalamus using *Nkx2.1-Cre x Shh* $^{lox/lox}$ mice (Fig. 2*B*). Loss of this source of Shh resulted in a failure of anterior and tuberal hypothalamic nuclei to develop, along with cells of the ID, whereas development of mamillary hypothalamus and prethalamus were unaffected.

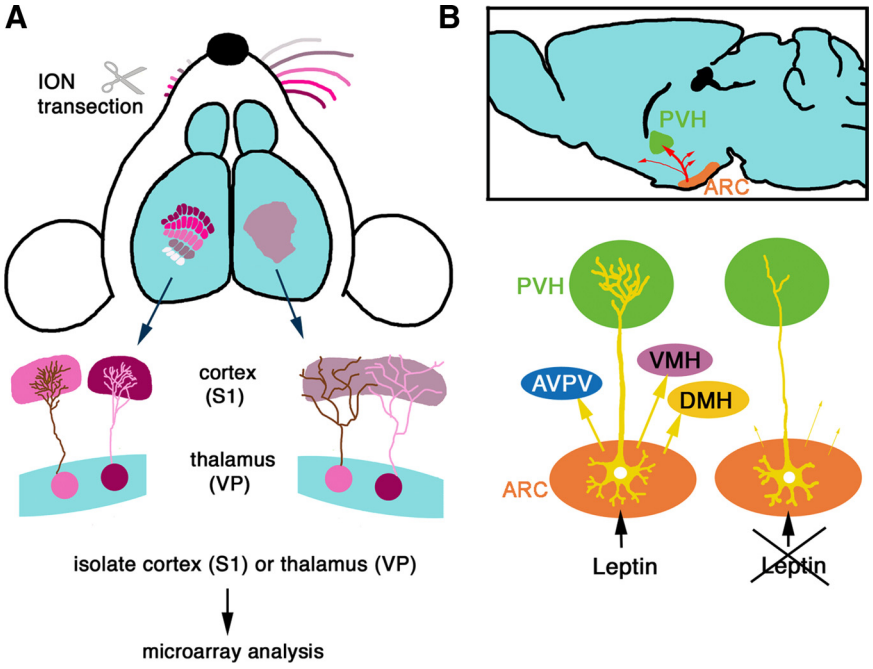

Figure 3. ***A***, Schematic for analysis of genes expressed in barrel cortex and thalamus that are regulated by sensory input. ***B***, Effects of leptin on development of axonal projection s of Agrp-positive neurons from ARC.

While very few genes are expressed exclusively in developing hypothalamus, it is possible using this genomic developmental atlas to identify many combinatorial patterns of gene expression that uniquely define various hypothalamic regions and cell types. For instance, *Lhx1* and *Lhx8*, which regulate neuronal specification in other brain regions (Zhao et al., 1999; Mori et al., 2004; Fragkouli et al., 2005; Zhao et al., 2007), are both expressed in the anterior hypothalamic neuroepithelium at embryonic day (E) 12.5. Later, at the time when hypothalamic neurogenesis ends (E16.5), *Lhx1* expression is restricted to the developing SCN (suprachiasmatic nucleus) and *Lhx8* expression is restricted to the central DMH (dorsomedial hypothalamic nucleus). By generating *Six3-Cre x Lhx1*$^{lox/lox}$ mice, in which *Lhx1* is selectively deleted from anterior hypothalamus, it was found that both terminal differentiation of SCN neurons and circadian behavioral rhythms are severely disrupted.

Similar efforts by other investigators have focused on analyzing development of the ventromedial hypothalamic nucleus (VMH), since it is linked to many innate behavioral responses, including feeding, fear, thermoregulation, and sexual activity, and can be readily and uniquely identified by expression of the nuclear hormone receptor *Nr5a1*. The precise neuronal circuits underlying these innate responses are largely unknown, in part because the cellular and molecular features of individual VMH neuronal subtypes have yet to be defined.

To this end, using laser-capture microdissection of neonatal VMH combined with microarray-based expression profiling (Fig. 2*C*), novel molecular markers of VMH in the newborn mouse were identified (Kurrasch et al., 2007). Many of these neuroendocrine markers exhibit distinct regional expression patterns in the newly formed hypothalamus. Top neonatal markers include transcriptional regulators such as *Vgll2*, *Nr5a1*, *Sox14*, *Satb2*, *Fezf1*, *Dax1*, *Nkx2–2*, and *COUP-TFII*. Interestingly, the highest expressed VMH transcript, the coregulator *Vgll2*, is completely absent in older animals. All VMH progenitors, as marked by steroidogenic factor 1 (SF-1; *Nr5a1*) expression, coexpress *Nkx2.1* on E11.5. Later in development *Nkx2.1* and SF-1 expression are non-overlapping. This is consistent with cellular studies in embryonic immortalized hypothalamic cells showing that *Nr5a1* potently represses *Nkx2.1* expression (Tran et al., 2003).

This new molecular toolkit has made it possible to investigate how neuronal subtypes of the VMH are specified and how they might contribute to innate behavioral responses. Floxed alleles of genes prominently and selectively expressed in developing VMH have been obtained and can be crossed with *Nr5a1* Cre-driver lines to selectively eliminate gene function in the VMH. These VMH mutant mice have been subsequently crossed with a *Nr5a1*-green fluorescent protein (GFP) knock-in reporter line that marks all major VMH projections. Strikingly, some of these GFP-positive projections exist as early as E11.5, including fibers to the habenular nucleus and thalamus via the ventral supraoptic commissure. Data assessing morphology, VMH projections, and behavior are presented on mice harboring conditional knock-outs of several genes, including *Nkx2.1*, *A2BP1*, *VGlut2*, and *Fbxw7*. These studies lead us to propose that the VMH is an important brain center for regulation of anxiety-related behavior.

Experience-dependent development of neural circuitry in thalamus and hypothalamus

The activity of thalamocortical axons (TCAs) has a critical role in shaping the receptive fields of neurons in layer IV of primary sensory cortex. Although this is most widely known and studied in the context of the ocular dominance columns of primate and carnivore visual cortex, more genetically tractable model organisms can also be used to investigate the mechanisms that regulate this process. In rodents, the arrangement of whiskers on the face is precisely mapped onto barrel-shaped receptive fields in layer IV of the somatosensory cortex. The barrel field contains spiny stellate cells, located predominantly around the thalamocortical axon-rich barrel centers (Fig. 3*A*). Each spiny stellate cell sends dendrites into a single barrel, and postsynaptic NMDA activity is critical for spiny stellate dendrite formation (Iwasato et al., 2000; Espinosa et al., 2009). Furthermore, serotonergic autoregulation has an important role in both regulating targeting of somatosensory TCAs and in barrel field formation, as determined by analysis of mice mutant for genes involved in serotonin biosynthesis or reuptake (Rebsam et al., 2002). Perturbations of the sensory periphery by nerve or whisker lesions during a critical period disrupt barrel structure, suggesting that early neural activity is essential for pattern formation in the barrel cortex (Lendvai et al., 2000). These results suggest that cell organization and dendrite formation of neurons in barrel cortex are dependent on TCA innervation and activity.

To reveal the molecular mechanisms that control barrel formation by thalamocortical input, gene expression profiles were obtained from the somatosensory cortex and thalamus of mice that had undergone neonatal whisker removal, and compared with profiles obtained from the contralateral unoperated hemisphere (Fig. 3*A*). Following this treatment, 103 transcripts are downregulated and 100 transcripts are significantly upregulated by >1.2-fold in the cortex of the experimental hemisphere attributable to abnormal input from the TCAs. Therefore, thalamocortical innervation of the barrel field affects gene expression patterns in developing somatosensory cortex, altering both the position of neuronal cell bodies and dendritic development. Functional analysis of these genes will provide more detailed insight into the molecular mechanisms that control the remodeling of dendritic arbors and receptive fields of cortical neurons in response to sensory input.

Dietary and endocrine signals can likewise profoundly affect the development of neural circuitry in the hypothalamus during the perinatal period, resulting in changes that can drastically affect behavioral homoeostasis throughout the later life of the individual. Links between the perinatal nutritional environment and metabolic phenotype have been known for decades; being born of either low or high birth weight confers an increased risk for obesity and diabetes. Manipulation of litter size also alters levels of the adipocyte-derived hormone leptin, which has been identified as a potent neurotrophic factor that functions during perinatal life to specify patterns of connectivity in the hypothalamus (Simerly, 2008).

During hypothalamic development, axons travel from the arcuate nucleus (ARC) to restricted targets and distribute to form synaptic contacts with neurons in the paraventricular nucleus of the hypothalamus (PVH), which contains cells known to mediate multiple aspects of energy balance (Fig. 3*B*). Neural projections from the ARC to functionally distinct parts of the PVH develop during a postnatal period when leptin levels are elevated, and ARC projection pathways are severely disrupted in leptin-deficient (*ob/ob*) mice (Fig. 3*B*) (Bouret et al., 2004). Treatment of *ob/ob* mice with leptin in adulthood does not restore the normal pattern of projections, but the pathways are largely rescued by neonatal leptin treatments. Thus, the postnatal leptin surge appears to be a key developmental signal that affects the architecture of hypothalamic circuits that mediate feeding during a discrete developmental critical period. Although leptin impacts both anorexigenic proopiomelanocortin (POMC) and orexigenic neuropeptide Y (NPY)-containing ARC projections, it does so through distinct intracellular signaling pathways, and manipulation of these pathways produces divergent developmental outcomes for POMC and NPY neurons. The combined use of retrogradely transported tracers and histochemical visualization of neuronal terminals on PVH neurons shows that leptin preferentially increases the density of Agouti-related peptide (Agrp)-containing inputs to preautonomic neurons in the PVH; however, development of POMC inputs to these neurons is largely leptin independent (Fig. 3*B*). These anatomical findings suggest that the ability of leptin to specify patterns of neuronal connectivity in the hypothalamus during development may differentially impact distinct components of autonomic regulation. Leptin appears to exert these actions through direct action on ARC neurons that include promoting neurite extension, specifying patterns of innervation in hypothalamic targets, and influencing cell type-specific alterations in synaptic density.

Conclusion

These studies highlight the considerable recent progress that has been made in the analysis of the development of the thalamus and hypothalamus. The availability of new tools to rapidly manipulate gene function and characterize mutant phenotypes in these structures implies that this progress should accelerate in the years ahead. Perhaps the most exciting potential future application of these developmental studies will come from the physiological and behavioral analysis of animals in which the development or function of defined neural subpopulations has been disrupted. Although the anatomical structure of both the thalamus and hypothalamus is quite well characterized, the anatomical–physiological relationship between individual nuclei and neuronal subtypes and specific behaviors is still generally unclear. Both thalamic and hypothalamic anatomy is highly complex, and it is rarely possible to produce selective surgical lesions that only affect individual neuronal subtypes. Identifying the molecular codes that determine specification of neuronal subtypes in these structures may ultimately allow selective manipulation of the function of individual neural subtypes, and a detailed analysis of their contribution to perception and behavior.

Further analysis of thalamic and hypothalamic development is also likely to have considerable importance for human health. In addition to the well established effects of early nutritional status in controlling development of hypothalamic circuitry, it is also likely that genetic defects in the development of specific cell subtypes in thalamus and hypothalamus may directly lead to disorders of perception, metabolism, and homeostasis; this has already been reported for congenital obesity of both Mendelian and polygenic origin (Holder et al., 2000; Traurig et al., 2009). Coming years are likely to see the identification of numerous different cases where defects in development of diencephalic neurons are found to directly cause a range of human diseases.

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