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Patterning, specification, and differentiation in the developing hypothalamus

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Abstract

Owing to its complex structure and highly diverse cell populations, the study of hypothalamic development has historically lagged behind that of other brain regions. However, in recent years, a greatly expanded understanding of hypothalamic gene expression during development has opened up new avenues of investigation. In this review, we synthesize existing work to present a holistic picture of hypothalamic development from early induction and patterning through nuclear specification and differentiation, with a particular emphasis on determination of cell fate. We will also touch on special topics in the field including the prosomere model, adult neurogenesis, and integration of migratory cells originating outside the hypothalamic neuroepithelium, and how these topics relate to our broader theme.

INTRODUCTION

The hypothalamus controls a wide range of homeostatic processes essential for life, including feeding, thirst, sleep, circadian rhythms, reproductive behavior, and more. This brain region, derived from the rostral diencephalon of the forebrain, is enormously anatomically complex. Where other brain regions such as the cortex or hippocampus have a clear overall structure of columns and parallel feed-forward loops, respectively, the hypothalamus has a densely interconnected patchwork of often quite ill-defined nuclei. Partially for this reason, the study of hypothalamic development has lagged behind that of many other brain regions, whose simpler anatomy facilitated the identification of factors that pattern them. However, as determinants of hypothalamic identity were studied in recent

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years, it has become increasingly clear that many of the same factors implicated in development of the cortex, retina, and other CNS regions play similar roles in the hypothalamus.

Shortly after the neural plate forms following gastrulation, diffusible morphogens begin patterning the developing nervous system, including the hypothalamus. In the beginning, these factors are generated outside of neural tissues, in mesodermal domains including the axial notochord and more anterior prechordal plate (PCP), as well as other more lateral tissues. Often, production of these same cues is later induced in neural tissue near or within the nascent hypothalamus, where dynamically shifting sources of morphogen production create ever-finer concentration gradients within the region. Sharp boundaries are translated from these gradients through induction of distinct and often mutually repressive sets of cell-autonomous transcription factors, progressively defining the fate of neural progenitors and emerging hypothalamic nuclei.[1,2]

In this review, we will first outline the contribution of several major growth and differentiation factor families in inducing, patterning, and sometimes specifying subdomains of the hypothalamus. We will then discuss how the pathways guiding neurogenesis, specification, and differentiation diverge in the heterogeneous hypothalamus to produce its variegated adult nuclei, with a focus on the transcriptional networks that control this process.

HYPOTHALAMIC INDUCTION AND SPATIAL PATTERNING

Wingless Family (Wnt) Signaling

The Wnts are a large family of secreted, lipid-modified glycoproteins long known to be involved in patterning during development, having originally been implicated in developmental segmentation by their homology to the Drosophila *Wingless* gene. Anterior-posterior (AP) patterning of newly induced neural plate is initiated by the posteriorizing morphogen Wnt8, secreted by lateral mesodermal precursors. Its effect is enhanced and sustained by nodal signaling in the posterior neural plate. Conversely, Wnt inhibition is necessary for anterior patterning, including the region that eventually gives rise to the hypothalamus. A variety of Wnt inhibitors are expressed in spatially and temporally dynamic patterns, first in the PCP (Figure 1) and later in the developing anterior brain. These inhibitors include sFRP1, sFRP2, sFRP3/Frzb1, Crescent/Frzb2, Dickkopf1, and Cerberus.[4,5]

After initial AP patterning, Wnt signaling in turn subdivides the forebrain. The domain receiving lower Wnt signaling gives rise to the telencephalon, which includes the cerebral cortex, hippocampus, striatum, amygdala, and associated structures. Higher Wnt signaling induces development of the diencephalon, including a more rostral domain that gives rise to the hypothalamus and prethalamus, and a more caudal domain that gives rise to the thalamus.[6] Telencephalic and rostral diencephalic neuroepithelium are hereafter distinguished by expression of the forkhead domain transcription factors FOXG1 and FOXD1, respectively.[7,8]

However, despite relatively high Wnt signaling in the diencephalon early on, inhibition of canonical Wnt signaling by Axin1 is essential for initial hypothalamic specification. In *Axin1*$^{-/-}$ mice, the ventral midline cells of the hypothalamus fail to develop, instead assuming a caudal floor plate identity.[9] Later roles of Wnts in hypothalamic development explain this seeming paradox. During the late gastrula stage in zebrafish, Wnt8b and Wnt1 secreted from the midbrain–hindbrain boundary pattern the forebrain and induce specification of posterior diencephalon, including the sensory thalamus and habenula. Overexpression of these Wnts or their receptors, or loss of the Wnt inhibitor *Axin1*, induce expanded expression of posterior hypothalamic markers such as *Emx2* at the expense of anterotuberal hypothalamic markers such as *Rx3*, suggesting a posteriorization of hypothalamic neuroepithelium.[9,10]

Consistent with this finding, *Wnt8b* is expressed in the mouse posterior hypothalamus (PH) beginning at ~ E8.5,[8,11] consistent with a role in patterning, but its function has not been directly explored in this context (Figure 2(e)). By E12.5, *Wnt8b* becomes restricted to the mammillary region, suggesting it may act to impart posterior hypothalamic identity in mammals as it does in zebrafish[8] (Figure 2(f)). WNT7a/7b become expressed selectively in prethalamic and hypothalamic GABAergic neuronal progenitors around the same time, suggesting a role in interneuron development, but their function is even less well-characterized than WNT8b[8] (Figure 2(F)).

Meanwhile, a gradient of WNT3 signaling further patterns the developing forebrain, including the hypothalamus (Figure 2(f)). High levels of WNT3 induce *Irx3* expression in the sensory thalamus, whereas low WNT3 signaling is permissive for more anterior *Six3* expression in the hypothalamus and prethalamus.[12] Mutual cross-repression of SIX3 and IRX3 demarcates the border between these regions, an effect that is reinforced by SIX3-mediated dampening of WNT responsiveness. Confirming the importance of these interactions, *Six3*$^{-/-}$ mice show a rostral expansion of caudal diencephalic markers at the expense of the hypothalamus and telencephalon, largely due to an anterior expansion of WNT1 expression in the roof plate of the forebrain.[1,13]

Sonic Hedgehog (SHH) Signaling

Concurrent with Wnts' central role in early AP patterning of the developing neural tube, the lipid-linked polypeptide signal SHH is a key secreted morphogen controlling neural dorso-ventral (DV) patterning. SHH is first expressed in the PCP (Figure 1), and is necessary for initial induction of the hypothalamus.[14,15] The ventral midline cells initially have a quasi-caudal floor plate identity, including expression of HNF3β but not the floor plate markers FP3 and FP4[16] (Figure 1). PCP-derived SHH goes on to induce transient *Shh* expression in the ventral diencephalic midline (and much of the rest of the developing hypothalamus) through a GLI-mediated signaling cascade that activates expression of transcription factors including SIX3, SOX2, and SOX3.[12,17] SOX2 and SOX3, members of the SOXB1 transcription factor family, directly activate and maintain *Shh* transcription in hypothalamic neuroepithelium.[17] SIX3 likewise targets the *Shh brain enhancer-2* to directly activate *Shh* transcription there.[18,19]

A subsequent downregulation of *Shh* expression is important for hypothalamic patterning. Indeed, early patterning of the ventral hypothalamus along the AP axis is achieved in part through downregulation of *Shh* expression in ventral tuberomammillary hypothalamus[15,20,21] (Figure 2(b)). For instance, studies in chick demonstrate cells giving rise to this region must downregulate *Shh* to adopt their proper fate through induction of Tbx2 expression, which in turn triggers Shh downregulation by upregulation of Bmp7 in the tuberomammillary hypothalamic neuroepithelium (see next section).

In contrast to the ventral tuberomammillary hypothalamus, *Shh* expression in the basal plate (BP), which is initially induced by PCP Shh and additional feed-forward Shh signaling from the ventral midline, is maintained through E14.5[22,23] (Figure 2(b)–(d)). This more dorsally located hypothalamic region begins immediately posterior and ventral to the presumptive suprachiasmatic nucleus (SCN) and terminates in the mammillary region[8,24] (Figure 2(b)–(d)). *Shh*(+) cells of the BP originate at the diencephalic/mesencephalic border, then migrate to their final destination in the hypothalamus.[21] BP SHH plays a critical role in hypothalamic patterning. Several studies have looked at loss of function mutants in which *Shh* is deleted from the BP. In these studies, the hypothalamus is still specified, but neurons fail to undergo further differentiation, resulting in the loss or reduction of several nuclear markers. In all mutant lines examined, both AP and DV patterning are disrupted, although the reported phenotypes are not identical among these studies, most likely because they used *Cre*-drivers with different spatial and temporal patterns of activity.[8,17,25,26]

BP Shh is also necessary to initiate sustained *Shh* expression in the zona limitans incerta (ZLI), a secondary *Shh*(+) organizer dividing the prethalamus and thalamus.[27,28] ZLI-derived Shh plays a central role in patterning the sensory thalamus, and is required for proliferation and differentiation of prethalamic progenitors[29,22,23] (Figure 2(b)–(d)). Importantly, ZLI *Shh* expression is preserved in Cre/lox studies where *Shh* was deleted in the BP, showing that the hypothalamic patterning roles of BP- and ZLI-derived Shh are distinct.

Bone-Morphogenetic Protein (Bmp) Signaling

BMP7 is the primary member of this family of TGF-β superfamily members that has been studied in the context of hypothalamic development. Surprisingly given the classical physical separation and antagonistic effects of BMPs and SHH in spinal DV patterning, many known actions of BMP7 in the hypothalamus revolve around cooperative interactions with SHH. For instance, during gastrulation PCP-derived BMP7 and SHH cooperate to induce expression of early general hypothalamic markers such as *Nkx2.1* in the ventral diencephalic midline, distinct from more caudal midline gene expression induced by notochord-derived SHH alone[16] (Figure 1).

During later stages of hypothalamic patterning, expression of *Bmp7* and *Shh* are transiently coexpressed in ventral tuberomammillary hypothalamus,[21] (Figure 1). BMP7 swiftly downregulates *Shh* expression here by activating expression of first *Tbx2* and subsequently *Gli3*, although it is unclear if TBX2 directly activates *Gli3* expression.[20,21] In chick this process begins around Hamburger and Hamilton (HH) stage 10, inducing complete elimination of *Shh* by HH stage 16 (roughly equivalent to E9.5–10.5 in mouse).[24] *Tbx2*

itself is in turn downregulated during stages 16–22; toward the end of this period, ventral tuberomammillary markers such as *Fgf10* and *Emx2* begin to be expressed in these now *Shh*(−) subdomains of the developing hypothalamus.

BMP7 signaling is also required for induction of *Pax7* expression in more dorsally located tuberomammillary progenitors, which occurs by HH stage 30. BMP7 accomplishes this by both downregulating *Shh* expression in ventral tuberomammillary hypothalamus, and by directly activating *Pax7* transcription via induction of Smad5 phosphorylation.[20] Interestingly, widespread expression of the Bmp antagonist *Chrdl1* is detected in chick hypothalamus at HH stage 13, with expression restricted to the anterioventral hypothalamic neuroepithelium by HH15 as Bmp expression becomes restricted to posterioventral hypothalamus. *Chrdl1* expression is upregulated in response to inhibition of Notch signaling, suggesting that Notch may play a role in early hypothalamic development by modulating BMP signaling,[30] although this has not yet been shown directly.

Nodal Signaling

Nodal proteins are other members of the TGF-β superfamily that play an important role in hypothalamic patterning. PCP-derived nodal signaling, most notably by cyclops, plays an essential role in initial hypothalamic specification in zebrafish[31] (Figure 1). Together with Shh and Bmp7, nodal signaling is essential for induction of early hypothalamic markers such as *Nkx2.1*; however, unlike in the telencephalon, Shh is not able to restore initial expression of the *Nkx2.1* homologue in zebrafish diencephalon in the absence of nodal, suggesting a separable role for nodal in early hypothalamic induction.[32]

On the other hand, cells defective in both Nodal and Shh signaling are unable to give rise to hypothalamic cells following transplantation into wild-type zebrafish, and Shh can restore some *nkx2.1* expression in the zebrafish ventral posterior hypothalamus (vPH) during early stages of hypothalamic development.[15,32] Together, these data suggest a cooperative role for Nodal and Shh in later stages of hypothalamic development, with Nodal enhancing the ability of cells to respond to Shh.

There also appears to be cross-talk between Nodals and Wnts in hypothalamic development, as the Wnt inhibitor Axin1 facilitates Nodal signaling.[9] Axin1 may thus alter the competence of hypothalamic progenitors to respond to both Nodal and indirectly, to Shh signaling, likely explaining the non-cell-autonomous requirement for Nodal signaling in patterning of the dorsal anterior hypothalamus (dAH) by Shh.[15] Conversely, cells of the zebrafish vPH require Nodal signaling cell-autonomously in order to activate *emx2* expression, whereas excessive Shh signaling inhibits *emx2* expression. Thus, Nodal must cooperate with Bmp7 to induce *emx2* in vPH—while Nodal acts as a cell-autonomous factor conferring competence to activate *emx2* expression, Bmp7 inhibits local *shh* to prevent Shh signaling from becoming excessive. Thus, Axin1 sits at the nexus of Wnt, Shh, Nodal, and Bmp signaling, from which it serves as a critical, integrative determinant of AP identity.

Fibroblast Growth Factor (FGF) Signaling

FGF family members do not appear to play a critical role in early stages of hypothalamic patterning. Irx3 is required for competence to respond to Fgf8 signaling, but is excluded

from the hypothalamus during this time by its cross-repressive interaction with Six3. The ZLI lies between the *Six3*(+) rostral diencephalon and *Irx3*(+) caudal diencephalon, demarcating the boundary of competence to Fgf8 signaling from the isthmic organizer and telencephalic roof plate.[33] That said, FGF family members do regulate cell proliferation at later stages of hypothalamic development, as discussed in later sections.

HYPOTHALAMIC NEUROGENESIS AND LATE PATTERNING

Hypothalamic neurogenesis occurs from ~ E10.5–16.5 in mice, with the great majority of hypothalamic neurons being generated between E12.5 and E14.5.[34,30,35] Birthdating studies using tritiated thymidine and BrdU indicated that neurons in lateral hypothalamic nuclei are typically born before medial nuclei, resulting in an 'outside-in' pattern of birth in the hypothalamus.[35] However, later research has contradicted this model. Looking at different parvocellular neuronal cell types within the neurosecretory dorsolateral anterior hypothalamus (dlAH), all were found to be born during the same approximate interval, despite occupying different medial-lateral locations within the hypothalamus.[36] Similar results have been reported for different neuronal subtypes in the arcuate nucleus (ARC),[37] suggesting that for progenitors in at least some regions of the hypothalamus, this 'outside-in' order of cell generation may not hold.

Growth and differentiation factors that pattern the developing hypothalamus also play important roles in later stages of hypothalamic cell fate specification. For example, while SHH is required for both early and late neurogenesis in much of the hypothalamus,[8,38] *Shh* downregulation is necessary for proliferation and expansion of the ventral tuberomammillary progenitor pool.[21] Meanwhile, canonical Wnt signaling, through its effector Lef1, plays an important role in both progenitor maintenance and in driving progenitor differentiation in the PH.[39,40] Notch signaling is also involved in progenitor maintenance in the hypothalamus, as it is elsewhere in the developing nervous system, but the specifics of its role in different parts of the hypothalamus has only recently begun to be understood.[30,41] In the rostral hypothalamus, Notch signaling works through lateral inhibition to regulate the progenitor population and generate new neurons. High levels of Notch signaling in AH upregulate *Hes* and *Hey* family genes while downregulating proneural genes such as *Ascl1* and *Nhlh1*. The genes *Tagln3*, *Chga*, *Robo2*, *Slit1*, and *Chrdl1* are then needed for terminal neural differentiation once precursor cells have exited mitosis.

Expression of a diverse assortment of transcription factors are induced by the intricate spatiotemporal code of exposure to morphogens discussed previously, which in turn confer intrinsic spatial identity upon hypothalamic progenitor cells. Patterning processes establish the anatomical DV and AP axes within the hypothalamus, limiting which nuclei can develop at any given set of coordinates. Consequently, many markers ubiquitously expressed in early hypothalamic progenitors become more restricted once neurogenesis begins. For instance, *Six3* and *Lhx2* are expressed in the anterior and tuberal hypothalamus (AH and TH), but not the PH. Meanwhile, *Rax* and *Nkx2.1* are expressed in the TH and PH, but only in limited regions of the AH.[42]

However, these still relatively broad domains of transcription factor expression become even more regionalized as neurogenesis progresses. Continuing with the previous examples, *Lhx2* becomes restricted to the dorsolateral aspects of AH (dlAH) while *Six3* becomes restricted to the ventral AH (vAH). Meanwhile, *Rax* expression is rapidly downregulated everywhere except the ventral TH, though *Nkx2.1* remains comparatively broadly expressed in the TH and PH.[8,42–45] By mouse E12.5, progressive restriction of expression of these and assorted other genes has already defined many nuclei found in the adult hypothalamus (Figure 3).

Developmental expression of over a thousand such mouse genes were documented in an extensive screen published by Shimogori et al in 2010. Among other findings, this study identified the intrahypothalamic diagonal/tuberomammillary terminal (ID/TT) complex, an excellent example of progressive regionalization in the hypothalamus. This region traverses most of the hypothalamus in the sagittal plane starting at E12.5 (Figure 3) and is characterized by uniform expression of Arx and patterned expression of Lim homeodomain factors *Lhx1*, *Lhx6*, *Lhx8*, and *Lhx9*. This largely nonoverlapping pattern of LIM homeodomain factor expression appears to define a number of hypothalamic nuclei involved in control of circadian rhythms and sleep homeostasis, such as the SCN, dorsomedial hypothalamic (DMH), and parts of the lateral hypothalamus (LH) (Figure 4). In at least two cases, these genes are required for proper development of the hypothalamic cell types in which they are expressed.[46,47] Such findings have already begun to facilitate the study of the development of individual hypothalamic nuclei, the topic we shall focus on for the remainder of the review.

DEVELOPMENT OF SPECIFIC HYPOTHALAMIC NUCLEI AND NEURONAL SUBTYPES

Dorsolateral Anterior Hypothalamus (dlAH): Paraventricular Nucleus (PVN), Supraoptic Nucleus (SON), and Anterior Periventricular Nucleus (APV)

The dlAH, or neuroendocrine hypothalamus, plays a central role in controlling systemic hormone secretion, with important implications for feeding and other homeostatic processes.[48,49] Although *Rax* expression in dlAH has been controversial,[43] fate mapping using Rax-CreER knock-in mice confirmed its previously reported low level expression beginning at E10.5.[8,50] *Six3*, *Six6*, and *Foxd1* also label the entire AH, including the dlAH, around this time.[8] In zebrafish dlAH, *fezf2* and *olig2* are respectively required for induction of orthologs for the early specific dlAH markers *otp* and *sim1*[51,52] (Figure 5(a)). *Fezf2* and *Olig2* are expressed in mouse dlAH by E11.5, suggesting that these functions may be preserved in mammals.[8,42] Furthermore, in zebrafish, Pac1 receptor signaling positively regulates levels of Otp protein, but not *otp* transcript.[51]

In contrast, early *Lhx2* expression suppresses neurosecretory specification through inhibition of *Otp* and *Sim1* (Figure 5(a)); the expression domains of these factors are expanded in *Lhx2*$^{-/-}$ AH.[44] *Lhx2* is also required to repress dlAH-specific genes in retina, even after retinal neurogenesis has begun. This implies that LHX2 acts as a fairly general repressor of dlAH identity in structures like the eye field that are derived from vAH hypothalamic neuroepithelium.[44]

Otp, *Sim1*, and the SIM1 binding partner *Arnt2* are expressed in mouse dlAH by E12.5, concurrent with downregulation of *Six3* and *Six6* in PVN and SON, but not APV.[8,45,53] SIM1/ARNT2 and OTP function in parallel to drive expression of several dlAH neuropeptides via their downstream factors *Brn2* and *Sim2*, as well as specifying A11 dopaminergic neurons of the dlAH[52,54–59] (Figure 5(a)). *Fezf2*, previously discussed as an upstream regulator of *Otp* expression, is similarly required for the formation of dopaminergic and neuropeptidergic lineages in zebrafish.[51,60]

However, in $Otp^{-/-}$ mice the dlAH exhibits additional developmental defects not observed in $Sim1^{-/-}$ mice, including ectopic expression of *Six3* and *Dlx1*, loss of calbindin expression, and defects in radial migration of neural progenitors.[54,57,59] This suggests that *Otp* is required at earlier stages of dlAH specification than *Sim1*. Conversely, SIM1 has additional later roles in dlAH, such as regulating longitudinal innervation of the spinal cord by A11 dopamine neurons in zebrafish and maintaining AH-specific gene expression in the postnatal mouse.[61,62] Accordingly, both early and late *Sim1* expression is essential for PVN function; both $Sim1^{+/-}$ and $CaMKII\text{-}Cre;Sim1^{lox/lox}$ mice are hyperphagic and obese.[62,63]

Acting downstream of *Sim1* and *Otp*, *Brn2* is required for expression of *Avp* and *Oxt* in PVN/SON, and *Crh* in PVN, whereas *Sim2* is required for expression of *Trh* in PVN/APV and *Sst* in APV[55,64,65] (Figure 5(a)). Sampling tissue from distinct *Sim2* and *Brn2* expressing subdomains of the developing *Sim1*(+) dlAH, a microarray-based screen identified additional *Sim1*-controlled genes, many of which are specifically expressed in cells expressing *Sim2* or *Brn2*.[66] This screen is thus also a starting point for identifying genes that act downstream of *Brn2* and *Sim2* in developing dlAH.

One of the few *Sim1*-regulated genes expressed throughout the entire domain of *Sim1* expression is *Uncx4.1*.[66] In $Uncx4.1^{-/-}$ mice, gross morphology of the PVN and expression of *Otp* and *Avp* is normal, although neuronal connectivity in the PVN is disrupted. No further gene expression analysis was reported in this study, but *Uncx4.1* is known to promote dopaminergic cell fate at the expense of glutamatergic cells in the midbrain.[67,68] Thus, given *Sim1*'s role in specifying hypothalamic dopamine neurons in zebrafish,[52] it is tempting to speculate that UNCX4.1 may act downstream of SIM1 to promote A11 neuron specification, in a pathway parallel to *Brn2*- and *Sim2*-dependent determination of dlAH neuropeptidergic cell fate.

Both extracellular and intracellular factors influence dlAH development downstream of *Sim2* and *Brn2*. Notch signaling plays a central role in this process. APV SST(+) cells are selectively depleted in $Hes1^{-/-}$ mice, and those that remain innervate the pituitary aberrantly, implicating *Hes1* in specification of this lineage[69] (Figure 5(a)). PVN and SON AVP(+) neurons also migrate improperly and ectopically express *Gad67* in $Hes1^{-/-}$ mice, suggesting a role in maturation.[69] *Fgf8* hypomorphs show a more specific defect, with processed OXT peptide expression lost in PVN and SON, OXT propeptide expression lost only in SON, and *Oxt* mRNA unaffected in both regions, suggesting a role for FGFs in promoting maturation of these cells.[70] AVP protein is also reduced in *Fgf8* hypomorphic PVN, possibly because of similar deficits in maturation.[71] Finally, *Uncx4.1* is required for proper pituitary innervation

by dlAH nuclei, whereas *Nhlh2* is required for *Pcsk* expression and production of mature peptide in PVN *Trh*(+) neurons.[67,72]

Unlike in most of the hypothalamus, the transcriptional network guiding specification of various PVN, SON, and APV lineages is sufficiently well understood to provide a solid starting point for building on our understanding of dlAH development. Identifying the factors that specify individual neuropeptidergic populations from hypothalamic progenitor pools delineated by *Sim2* and *Brn2* expression is a topic of particular interest, particularly given recent findings suggesting that the fate of dopamine and SST neurons in adult rat dlAH can be bidirectionally changed by manipulating the circadian cycle the animal experiences, with significant effects on stress behavior.[73] This suggests that developmental pathways regulating cell fate in the dlAH may remain active into adulthood, imparting plasticity to this system that can profoundly alter behavior in response to environmental changes.

Ventral Anterior Hypothalamus (vAH): The Suprachiasmatic Nucleus (SCN)

The best-characterized nucleus of the vAH is the SCN, which functions as the body's central light-entrained circadian clock.[74,75] In mouse, the ventral hypothalamic markers *Rax* and *Lhx2*, and anterior markers *Foxd1* and *Nkx2.2*, are all expressed early in SCN, but downregulated as development proceeds. Other anterior markers, such as *Six3* and *Six6*, remain expressed throughout development as they become progressively more restricted to the vAH. *Fzd5* is expressed early in the *Rax*/*Six3*(+) region, possibly implicating Wnts in regulating initial SCN specification. *Fzd5* is later downregulated coincident with induction of *Dlx2* (specifying GABAergic fate), *Lhx1* (the earliest specific marker of developing SCN), and *Lhx8* in the anterior ID domain (Figure 3) that gives rise to the SCN[8,42,45,50] (Figure 4). Induction of other selective SCN markers follows initial expression of the LIM and pro-GABAergic factors, including *Rora*, *Id4*, and *Igfbp5* at E14.5 and *Rorb*, *Nr1d1*, *Vipr2*, and *Sema6d* at E16.5. By E16.5, *Lhx8* expression is downregulated in the vAH, but persists in the presumptive DMH nucleus.[8,45]

In addition to the SCN, the anterior domain of the ID likely gives rise to other regions of the vAH, such as the subparaventricular, retrochiasmatic, and anterior nuclei (Figure 4). For example, the prominent ID marker *Arx* is present throughout most of the vAH despite being largely excluded from the SCN for most of the region's development.[8,42] However, the vAH is poorly characterized both developmentally and functionally outside of the SCN, and will not be discussed further in this review.

Lhx2 is required for specification of SCN and other vAH cell fates (Figure 5(b)); $Lhx2^{-/-}$ mice lack expression of multiple ventral anterior markers at E12.5, including *Lhx1* and *Arx*.[44] *Six3* and *Six6* are also required for SCN specification (Figure 5(b)), as *Nestin-Cre;*$Six3^{lox/lox}$ and $Six6^{-/-}$ mice both fail to form a morphologically recognizable SCN or express SCN-specific markers.[45,76] Corresponding to this morphological defect, behavioral rhythms are also profoundly disorganized in $Six6^{-/-}$ mice, the only one of the three mutants that survive long enough to assess behavior.[76] Although *Nestin-Cre;*$Six3^{lox/lox}$ mice likely have some disruption of the BP domain of *Shh* expression that is required for expression of AH markers,[8,18,19] mosaic loss of *Six3* function in the SCN of a subset of *Nestin-*

Cre;Six3$^{lox/lox}$ mice led to a specific loss of *Lhx1* expression only within the *Six3*-deficient region. This suggests that *Six3* has a cell-autonomous role in SCN specification separable from its effects on control of *Shh* expression.[45]

Downstream of *Six3* and *Six6*, *Lhx1* is a master regulator of mammalian SCN terminal differentiation[46] (Figure 5(b)). *Six3-Cre;Lhx1*$^{lox/lox}$ SCN retains expression and proper regionalization of most markers initially expressed prior to E16.5, but neuropeptides with important roles in adult circadian function including *Vip*, *Grp*, *Avp*, *Prok2*, *Enk*, and *Nms* are lost.[46] The relatively normal development of *Lhx1*-deficient SCN from E11.5–E16.5 suggests possible compensation by one or more factors during this period; *Lhx8* is one possible candidate. Lhx1 directly regulates *Vip* and likely *Nms*, *Prok2*, and *Enk*, but perhaps not *Grp* and *Avp*, as they lack conserved Lhx1 binding sites in their proximal enhancers.[46,77] *Creb3l1*, another Lhx1-regulated gene, is a more likely direct regulator of *Avp* downstream of Lhx1, though the role of CREB3L1 in SCN development has not yet been assessed.[77,78] Although its precise composition remains to be worked out, this LHX1-dependent transcriptional network is essential for adult SCN function, as *Six3-Cre;Lhx1*$^{lox/lox}$ mice show assorted defects in circadian entrainment of activity rhythms, including fragmentation or total arrhythmicity in the absence of external light cues.[46]

Further downstream, the role of *Rora* in SCN development has also been examined; *Rora*$^{sg/sg}$ loss-of-function mice surprisingly have a morphologically wild-type SCN with normal *Vip* and *Avp* expression, suggesting *Rora* may be dispensable for SCN development. However, the molecular characterization of *Rora*$^{sg/sg}$ SCN tested only these two molecular markers, and it is possible that subtle deficits in SCN development went undetected.[45] Currently, the only factor known to control SCN differentiation downstream of LHX1 is FGF8, which is required for SCN-specific expression of *Avp*. This may reflect a role for FGF8 in promoting maturation of this population, as FGF8 appears to do for AVP+, PVN neurons.[70,71] Looking ahead, dissecting the transcriptional networks upstream and downstream of *Lhx1* is a promising approach for better understanding not only SCN differentiation, but also the adult function of the nucleus. Already, the *Six3-Cre;Lhx1*$^{lox/lox}$ mouse presents a unique opportunity to study interactions among SCN signals by disrupting many of them simultaneously with a single gene deletion, and it has been found that *Lhx1* expression is directly regulated by light in adulthood, suggesting a possible role for LHX1 in mediating environmental control of neuropeptide signaling.[46,77]

DORSOMEDIAL HYPOTHALAMUS (DMH)

The DMH is reciprocally interconnected to many different hypothalamic regions, and functions at least in part as an association area that integrates information from many different modalities. A well-studied but highly controversial example is its contribution to food entrainment of circadian rhythms.[79–82] The DMH arises from a *Rax*(−) domain of the *Nkx2.1*(+) hypothalamic ventricular zone (HVZ), a region also characterized by low *Six3* and expression of *Otp* and *Nkx2.2*.[8,42,43] By E12.5, the ID domain that gives rise to the DMH expresses *Dlx2*, *Arx*, *Lhx1*, and *Lhx8*, much like developing SCN (Figures 3 and 4). But complementarily to SCN, by E16.5 *Lhx8* is preserved and *Lhx1* is downregulated in

DMH, with weak *Lhx8* expression preserved through adulthood in the dense ventromedial core subdomain of DMH.[8]

No studies have focused solely on DMH cell fate, though some broader studies included characterization of DMH phenotypes. NKX2.1 is crucial for early specification of the entire TH and PH (Figure 5(c)and (e)), and *Nkx2.1*$^{-/-}$ DMH is hypocellular and fused with ventromedial hypothalamus (VMH).[83] Conversely, RAX may suppress selection of DMH fate within TH (Figure 5(c)), as *Shh-Cre;Rax*$^{lox/lox}$ mice ectopically express the DMH markers *Dlx2*, *Gad67*, and *Lhx1* in a portion of the VMH at E12.5.[43]

Otp may be necessary to specify certain DMH lineages (Figure 5(c)); for instance, *Otp*$^{-/-}$ DMH appears to lack *Sst* expression,[54] though this was not specifically discussed in the original manuscript. Meanwhile, DLX1 is necessary for normal development of A12 dopamine neurons, some of which are located in the DMH[84] (Figure 5(c) and (d)). The transcription factors that specify DMH from tuberal hypothalamic neuroepithelium and guide its terminal differentiation are currently unknown. In fact, it is unclear whether DMH's dense ventromedial core and diffuse dorsolateral shell subdivisions even represent unitary or separable developmental compartments. *Lhx8* is a factor of particular interest in this context.

Ventromedial Hypothalamus (VMH)

The VMH is a multifunctional nucleus, including major roles regulating feeding and reproductive behavior.[85,86] VMH is derived from the *Rax*(+)/*Nkx2.1*(+)/*Six3*-high HVZ, which is also *Otp*(−) and *Nkx2.2*(+).[8,42,43] As discussed previously for the DMH, *Nkx2.1* is necessary for specification of VMH, whereas *Rax* prevents the VMH lineage from assuming a DMH-like fate[43,83] (Figure 5(d)). *Ascl1* (also known as *Mash1*) is broadly expressed here, and *Ngn3* is expressed within a more restricted region. Basic helix-loop-helix transcription (bHLH) factor activity, but not these specific factors per se, are required for VMH neurogenesis (Figure 5(d)); in *Ascl1*$^{-/-}$ mice, most VMH neurons are lost excepting a small pool derived from a residual *Ngn3*(+) region, but a *Ngn2* knock-in at the *Ascl1* locus (*Ascl1*$^{Ngn2/Ngn2}$) rescues this defect.[87] Notch signaling may regulate *Ascl1* expression as it does in ARC, but this has not been directly examined.[41] *Nr5a1* (also known as *Sf-1*) is the earliest selective VMH marker, expressed in all VMH neurons exiting neurogenesis; however, *Nr5a1* becomes restricted to dorsal-medial (dm)VMH by E14.5, when a distinct *Nr5a1*(−) cell cluster emerges in ventro-lateral (vl)VMH.[8,88] By adulthood, VMH is even further subdivided, between *Nr5a1*(+) dmVMH, *Nkx2.1*(+) vlVMH, and *Isl1*/*ERα*(+) vmVMH.[89]

Both *Ascl1* and *Ngn3* are required for specification of VMH *Nr5a1*(+) neurons (Figure 5(d)); their numbers are dramatically decreased in both *Ascl1*$^{-/-}$ and *Ngn3*$^{-/-}$ mice. This effect is specifically mediated by *Ascl1*, as *Ascl1*$^{Ngn2/Ngn2}$ knock-in mice show only very modest rescue of *Nr5a1* expression.[87,90] A screen of factors upregulated in neonatal mouse VMH and downregulated in adulthood uncovered a number of differentially expressed genes, including *Vgll2*, *Nr5a1*, *Sox14*, *Satb2*, *Fezf1*, *Dax1*, *Nkx2.2*, and *Nr2f2*. The roles of most of these factors in regulating VMH development largely remain unclear, though in

zebrafish hypothalamus, morpholinos targeting *a2bp1*, *fezf1*, *satb2*, or *sox14* modestly downregulate expression of *nr5a1*.[91]

Nr5a1 is a master regulator of VMH terminal differentiation (Figure 5(d)). Although animals lacking *Nr5a1* show unchanged VMH cell numbers, all of its subdivisions are grossly disorganized.[92,93] The pan-VMH marker *Bdnf* and subdomain-specific markers including *Nkx2.1*, *Isl1*, *ERα*, *Npy*, and *Gal* are all selectively downregulated and/or aberrantly distributed in mutant VMH, and GABAergic markers that are normally excluded from VMH are ectopically expressed.[94–96] Furthermore, development of VMH efferent projections to targets both inside and outside of the hypothalamus are compromised.[96,97] Consistent with these developmental defects, $Nr5a1^{-/-}$ mice have profound behavioral defects; these mutants are hyperphagic, obese, anxious, and infertile.[98–100]

For the future, identifying the molecular cues that functionally subdivide the VMH downstream of *Nr5a1* is a topic of particular interest. One promising avenue is the study of genes that directly regulate NR5A1 function, such as DAX1, which is coexpressed with *Nr5a1* during VMH development. DAX1 inhibits NR5A1 function through direct protein–protein interaction, and DAX1 mutations cause defects in human sexual maturation that likely partially result from disrupted VMH differentiation.[101–103] Another negative regulator of NR5A1 is FOXO1; while the developmental expression pattern of this factor is unclear and its effects on VMH gene expression have not been explored, $Nr5a1\text{-}Cre;Foxo1^{lox/lox}$ mice are lean, with heightened leptin and insulin sensitivity, the polar opposite of the phenotype seen in *Nr5a1* mutants.[98,104] Genes whose expression delineates *Nr5a1*(−) domains of the VMH in adulthood, such as *Isl1* and *Nkx2.1*, are also likely candidates for specifying these respective subdomains from *Nr5a1*(+) VMH progenitors.[89]

Arcuate Nucleus (ARC)

The ARC serves as a crucial regulator of feeding homeostasis, as well as dictating the release of most systemic hormones not controlled by the dlAH.[105,106] The ARC is derived from the same *Rax*(+)/*Nkx2.1*(+)/*Six3*-high zone as VMH at E12.5, and both *Nkx2.1* and *Rax* are required for ARC specification[43,83] (Figure 5(e)). However, *Otp* and *Dlx* family genes are also expressed early in ARC development, though *Nkx2.2* and *Arx* are not.[8,42,84]

As in VMH, *Ascl1* and to a lesser extent *Ngn3* are present in ARC progenitors and required as general bHLH factors for neurogenesis[43,83,87] (Figure 5(e)). Notch signaling inhibits ARC neurogenesis, as conditional deletion of the transcription factor *Rbpj*, which mediates Notch-dependent gene expression through interaction with the Notch intracellular domain, increases *Ascl1* expression and *Pomc*(+) neuron number at E13.5, leading to a corresponding increase in the number of multiple ARC neuropeptidergic populations at E18.5.[41]

It has been reported that *Ascl1* (but not other bHLH factors) drives not only expression of the early marker *Pomc* in ARC progenitors at E10.5[87,37] (Figure 5(e)), but also transient expression of *Nr5a1*, which is downregulated in the ARC and becomes specific to the VMH shortly thereafter.[87] However, other studies do not detect *Nr5a1* expression in ARC even very early in development,[8] and fate mapping with a knock-in *Nr5a1-Cre* driver does not

label ARC neurons.[107] One possible explanation for these divergent results comes from the finding that the *Nr5a1* paralog *Nr5a2* is expressed at high levels in adult ARC kisspeptin(+) (KISS) neurons[108] and more broadly in ARC as late as E14.5.[109] It is thus possible that *Nr5a2* expression may instead have been detected with the anti-NR5A1 antibody used in the McNay et al, 2006 study, a possibility which could be readily tested by fate mapping using an *Nr5a2-Cre* driver. In any case, it is uncontroversial that *Ascl1*, which as a general neurogenic bHLH factor is required for specification of many neuronal subtypes, is also required for specification of the *Pomc*(+) lineage (Figure 5(e)). $Ascl1^{-/-}$ mice lack *Pomc* expression at all time points examined, whereas $Ascl1^{Ngn2/Ngn2}$ knock-in mice recover normal *Pomc* expression by E12.5.[87]

Partial loss of *Ngn3* expression in $Ascl1^{-/-}$ ARC, and prominent *Ngn3* expression in both ARC progenitors and postmitotic neurons, suggest that *Ngn3* acts downstream of *Ascl1* in its regulation of ARC development[87,90] (Figure 5(e)). Much like *Ascl1*, *Ngn3* is also required for the early *Pomc*/*Nr5a* expression in many ARC neurons at E10.5, but is partially dispensable for initial specification of the adult *Pomc*(+) lineage, as cell number partially recovers by E15.5. The very different *Pomc* specification phenotypes observed early and late in the development of both *Ascl1* and *Ngn3* mutants are consistent with developmentally phased control of ARC *Pomc* expression by two independent, conserved enhancers,[110] only one of which contains target sites for early-expressed transcription factors such as NKX2.1.[111,112] The factors required for specification of the adult *Pomc*(+) lineage remain relatively poorly understood, despite the central anorexigenic role played by these neurons in feeding homeostasis,[105,113,114] though maternal dietary and hormonal signals are known to influence *Pomc*(+) neuronal specification.[115] Thoroughly understanding this process is especially important in light of the highly plastic fate of early *Pomc*(+) cells; fate mapping studies using the mouse *Pomc-Cre* allele showed that *Pomc* is expressed early in the development of not only adult *Pomc*(+) neurons, but also other ARC lineages,[37] including the orexigenic *Npy*/*Agrp*(+) neurons that directly oppose the action of adult POMC neurons on feeding.[105,116]

Downstream of the early bHLH transcription factors, *Nhlh2* and *NeuroD1* remain downregulated in most $Ngn3^{-/-}$ *Pomc*(+) neurons past E15.5, despite the partial recovery in *Pomc*(+) cell number in $Ngn3^{-/-}$ mice seen at this stage. This suggests that *Ngn3* may also control terminal differentiation of ARC neurons, as *Nhlh2* directly drives ARC expression of *Pcsk1*/*2*, enzymes required for proteolytic processing of αMSH from the POMC propeptide.[72,117,118] Terminal differentiation of these cells is crucial for maintenance of normal metabolic homeostasis; $Nhlh2^{-/-}$ mice exhibit a rare adult-onset obesity phenotype, likely caused by incomplete differentiation of *Pomc*(+) ARC neurons.[119] Finally, although *Neurod1* has not been functionally studied in the ARC, it is able to drive *Pomc* expression in concert with the pituitary transcription factor *Ptx1* in cultured cells,[120] and thus may also play a role in these neurons' differentiation in the ARC. The discovery of functionally distinct *Pomc* neurons that respond differentially to metabolic cues such as leptin, insulin, and serotonin makes the terminal differentiation of these cells particularly interesting.[121,122] Whether the later-acting bHLH factors *Nhlh2* and *NeuroD1*, or other factors, are responsible for this functional divergence is currently unclear.

In contrast to their effects on *Pomc*(+) neuron differentiation, *Ascl1* and *Ngn3* suppress terminal differentiation of ARC *Npy*(+) and A12 dopamine cells (Figure 5(e)), even though *Ascl1* is required for their initial generation.[87,90] *Bsx* is crucial for terminal differentiation of *Npy*/*Agrp*(+) neurons (Figure 5(e)); while the *Bsx*(+) lineage is preserved after *Bsx* deletion, ARC *Npy* and *Agrp* expression are selectively and severely downregulated.[123] *Dlx1* promotes specification of the A12 dopamine lineage in ARC, similar to its role in DMH[84] (Figure 5(d) and (e)). In addition, BMP signaling is required specifically for ARC A12 neuron development (Figure 5(e)), as these cells are selectively lost in the ARC but not DMH of *Olig1-Cre;Bmpr1a*$^{lox/lox}$ mice that lack the receptor in ARC progenitors.[124]

The development of GHRH neurons is perhaps the best understood of all ARC cell types. ASCL1 promotes development of *Ghrh*(+) neurons in ARC[87] (Figure 5(e)). Unlike most *Ascl1*-regulated ARC lineages, NGN3 does not appear to regulate development of the *Ghrh*(+) lineage; instead, ASCL1 specifies these neurons by regulating *Gsh1*, an essential factor for ARC *Ghrh* expression[90,125] (Figure 5(e)). *Hmx2* and *Hmx3* are also redundantly required for specification of GHRH neurons[126] (Figure 5(e)). The requirement of *Gsh1* and *Hmx2*/*3* for proper GHRH neuron development is ARC-specific; VMH *Ghrh* expression is unaffected by loss of these factors, and only ARC GHRH neurons contribute to neuroendocrine deficiencies that cause the dwarfism observed in *Gsh1*$^{-/-}$ and *Hmx2*$^{-/-}$;*Hmx3*$^{-/-}$ mice.[125,126] Meanwhile, ARC expression of *Sst* requires *Otp*, ARC expression of *Gal* requires either *Hmx2* or *Hmx3*, and ARC *Kiss* expression requires *Nr5a2*[54,108,126] (Figure 5(e)).

Lateral Hypothalamus (LH)

The LH regulates many different behaviors, perhaps most notably arousal.[127] Very little is known about factors determining cell fate in the LH, including whether the diffuse morphology and heterogeneous gene expression of the region reflects a unitary developmental unit or, more likely, many developmentally distinct compartments. *Foxb1* is transiently expressed in progenitors that give rise to many LH neurons, at least some of which migrate from prethalamus, and parts of LH are likely derived from *Lhx9*(+) and *Lhx6*(+) domains of the ID/TT complex.[8,128]

Neurons expressing the neuropeptide gene hypocretin/orexin (*Hcrt*) are the primary lineage whose development has been studied in LH, due to their prominent role in stabilizing sleeping and waking states.[129,130] LHX9 does not appear to directly regulate *Hcrt* expression, but in *Lhx9*$^{-/-}$ LH a third of *Hcrt*(+) cells are lost, suggesting a role of *Lhx9* in specification, differentiation, or survival of this subset.[47] More generally, *Shh* is required for specification of all *Hcrt*(+) neurons, as well as most *Mch*(+) neurons.[25]

Posterior Hypothalamus (PH), Including Zona Incerta (ZI), Tuberomammillary Nucleus (TMN), Premammillary Nucleus (PMN), Medial Mammillary Nucleus (MMN), Lateral Mammillary Nucleus (LMN), and Supramammillary Nucleus (SMN)

The nuclei of the PH have prominent roles in memory formation, among other functions.[131] Much like the LH, development of the PH is poorly understood. *Rax* is transiently expressed in a subset of PH progenitors, though expression here is weak.[8,50] Many genes expressed

early in mouse dlAH are simultaneously expressed in PH, including *Fezf2*, *Otp*, and *Sim1*; however, other factors including *Emx2*, *Foxb1*, and the broad tuberal-posterior marker *Nkx2.1* distinguish the PH expression pattern from dlAH.[8,42] *Nkx2.1* is required for very early stages of PH specification, as PMN, MMN, and SMN are essentially absent in *Nkx2.1*$^{-/-}$ mice[83] (Figure 5(f)). Zebrafish MO studies show that Fezf2 is required for expression of *otpa*/*otpb* and *emx2*, but not *foxb1.2* (Figure 5(f)). However, Otpa/b in turn inhibits *fezf2* expression, Foxb1.2 inhibits *fezf2* and *sim1a* expression, and Fezf2 inhibits *foxb1.2* and *sim1a* expression (Figure 5(f)). This leads to stratification of the PH, with *fezf2* ventral, *foxb1.2* dorsal, and *otpa*/*otpb* lateral in mostly nonoverlapping domains. *sim1a* is coexpressed in large portions of all three domains, and extends somewhat beyond them anteriorly.[132] All of these factors are expressed in developing mouse PH, and may serve important developmental roles in mammals as well.[8,42]

The mouse PH is further subdivided by expression of multiple transcription factors beginning at E11.5. *Lhx6* labels TT, which gives rise to GABAergic neurons of the ZI and PMN. *Lef1* in turn labels PMN, *Foxb1* labels MMN, and *Irx5* selectively labels SMN.[8] Nonoverlapping expression domains of these genes are conserved in zebrafish PH.[132] *Foxb1* is essential for MMN specification, which is completely missing in *Foxb1*$^{-/-}$ mice, disturbing organization of the overall PH[133] (Figure 5(f)). Loss-of-function studies have not been carried out for the other factors.

Factors controlling the selection of a few particular fates have been studied in zebrafish PH. *fezf2* and Pac1 signaling act through *otpb* to drive dopaminergic cell fate, primarily in the PH.[51,60] *vip*(+) cells are generated in the *sim1a*(+)/*otpa*/(+)*otpb*(+) domain, and MO studies showed that these factors (as well as Fezf2) are required for PH *vip* expression.[132] Meanwhile, *uts1*(+) cells are generated in the *fezf2*(+)/*sim1a*(+) region; both factors are required for *uts1* expression.[132] However, it is unclear whether some of these findings in zebrafish PH apply to vertebrates more broadly, as data on whether most of these lineages are present in mammalian PH is contradictory. While neither *Vip* nor *Ucn1* (the mammalian *uts1* ortholog) are detectable anywhere in mouse PH by ISH, colchicine-enhanced immunohistochemistry experiments in rat have detected VIP and UCN1 in SMN, and UCN1 in LMN.[42,134,135] Lineage tracing studies in mouse present the surest option for resolving this confusion.

Remaining studies of PH development have largely focused on formation of the mammillothalamic and mammillotegmental tracts. *Sim1* and *Sim2* are required in partially redundant pathways for formation of both; unlike in dlAH, these effects are independent of *Arnt2*.[136] Furthermore, mammillary *Foxb1* is required for mammillothalamic tract formation, whereas *Pax6* is required in cells that follow the mammillothalamic axons to their point of bifurcation from the mammillotegmental tract near the ZI.[137–139] Meanwhile, pan-neuronal deletion of *Pitx2* in *Nestin-Cre;Pitx2*$^{lox/lox}$ mice causes both failure of mammillothalamic tract elongation and loss of *Pax6*(+) cells, with more subtle mammillotegmental tract defects.[140] No genes are known to selectively regulate mammillotegmental tract formation.

CONCLUSION

The development of the hypothalamus remains poorly understood, with large and obvious gaps in the literature at every developmental stage. This is in large part due to the enormous complexity of its anatomy, gene expression, and functionality. However, going forward, large-scale studies of gene expression in developing hypothalamus[8,42,66,91,109] provide a springboard for new avenues of research. The relevance of this work is substantial and only continues to grow, as chronic diseases affecting physiological processes controlled by the hypothalamus, such as metabolic and sleep disorders, continue to increase in prevalence (Boxes 1–3).

BOX 1

THE PROSOMERE MODEL AND HYPOTHALAMIC DEVELOPMENT

The prosomere model of forebrain development is a framework for understanding patterning of the vertebrate forebrain, including the hypothalamus, and has undergone multiple modifications. Originally, the theory proposed that six segmented domains, or prosomeres (p1–p6), compose the embryonic forebrain: the diencephalon, including the epithalamus (p1), thalamus (p2), and prethalamus (p3), and the secondary prosencephalon (p4–p6), including the hypothalamus and telecephalon.[141] In 2003, the model was updated to accommodate new gene expression data.[32,142,143] Prosomeres p4–p6 were eliminated and the secondary prosencephalon (SP) was redefined as a unitary compartment, containing both the telencephalon and hypothalamus[143,144]. This version of the model places the eye field as the rostral most domain of the neuraxis, as it lies immediately anterior to the PCP.[143] The latest version of the model, released in 2013, places the hypothalamus and preoptic area as the most anterior regions of the neuraxis.[144] This stands in sharp contrast to other models of forebrain development, which place the rostral pole of the telencephalon in this position.[145]

The prosomere model makes several testable predictions: (1) Given the postulated alignment of the neuraxis, the telencephalon should be dorsal, not rostral, to the hypothalamus. (2) Domains of gene expression should be contiguous and symmetrical within, but not between, individual prosomeres. Importantly, this includes the telencephalon and hypothalamus, which the model has grouped together as a common developmental unit in the SP. (3) Altered expression of morphogens and transcription factors that pattern the forebrain should trigger similar changes within, but not between, prosomeres.

Although this model is widely referenced and found in many neural development textbooks, many of its predictions have not withstood close scrutiny. Addressing the first prediction, physical separation between the structures ancestral to the telencephalon and dlAH long predates vertebrate evolution, as revealed by the annelid worm *Platynereis dumerilii*. In these animals, structures homologous to the vertebrate telencephalon develop rostral to the primordial NH.[146–148] Furthermore, these animals' lateral eyes develop from epidermal cells, rather than extending outward from an involuted neural tube, as in vertebrates.[149,150] This implies that the basic forebrain layout is inconsistent with the prosomere model, and that the mechanisms establishing this layout both predate

the emergence of vertebrate-specific patterning mechanisms, and may be generated in part by notochord-independent signals.

Second, when gene expression patterns reported to mark prosomere borders are carefully examined, it becomes less clear that these actually delineate the regions they are claimed to mark. In no small part, this stems from the fact that the gene expression data used to formulate the model comes from time points as late as E15.5, when hypothalamic neurogenesis is largely complete. On multiple occasions, this has led to claims being revised or withdrawn as expression at earlier developmental stages is more extensively characterized. For example, in 2003, it was stated that *Arx* indicates the border of P3 and the SP as its expression is limited to prethalamus[143]; however, later studies identified a domain of *Arx* expression extending well into the hypothalamus by E11.5.[8] Many other gene expression domains also clearly traverse the borders of the prethalamus (p3) and hypothalamus (SP) by E11.5, including *Isl1*, *Foxd1*, *Prox1*, *Emx2*, and *Lhx5*.[8] Such expression patterns are more consistent with a model in which the hypothalamus and prethalamus form a common developmental unit.

The prosomere model makes specific predictions about the developmental consequences of disrupting genes expressed in individual prosomeres. For example, it has been claimed that loss of the SP in $Six3^{-/-}$ and $Hesx1^{-/-}$ mice supports the claim that the SP is a single histogenic field.[143] However, subsequent studies did not support this interpretation. In $Six3^{-/-}$ mice, the *Six3* (+) prethalamus also fails to develop properly; furthermore, both the prethalamus and PH are rescued when *Wnt1* is deleted along with *Six3*,[151] suggesting that *Six3* is essential only for development of the telencephalon and AH.

A more recent study revealed that mice deficient for *Lhx2*, normally expressed in progenitors of the eye field and ventral hypothalamic neuroepithelium, show ectopic expression of genes specific to both the PVN and thalamic eminence in the normally *Lhx2* (+) regions.[44] *Lhx2* is required to repress these genes even after the onset of retinal neurogenesis. The thalamic eminence adjoins the PVN along the diencephalic–telencephalic border,[8] and the most parsimonious explanation for these results is that these structures, along with the vAH and eye field, derive from a common pool of progenitors. This is hard to reconcile with the prosomere model, which holds that the thalamic eminence is part of p3, not the SP compartment containing PVN and the rest of the hypothalamus.[143]

Finally, loss of key developmental regulators expressed in both hypothalamus and telencephalon often results in very different phenotypes in the two regions. For instance, loss of *Ascl1a* causes loss of *Dlx1/2* and *Gad1b* in prethalamus and hypothalamus, but not telencephalon. Loss of *Dlx1/2* has a similar effect on *Ascl1a* expression.[152]

Taken together, these data are consistent with a more classical model of forebrain organization, in which the hypothalamus lies ventral to the prethalamus, and both structures reside posterior to the developing telencephalon, which originates from a separate developmental compartment. Now that large numbers of mouse lines are available for conducting cell lineage analysis and conditional inactivation of candidate developmental regulatory genes, along with the ability to reconstruct gene expression

patterns at high resolution in 3D, it is likely that these long-unsettled questions of vertebrate forebrain organization may soon be resolved once and for all.

BOX 2

HYPOTHALAMIC PROLIFERATIVE ZONE (HPZ)

Tanycytes are a radial glia-like cell population lining portions of some ventricles in the brain. A population of tanycytes lining the ventral portion of the third ventricle at the coronal plane of the TH forms a recently discovered neurogenic niche: the HPZ. Lineage tracing revealed that ventral β2-tanycytes of postnatal mice are a diet-responsive neurogenic unit that populates the median eminence (ME), whose proliferative activity is required for metabolic homeostatic plasticity in response to diet.[153] Although this study yielded little evidence of neurogenesis among other tanycyte subpopulations, other studies have suggested that more dorsally located α-tanycytes become neurogenic when exposed to FGFs, and may be neurogenic in adulthood.[154–156] Reviews discussing these results in more detail can be found elsewhere.[157,158]

As the HPZ is the only anatomically well-defined neurogenic niche in the adult hypothalamus, the study of factors controlling tanycytic cell fate serves as a potential model system for understanding control of hypothalamic neurogenesis and very early events in cell type specification. HPZ tanycytes are morphologically reminiscent of radial glia, and express genes characteristic of hypothalamic progenitors, including *Rax*, *Lhx2*, and Notch pathway components.[8,153] Both RAX and LHX2 have been shown to be required for tanycyte differentiation. $Rax^{+/-}$ haploinsufficient mice show partial conversion of tanycytes to the nonneurogenic ependymal cell lineage,[159] whereas mice selectively lacking RAX in early hypothalamic progenitors lack expression of tanycyte-specific genes altogether.[160] *Lhx2* is required to maintain expression of *Rax* (along with other tanycyte-specific genes) in both differentiating and mature tanycytes,[160] which tallies with previous work showing Lhx2-dependent regulation of *Rax* in other anterior neural structures.[44,161] Interestingly, although *Lhx2*-deficient tanycytes retain radial morphology, they ectopically activate expression of markers of ependymal cells such as *Foxj1* and become multiciliated,[160] suggesting that LHX2 may promote tanycyte specification while simultaneously repressing ependymal fate.

BOX 3

A CASE STUDY OF EXTRA-HYPOTHALAMICALLY DERIVED CELLS: GNRH NEURONS

In contrast to the great majority of hypothalamic neurons, which arise locally from progenitors within the hypothalamus,[162] gonadotropin-releasing hormone (GNRH) neurons originate in the olfactory placode at E11.5 and migrate into AH and TH from E12.5–15.5.[163,164] GNRH expression in these cells steadily increases from E12.5–19.5, and expression positively correlates with distance from the olfactory bulb, suggesting that

both developmental age and position within the brain jointly contribute to differentiation and maturation of these neurons.[165]

Both FGF8 signaling through FGF receptor 1 and *Sox2* expression are required for GNRH(+) neurogenesis in the olfactory placode.[166–168] Immature GNRH(+) neurons express *Msx1* and *Oct6*, which directly suppress *Gnrh* expression and possibly maturation more generally.[169,170] They also express GRG4, a cofactor that maintains *Gnrh* suppression through interactions with binding partners, along with TYRO3 and AXL, which stimulate migration toward the hypothalamus.[171–173] Once the developing neurons reach the cribriform plate, they must upregulate expression of *Lhx2* in order to cross.[174] It would be interesting to determine whether *Lhx2* induction directly leads to downregulation of *Axl* and induction of *Mer* (both factors capable of regulating migration) as these neurons mature,[172] as this would explain the requirement for *Lhx2* in the transition of GNRH(+) neurons to the next stage of migration. Finally, numerous factors are required for *Gnrh* expression in mature neurons. These include *Oct1*, *Gata4*, *Otx2*, *Brn2*, *Dlx1/2*, and *Six6*, which directly promote GNRH expression; *Grg5* and *Pbx1*, cofactors for GNRH activation; and *Necdin*, which relieves *Msx1* suppression of *Gnrh*.[169,173,175–183]

After decades of heroic effort, more transcription factors have been implicated in GNRH(+) neuron development than for any other hypothalamic cell type. However, a large subset of these genes have only been studied in immortalized cell lines. Validating their roles *in vivo* would be a useful stepping stone to confirming their contributions to GNRH(+) neuron specification and differentiation. A search for other hypothalamic lineages derived from extra-hypothalamic precursors is also likely to be fruitful, and eminently feasible using Cre lines driven by regulatory elements from genes such as *Foxg1*, that are expressed early in other brain regions and selectively excluded from the developing hypothalamus.[184]

AN INDEX OF ANATOMICAL ABBREVIATIONS USED IN THE TEXT

AH	anterior hypothalamus;
AP	anterior-posterior
ARC	arcuate nucleus
APV	anterior periventricular nucleus
BP	basal plate
dlAH	dorsolateral anterior hypothalamus (neurosecretory hypothalamus)
DMH	dorsomedial hypothalamus
DV	dorso-ventral
E[x]	embryonic day x in mouse

HH	Hamburger-Hamilton developmental stage in chick
HPZ	hypothalamic proliferative zone
HVZ	hypothalamic ventricular zone
ID	intrahypothalamic diagonal
LH	lateral hypothalamus
LMN	lateral mammillary nucleus
ME	median eminence
MMN	medial mammilary nucleus
p[x]	prosomere x
PCP	prechordal plate
PH	posterior hypothalamus
PMN	premammillary nucleus
PVN	paraventricular nucleus of the hypothalamus
SCN	suprachiasmatic nucleus
SMN	supramammillary nucleus
SON	supraoptic nucleus
SP	secondary prosomere
TH	tuberal hypothalamus
TMN	tuberomammillary nucleus
TT	tuberomammillary terminal
vAH	ventral anterior hypothalamus
VMH	ventromedial hypothalamus
vPH	ventral posterior hypothalamus
ZI	zona incerta
ZLI	zona limitans intrathalamica.

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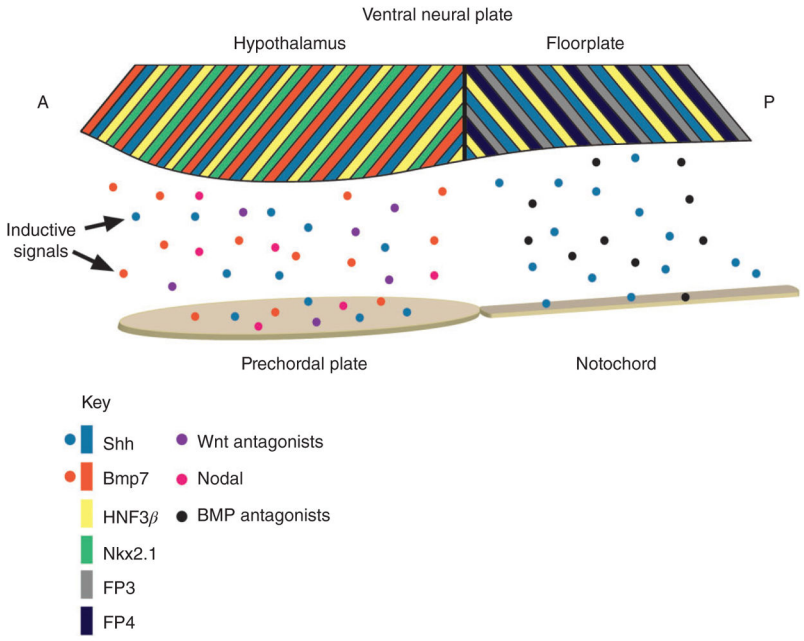

FIGURE 1.

Schematic showing the inductive signals that act on the ventral neural plate. The posterior neural plate receives inductive signals from the notochord to become floor plate, whereas the more anterior neural plate that goes on to become the hypothalamus receives a different set of inductive signals from the prechordal plate. Circles represent signaling from the prechordal plate/notochord, whereas the diagonal bars in the neural plate represent local gene expression. It should be noted that the juxtaposition of the prechordal plate and the hypothalamic neural plate is transient. These structures migrate out of register with each other. The prechordal plate reaches its final rostral position in advance of the hypothalamic neural plate, which migrates over and rostrally past the prechordal plate. Thus by the time the floor plate and the hypothalamic neural plate are specified, the prechordal plate actually resides caudal to the hypothalamic neural plate.[3] A, anterior; P, posterior

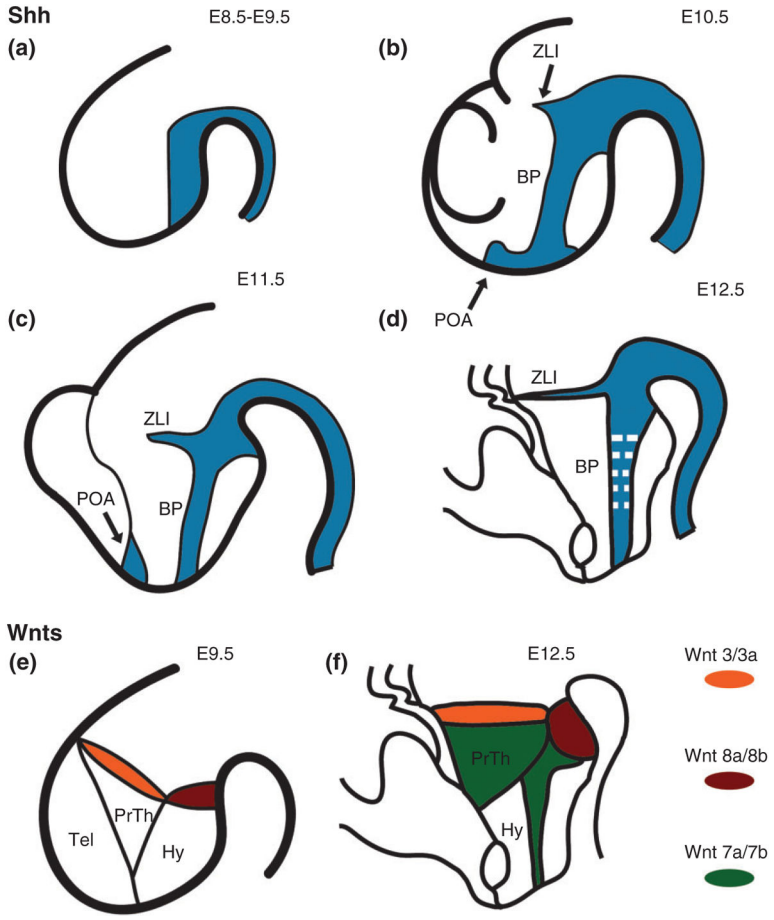

FIGURE 2.

Sagittal view of morphogen expression over the course of development in the mouse forebrain, emphasizing the hypothalamus. (a–c). Forebrain *Shh* expression from E8.5 through E11.5. (d). Hypothalamic *Shh* expression at E12.5. White bars represent areas of diffuse *Shh* expression. (e) Wnt expression in the forebrain at E9.5. (f). Wnt expression in the hypothalamus at E12.5. *Wnt 7a/7b* is expressed in interneuron progenitors only while *Wnt 3/3a* and *Wnt 8a/8b* are expressed more broadly. BP, basal plate; ZLI, zona limitans intrathalamica; POA, preoptic area; Tel, telencephalon; PrTh, prethalamus; Hy, hypothalamus.

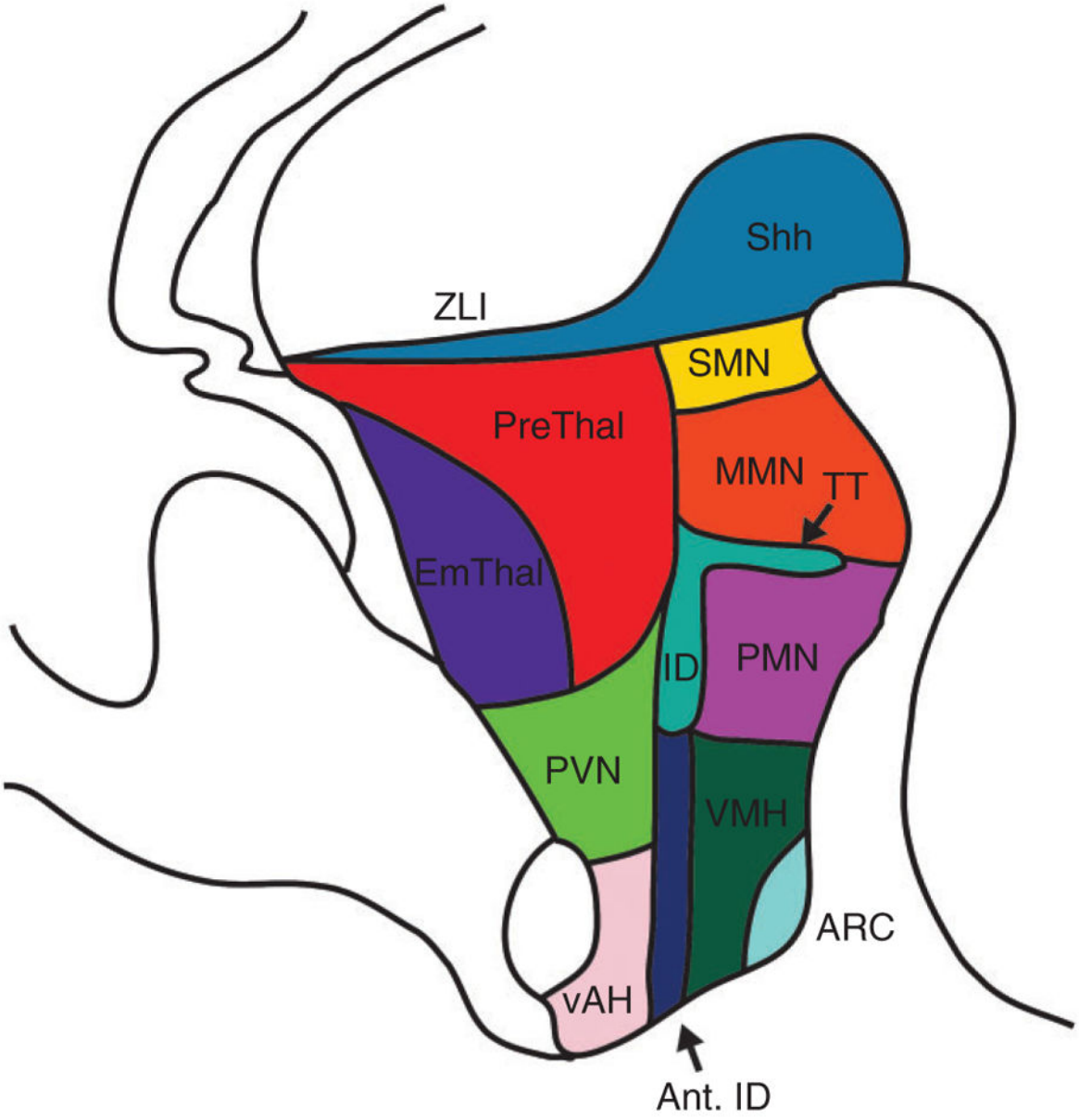

FIGURE 3.

Sagittal map of nuclei in the mouse hypothalamus at E12.5 at the level of the third ventricle. ZLI, zona limitans intrathalamica; PreThal, prethalamus; EmThal, thalamic eminence; PVN, paraventricular nucleus; vAH, ventral anterior hypothalamus; ant. ID, anterior intrahypothalamic diagonal; ID, intrahypothalamic diagonal; ARC, arcuate nucleus; VMH, ventromedial hypothalamus; PMN, premammillary nucleus; TT, tuberomammillary terminal; MMN, mammillary nucleus; SMN, supramammillary nucleus.

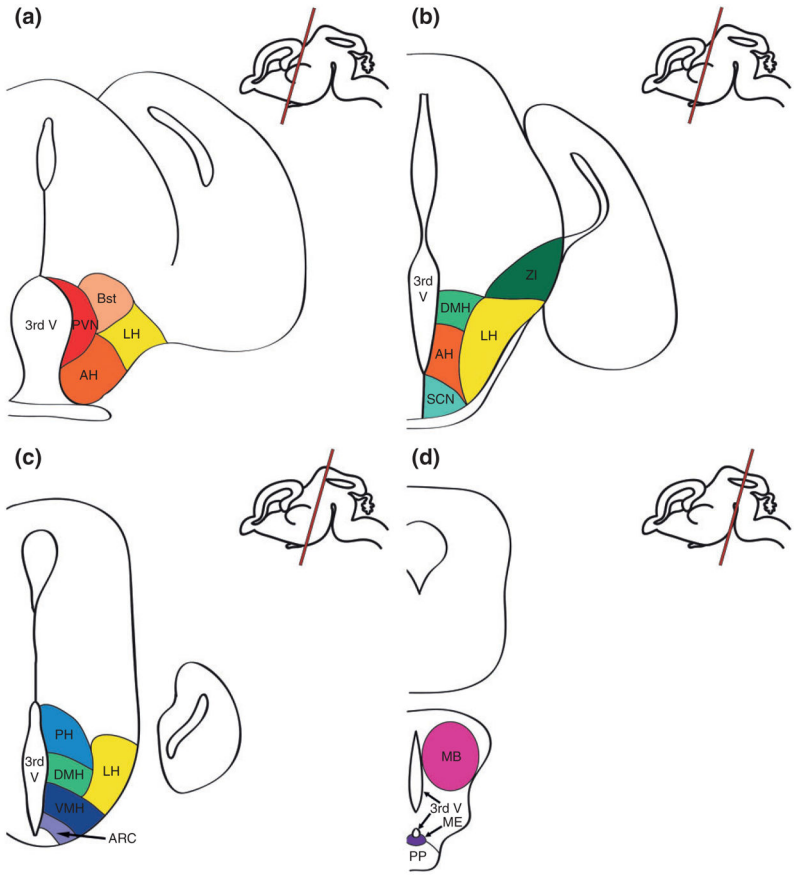

FIGURE 4.

(a–d) Coronal diagrams of the nuclei of the developing hypothalamus at ~E15.5. Insets in the upper right-hand corners indicate the approximate position of these coronal sections in the sagittal plane. Note that the angle of the cut shown in the developing hypothalamus sometimes places nuclei normally present on different coronal planes in typical adult brain sections on the same plane in our diagram (e.g.: SCN and DMH in panel b). Abbreviations indicating divisions of the hypothalamus larger than single nuclei as described in the text (e.g.: AH and PH) here show the disposition of poorly defined and/or less well-studied nuclei within those regions. For example, though the PVN and SCN are derived from the broader AH, in this figure they are shown separately and the region labeled AH only encompasses less-studied regions we do not discuss in detail, such as the periventricular, subparaventricular, and retrochiasmatic nuclei. SCN, suprachiasmatic nucleus; DMH, dorsomedial hypothalamic; PVN, paraventricular nucleus; AH, anterior hypothalamus; PH, posterior hypothalamus.

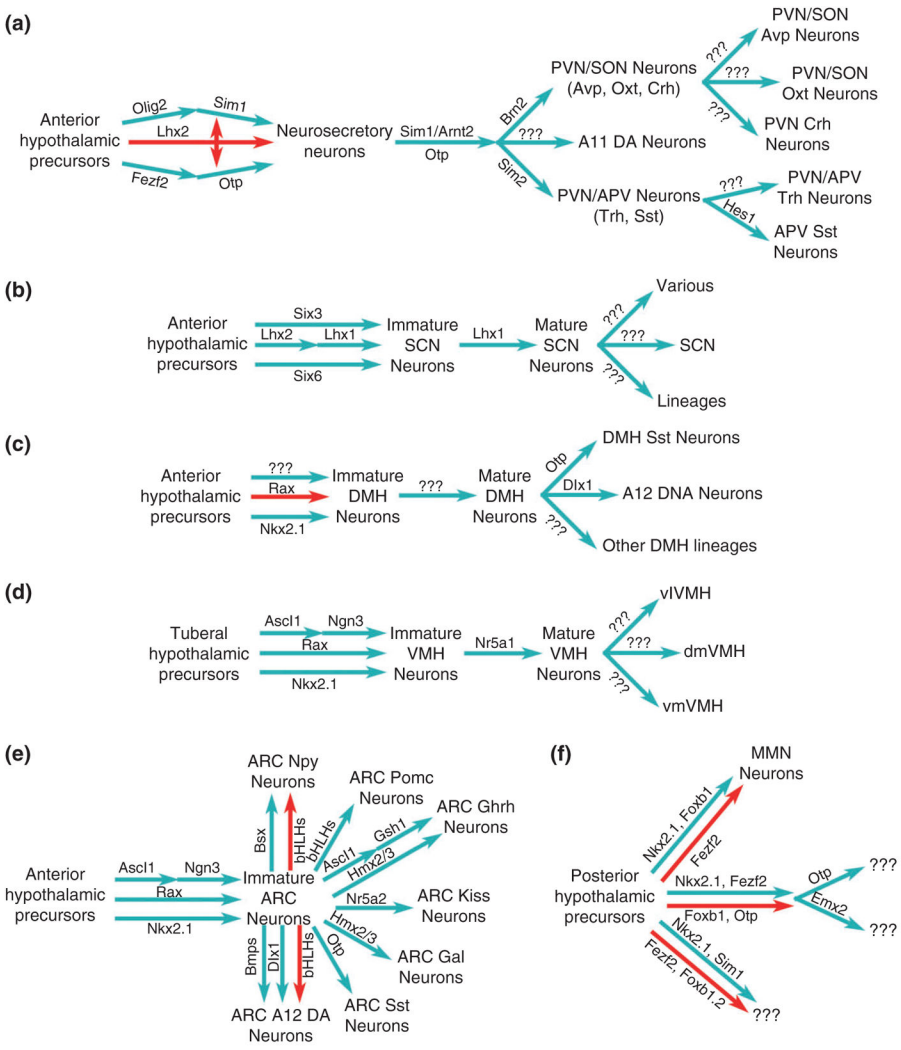

FIGURE 5.

Factors controlling specification and differentiation within specific nuclei or regions of the developing hypothalamus. Regions depicted include the dlAH (a), SCN (b), DMH (c), VMH (d), ARC (e), and PH (f). Blue arrows indicate factors that direct cells within the region toward the fate pointed at by its arrowhead, whereas red arrows indicate factors that inhibit the indicated fate. Serial arrows show that the downstream arrow is dependent upon the upstream arrow for its expression, directly or indirectly. Parallel arrows represent factors that act in concert to direct cells toward a common fate, but are not known to be directly dependent on one another for their expression. dlAH, dorsolateral anterior hypothalamus; SCN, suprachiasmatic nucleus; DMH, dorsomedial hypothalamic; VMH, ventromedial hypothalamus; ARC, arcuate nucleus; PH, posterior hypothalamus.