Molecular genetics of the developing neuroendocrine hypothalamus

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Abstract

Formation of the mammalian endocrine system and neuroendocrine organs involves complex regulatory networks resulting in a highly specialized cell system able to secrete a diverse array of peptide hormones. The hypothalamus is located in the mediobasal region of the brain and acts as a gateway between the endocrine and nervous systems. From an endocrinology perspective, the parvicellular neurons of the hypothalamus are of particular interest as they function as a control centre for several critical physiological processes including growth, metabolism and reproduction by regulating hormonal signaling from target cognate cell types in the anterior pituitary. Delineating the genetic program that controls hypothalamic development is essential for complete understanding of parvicellular neuronal function and the etiology of congenital disorders that result from hypothalamic-pituitary axis dysfunction. In recent years, studies have shed light on the interactions between signaling molecules and activation of transcription factors that regulate hypothalamic cell fate commitment and terminal differentiation. The aim of this review is to summarize the recent molecular and genetic findings that have advanced our understanding of the emergence of the known important hypophysiotropic signaling molecules in the hypothalamus. We have focused on reviewing the literature that provides evidence of the dependence on expression of specific genes for the normal development and function of the cells that secrete these neuroendocrine factors, as well as studies of the elaboration of the spatial or temporal patterns of changes in gene expression that drive this development.

1. Introduction

The hypothalamus influences a broad spectrum of physiological functions, including pituitary hormone synthesis and secretion, autonomic nervous system activity, energy intake and expenditure, body temperature, reproduction and behavior. Despite its physiological importance, we are only beginning to understand the molecular mechanisms underlying neural differentiation and development within the hypothalamus and the ontogeny of its connections with the pituitary. The hypothalamic parvicellular neurosecretory neurons are of particular interest due to their role in controlling anterior pituitary (AP) hormone secretion. For this reason, many studies have focused on the signaling molecules and transcription factors that control hypothalamic morphogenesis and the emergence of the seven known parvicellular neurosecretory neuronal subtypes (described in detail below). While much of the early research into hypothalamic development and function has been conducted in rats, recent advances in murine transgenesis and mutagenesis techniques have established mice as the principal model for analysis of central nervous system (CNS) development. Therefore, in this review we have focused primarily on rodent hypothalamic development but also key findings from other developmental models, including chick and zebrafish, which have contributed to our understanding of this field.

2. Functional anatomy of the neuroendocrine hypothalamus

The vertebrate hypothalamus is located ventral to the thalamus and dorsal to the pituitary gland, at the mediobasal region of the CNS. It extends from the optic chiasm (located anteriorly) to the mammillary body (located posteriorly) and is organized into four distinct rostral-to-caudal regions: preoptic, anterior, tuberal, and mammillary. It is also divided into three medial-to-lateral areas: periventricular, medial and lateral. The periventricular hypothalamus contains four distinct cell clusters: the paraventricular nucleus (PVN), arcuate nucleus (ARC), suprachiasmatic nucleus (SCN), and the periventricular nucleus (PeVN; **Figure 1A**). The medial hypothalamic zone is comprised of the medial preoptic nucleus, the anterior hypothalamus (AH), the dorsomedial nucleus, the ventromedial nucleus (VMN) and the mammillary nuclei. The lateral hypothalamus consists of the preoptic area (POA) and hypothalamic area.

Located throughout hypothalamus are hypothalamic neurosecretory cells that are divided into two populations: the parvicellular and magnocellular neurosecretory systems. The former consists of neurons controlling the release of specific AP neurohormones: thyrotropin-releasing hormone (TRH; located within the medial part of the medial parvicellular subdivision of the PVN), corticotropin-releasing hormone (CRH; located within the lateral part of the medial parvicellular subdivision of the PVN), growth hormone-releasing hormone (GHRH; located within the lateral part of the ARC), somatostatin (SS; located within the PeVN), gonadotropin-releasing hormone (GnRH; located within the medial POA), dopamine (DA; located within the medial part of the ARC and detected by the enzymatic activity of tyrosine hydroxylase) and, the recently discovered gonadotropin-inhibiting hormone (GnIH; located within the dorsomedial nuclei in rodents; **Figure 1B**). The magnocellular neurosecretory system consists of neuronal cells secreting two neurohormones, vasopressin (AVP) and oxytocin (OT), whose axons project directly into the posterior pituitary (neurohypophysis) and release peptides systemically in response to various homeostatic cues (osmotic, cardiovascular and reproductive). The primary focus of this review is the development and function of the parvicellular neurons. For in-depth information and discussion on the magnocellular neurosecretory system we refer the reader to the paper by (Caqueret *et al.*, 2006).

3. Hypothalamic induction and the role of signaling pathways

The hypothalamus develops from the ventral region of the diencephalon (Figdor and Stern, 1993) and, in the mouse, its primordium is morphologically evident from approximately 9.5 days post coitum (dpc; where 0.5 dpc is defined as noon of the day on which a copulation plug is present). Developmental studies performed in mice, chick and zebrafish indicate that sonic hedgehog (SHH) signaling plays an important role in the induction and early patterning of the hypothalamus (Manning *et al.*, 2006; Mathieu *et al.*, 2002; Szabo *et al.*, 2009). Secretion of SHH from the murine axial mesendoderm, from 7.5 dpc, is essential for correct patterning of the anterior midline. In humans as well as in mice, mutations in the *SHH/Shh* gene (and several other components of this pathway) result in holoprosencephaly due to a failure of hypothalamic anlagen induction and optic field separation (Chiang *et al.*, 1996; Schell-Apacik *et al.*, 2003). Increased SHH activity leads to ectopic expression of

hypothalamic markers in zebrafish, suggesting that SHH signaling has an instructive rather than a permissive role in shaping the hypothalamus (Barth and Wilson, 1995; Hauptmann and Gerster, 1996; Rohr *et al.*, 2001). Studies in chick have shown that once the hypothalamic primordium is established, down-regulation of *Shh* is critical for the progression of ventral cells into proliferating hypothalamic progenitors, at least within the ventral tubero-mammillary region (Manning *et al.*, 2006). In addition, *Shh* down-regulation is mediated, to some extent, by local production of Bone Morphogenetic Proteins (BMPs), which belong to the transforming growth factor-beta (TGF β) super-family of signaling proteins (Manning *et al.*, 2006). This antagonism between SHH (ventral gradient morphogen) and BMP (dorsal gradient morphogen) in the hypothalamus is reminiscent of their opposing actions in dorsal-ventral patterning of the neural tube. However, in the developing hypothalamus this incorporates a temporal aspect (SHH early - BMP late) that appears necessary for establishing region-specific transcriptional profiles (Ohshima *et al.*, 2008; Patten and Placzek, 2002). Although axial secretion of another member of the TGF β super-family, NODAL, is also necessary for hypothalamic induction, the early lethality of *Nodal* mutants has precluded detailed assessment of its role in hypothalamic development in mice (Brennan *et al.*, 2001; Conlon *et al.*, 1994; Varlet *et al.*, 1997). Genetic studies in zebrafish have shown that the Wnt signaling pathway is required for specification of the hypothalamic anlagen, its regionalization and neurogenesis (Kapsimali *et al.*, 2004; Lee *et al.*, 2006). Together, these studies have shown that hypothalamic induction and pattern formation depends on the activities of major protein signaling pathways involved in patterning, regional identity and cell fate determination.

4. Patterning the hypothalamic primordium

Embryonic neurogenesis in vertebrates follows a stereotypical progression that begins with the generation of the neural tube, which is composed of a pseudostratified columnar epithelium of cycling stem cells. As a general rule, these neuronal precursors acquire distinct positional identities, commit to a neuronal fate, exit mitosis, migrate away from the periluminal progenitor zone and terminally differentiate. A large body of evidence, gained principally from mouse and chick embryos, has established that transcription factors belonging to the homeodomain and basic Helix-Loop Helix (bHLH) families play a major role in neurogenesis (reviewed

in (Guillemot, 2007)). Regionally restricted expression of these factors is induced in response to local signaling cues (see above), establishing a transcription factor “code” that directs the generation of distinct neuronal cell types at each neuroaxial level. Mouse mutagenesis has identified several transcription factor pathways critical for the development of the parvicellular neurons in the POA, PVN, PeVN, VMN and ARC, which together provide the foundation for a rudimentary “hypothalamic transcription factor code” and are outlined below.

***Sim1/Arnt2-Brn2* pathway:** The bHLH-PAS transcription factor SIM1 is expressed in the incipient PVN, supraoptic nucleus (SON), and anterior PeVN (aPeVN) from 10.5 dpc and is maintained in these regions into postnatal development (Caqueret *et al.*, 2006; Michaud *et al.*, 1998). Homozygous *Sim1* mutants die shortly after birth and exhibit significant hypoplasia of the anterior hypothalamus. Histological and molecular marker analysis has revealed that these mutants lack virtually all neurons of the SON and PVN, including those expressing TRH and CRH. SS neurons in the aPeVN and other populations of TRH neurons in the lateral hypothalamus and in the POA region are also missing. Interestingly, mutant mice lacking the *Sim1* dimerisation partner ARNT2 have a strikingly similar phenotype, indicating that these proteins function cooperatively in the AH (Hosoya *et al.*, 2001; Keith *et al.*, 2001). A key downstream target of SIM1/ARNT2 is *Brn2*, which encodes a POU domain transcription factor and is required for the differentiation of CRH (as well as OT and AVP) neurons of the PVN/SON. *Brn2* expression in the prospective PVN/SON region is absent in *Sim1* and *Arnt2* mutants, indicating that *Brn2* is regulated by SIM1/ARNT2, although it is not currently known if this is a direct or indirect interaction.

***Otp*:** The homeobox gene *Orthopedia* (*Otp*) is expressed in neurons giving rise to the PVN, SON, aPeVN and ARC throughout their development. *Otp* mutants die as neonates and fail to generate the parvicellular and magnocellular neurons of the anterior PeVN, PVN, SON, and ARC (Acampora *et al.*, 1999; Wang and Lufkin, 2000). These defects are associated with reduced cell proliferation, abnormal cell migration, and failure of terminal differentiation. Like the *Sim1* and *Arnt2* mutants, *Otp* null embryos fail to maintain *Brn2* expression. However, OTP does not appear to directly interact with SIM1 or ARNT2 (Caqueret *et al.*, 2006) and SIM1 and OTP do

not regulate each other's expression (Acampora *et al.*, 1999), suggesting that OTP and SIM1/ARNT2 operate in parallel or convergent pathways.

Nkx2.1: During early development of the CNS, signals produced from the anterior axial mesendoderm induce expression of the homeodomain transcription factor gene *Nkx2.1* (also known as *Tebp*) in the overlying presumptive hypothalamus (Ericson *et al.*, 1998; Kimura *et al.*, 1996). *Nkx2.1* mutant mice die at birth and, in addition to lung and thyroid defects, exhibit profound abnormalities in the ventral hypothalamus, including agenesis of the ARC and VMN. Interestingly, null mutants also fail to generate the Rathke's pouch (which does not express *Nkx2.1*), confirming the ventral diencephalon/infundibular recess is essential for induction of the AP (Kimura *et al.*, 1996; Takuma *et al.*, 1998).

Sf1: The *Sf1* gene encodes an orphan nuclear hormone receptor that is required for normal development of the gonads and adrenals and function of pituitary gonadotropes (Ingraham *et al.*, 1994; Shinoda *et al.*, 1995). Within the CNS, *Sf1* is specifically expressed within the VMN and is required for multiple phases of VMN development. Analysis of *Sf1* null embryos indicates that this transcription factor is initially involved in the survival and migration of VMN precursors from the ventricular zone and at later stages is required for aggregation and condensation of the VMN nucleus and terminal differentiation.

Hmx2/Hmx3: Two closely related homeobox genes, *Hmx2* and *Hmx3*, are expressed in overlapping domains of the ventral hypothalamus from 10.5 dpc (Wang *et al.*, 2004). While single gene mutants do not have any discernable hypothalamic phenotype (although it bears noting that ear development is affected), *Hmx2;Hmx3* null mice exhibit postnatal dwarfism and a severe deficiency of GHRH neurons in the ARC, but not the VMN (Wang *et al.*, 2004). Expression of the homeobox gene *Gsh1*, which overlaps with *Hmx2* and *Hmx3* and is required for *Ghrh* expression, is also absent in *Hmx2;Hmx3* null embryos. Neuronal cell numbers in the ARC are not significantly different in double mutants indicating that, despite their widespread expression, *Hmx2* and *Hmx3* are not required for early determination of neuroprogenitors in this region of the hypothalamus.

***Mash1*:** MASH1 is a proneural protein that belongs to the bHLH family of transcription factors and is required for neurogenesis and subtype specification in many regions of the CNS (Parras *et al.*, 2002). *Mash1* is expressed throughout the ventral retrochiasmatic neuroepithelium from 10.5-12.5 dpc. *Mash1* null embryos exhibit hypoplasia of the ARC and VMN nuclei due to neurogenic failure and increased apoptosis (McNay *et al.*, 2006). Using a knock-in strategy, McNay *et al.*, (2006) elegantly showed that this phenotype could be rescued by ectopic expression of *Ng2*, which is also a member of the bHLH proneural gene family. *Mash1* also appears to have a role in subtype specification (that cannot be rescued by *Ng2*), and is absolutely required for expression of *Gsh1* and the subsequent generation of *Ghrh*-expressing neurons.

***Sox3*:** *Sox3* is a member of the SOX (Sry-related HMG box) family of transcription factor genes and is located on the X chromosome (Lefebvre *et al.*, 2007). This gene was initially implicated in hypothalamic development from clinical and genetic studies of families with the male-specific congenital disorder X-linked Hypopituitarism (XH). XH males have GH deficiency and, in some cases, additional pituitary hormone deficiencies as well as intellectual disability (Solomon *et al.*, 2002). Magnetic resonance imaging analysis of affected males has revealed abnormalities of the hypothalamic region including ectopic posterior pituitary and thin pituitary stalk, indicating that XH results primarily from a hypothalamic defect (Woods *et al.*, 2005). Interestingly, XH is associated with duplications and mutations in *SOX3*, suggesting that over-expression and loss-of-function mutations result in a similar developmental defect (Solomon *et al.*, 2002; Woods *et al.*, 2005). Although the mechanism by which altered *SOX3* dosage causes XH is not fully understood, genetic studies in mice have provided some clues. *Sox3* null animals exhibit multiple pituitary hormone deficiency, variable dwarfism and CNS abnormalities, indicating that SOX3 function is broadly conserved in mice and humans (Rizzoti *et al.*, 2004; Weiss *et al.*, 2003). Importantly, *Sox3* is expressed in the developing hypothalamus (see below) but has minimal expression in the AP, suggesting that hypothalamic (and not AP) dysfunction is the primary cause of pituitary hormone deficiency in *Sox3* mutants.

Studies from our laboratory have shown that *Sox3* is expressed in the hypothalamus from inception to maturity suggesting that it may have multiple roles in hypothalamic

development and function (**Figure 2** and data not shown). Analysis of *Sox3* mutants has indicated that early expression in the ventral diencephalon/infundibular recess (at 10.5 dpc) is required for normal induction and morphogenesis of the AP, but, remarkably, not AP function (Rizzoti *et al.*, 2004). From approximately 12.5 dpc, *Sox3* expression is restricted to multiple hypothalamic regions/nuclei including the hypothalamic neuroepithelium, median eminence, ARC, PVN, medial POA and VMN (unpublished data). Interestingly, all of these nuclei contain parvicellular neuronal subtypes. It is therefore possible that the multiple pituitary hormone deficiencies in *Sox3* null mice (and some XH patients) may reflect a specific requirement for *SOX3* in the generation and/or maintenance of some, if not all, parvicellular neuronal subtypes. Alternatively, or in addition, defective development of the median eminence, which also expresses *Sox3* (Rizzoti *et al.*, 2004) and our unpublished data), may compromise the functional connection to the portal vasculature, resulting in altered regulation of AP hormone synthesis and secretion by parvicellular neuronal factors.

5. Birthdate analysis of hypothalamic nuclei

Detailed birth-dating studies of hypothalamic nuclei have been performed in rats, and to a lesser extent, in mice (Markakis, 2002; Markakis and Swanson, 1997). For extensive discussion of these reports we refer the reader to the excellent review by (Markakis, 2002). The general conclusion arising from birth-dating analyses is that the hypothalamus matures “from outside to inside” such that the lateral nuclei are generated before those located at more medial positions. This developmental sequence is opposite to that occurring in the cerebral cortex, where nascent neurons migrate past older neurons as they move radially towards the pial surface (from “inside to outside”) (Misson *et al.*, 1991). The order in which hypothalamic nuclei are generated may reflect, to some extent, a passive process by which the third ventricle is progressively reduced in volume due to accumulation of nascent neurons in a lateral to medial sequence. This is supported by gene expression analysis of the developing anterior hypothalamus, whereby it has been revealed that laminar patterns of gene expression may correspond to distinct waves of neurogenesis (Caqueret *et al.*, 2006). However, birth-dating studies of the six parvicellular neural subtypes suggests that this model is an oversimplification as peak generation of parvicellular neurons occurs

before the peak generation of their cognate nuclei (Markakis and Swanson, 1997). These observations imply that nascent parvicellular neurons exhibit a delayed migratory phase. Apart from the exceptional case of GnRH neurons, which undergo extensive migration from their source in the olfactory placode (Verney *et al.*, 1996), this area is poorly understood. Perturbation of this migratory pathway could contribute to the altered distribution of anterior hypothalamic neurons in *Sim1* mutant embryos (see above) (Caqueret *et al.*, 2006) although further studies are required to determine the precise mechanism. A second intriguing finding from parvicellular birth-dating studies is that there is no obvious correlation between the time at which the neurons are born and the neuronal subtype (in rats the peak parvicellular neuron generation occurs at 12.5-13.5 dpc, regardless of cell type). It therefore appears that, apart from GnRH, the parvicellular neurons are generated concurrently from the ventricular neuroepithelium that spans the hypothalamic region. Almost nothing is known about the coordination of progenitor cell selection and lineage commitment in the hypothalamus but it seems possible that similar genetic mechanisms to those employed in other CNS regions (e.g. Notch signaling) may be utilized.

6. Generation and function of parvicellular hypophysiotropic factors

Hypothalamic control of the AP became an accepted principle and the entire field took a major step forward with the discovery that (pyro)Glu-His-Pro(amide), synthesized in the hypothalamus, acted as a releasing factor for TSH (Guillemin *et al.*, 1963). Along with the discovery of additional hypophysiotropic factors, subsequent research has focused on better understanding of the expression of these factors in the hypothalamus and the mechanisms by which they exert physiological activity at the pituitary. The developmental sequence of expression of the known hypothalamic hypophysiotropic factors has been investigated in numerous species including rat, mouse, human and chicken (**Table 1**).

7. Origin and birthdate of neuroendocrine hypophysiotropic factors

The availability of genetically engineered mouse models has added a new dimension to studies of the ontogeny of parvicellular neuronal subtypes. In recent years, a clearer picture has emerged of the precise steps in development and the factors involved in the differentiation of and acquisition of function by cells that secrete

hypothalamic releasing factors. Below we outline some of the key advances in this field.

7.1. GnRH

A total of 14 forms of GnRH have been described (for review see (Wray, 2002)), with the physiologically most important form being GnRH-1 (referred to here as GnRH). GnRH is a central regulator in the hypothalamic–pituitary–gonadal axis and is produced by neurosecretory cells located throughout the basal hypothalamus including the preoptic nucleus and AH. The release of GnRH triggers the synthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which regulate gonadal steroidogenesis and gametogenesis (for review see (Lee *et al.*, 2008)).

Unlike all other parvicellular neurons that arise from within the hypothalamic anlagen, GnRH neurons originate in the olfactory placode (Wray, 2002) and migrate through the ventral forebrain. In mice, GnRH neuron migration terminates in the medial septum, POA and anterior hypothalamic regions (Wray, 2001). Recent evidence indicates that the initial population of GnRH neurons (9.5-10.5 dpc) are generally located rostral to later-born (11.5-12.5 dpc) GnRH neurons and that the GnRH neurons located at different rostral-caudal positions may be functionally distinct (Jasoni *et al.*, 2009). Several extracellular cues that direct the emergence and migration of nascent GnRH neurons have been identified which include Fibroblast Growth Factor 8 (Chung *et al.*, 2008), hepatocyte growth factor (Giacobini *et al.*, 2007) and secreted-class 3 semaphorins (Cariboni *et al.*, 2007). Of particular interest is semaphorin-4D (Sema4D) which belongs to the semaphorin protein family group of axon/cell guidance proteins and is expressed along GnRH migratory route (Tran *et al.*, 2007). The Sema4D receptor, PlexB1, is expressed in migratory cells that are exiting the olfactory placode (Giacobini *et al.*, 2004). *PlexB1* deficient mice exhibit aberrant migration of the principal GnRH fibers that project to the ME (Giacobini *et al.*, 2008) confirming the importance of Sema4D/PlexB1 interaction during GnRH cell migration.

A number of transcription factors have been implicated in GnRH differentiation such as GATA-4 and Activator Protein-2 α (AP-2 α). GATA-4, a member of the GATA family of zinc finger-domain transcription factors, binds to the GnRH enhancer and regulates GnRH gene transcription (Lawson *et al.*, 1996). In the 13.5 dpc mouse brain, GnRH neurons express GATA-4 along their migration from the olfactory placode into the brain (Lawson and Mellon., 1998). The Activator Protein transcription factors are critical regulators of gene expression during embryogenesis. AP-2 α has been detected in olfactory placode epithelium (Mitchell *et al.*, 1991). It has been reported that GnRH neurons express AP-2 α as they migrate into the forebrain (Kramer *et al.*, 2000).

7.2. GnIH

GnIH was recently discovered in the Japanese quail and acts directly on the pituitary to inhibit gonadotropin release (Tsutsui *et al.*, 2007; Tsutsui and Ukena, 2006). The identification of GnIH arose when neurons immune-positive for the molluscan cardioexcitatory neuropeptide Phe-Met-Arg-Phe-NH₂ (FMRFamide, (Price and Greenberg, 1977) were found in the vertebrate nervous system to contain an unknown, but similar, neuropeptide (Raffa, 1988). In the amphibian brain, some of these neurons were seen to project to the hypothalamic region close to the pituitary (Raffa, 1988; Rastogi *et al.*, 2001). In turn, in the Japanese quail brain, clusters of these distinct neurons were seen localized in the PVN in the hypothalamus, with wide distribution in the diencephalic and mesencephalic regions and the most prominent fibers within the ME (Tsutsui *et al.*, 2007). Recent studies have confirmed the effects of GnIH in rodents and sheep (Ducret *et al.*, 2009; Johnson *et al.*, 2007; Kriegsfeld *et al.*, 2006; Murakami *et al.*, 2008). Birth-dating and neuronal migration, however, have yet to be examined.

7.3. DA

DA is a catecholamine neurotransmitter, which in the pituitary is primarily involved in the inhibition of prolactin (PRL) release. In order to detect DA and the cells that produce it, tyrosine hydroxylase (the rate-limiting enzyme in synthesis of dopamine) expression is used as a surrogate marker. Secretion of PRL is regulated by three populations of hypothalamic dopaminergic neurons, originally identified in rats

(DeMaria *et al.*, 1999): (1) the tuberoinfundibular (TIDA) dopaminergic neurons, arising from the dorsomedial ARC and project to the external zone of the median eminence (Bjorklund *et al.*, 1973); (2) tuberohypophysial (THDA) dopaminergic neurons, arising from the rostral ARC and project into the hypothalamic-hypophysial tract and into the intermediate and neural lobes of the pituitary gland (Fuxe, 1964); and (3) the periventricular hypophysial (PHDA) dopaminergic neurons, arising from the more rostral PeVN and their axons terminate within the intermediate region of the pituitary gland (Goudreau *et al.*, 1992). The PHDA neuronal populations control basal regulation of PRL secretion. Early immunohistochemical detection show the first appearance of dopaminergic neurons at 11.5 dpc in the rat (Daikoku *et al.*, 1984).

Insight into the role of specific transcription factors in the development and differentiation of dopamine neurons, specifically the THDA and PHDA subtypes is limited. The LIM-homeodomain transcription factor *Lmx1a* has been shown to play critical roles in the determination of midbrain dopaminergic neurons during brain development (Failli *et al.*, 2002). More recently, it was identified that *Lmx1a* is expressed at high levels within the posterior hypothalamic area, ventral pre-mammillary nucleus, sub-thalamic nucleus, ventral tegmental area, compact part of the substantia nigra and parabrachial nucleus from birth to adulthood (Zou *et al.*, 2009). However, the exact role of *Lmx1a* in the dopaminergic neurons that regulate secretion of prolactin is yet to be determined. *Otp* has also been found to be a key determinant of hypothalamic differentiation, including the DA neurons (Blechman *et al.*, 2007). Recent studies have begun to uncover the factors that regulate OTP expression and function. In zebrafish, Blechman *et al* (2007) have shown that *Otp* is transcriptionally regulated by the zinc finger-containing transcription factor Fezl. Furthermore, epistasis and cell culture experiments indicate that signaling via the G-protein-coupled receptor PAC1 increases the level of OTP protein by promoting OTP synthesis. Further research into the role of transcription factors, such as *Lmx1a* and *Otp*, on postnatal maturation, survival and/or function of midbrain dopaminergic neurons will help to provide a better understanding of the complexity of PRL inhibition and its regulation of secretion.

7.4. GHRH

GHRH stimulates the release of growth hormone (GH) from the pituitary. GHRH is expressed during the later stages of development and is essential for the expansion of somatotropes. Hypophysiotropic GHRH neurons are confined to the ventrolateral part of the ARC (Niimi *et al.*, 1990; Sawchenko *et al.*, 1985) and first appear at 11.5 dpc in rat (Markakis and Swanson, 1997).

The development and transcriptional control of GHRH neurons has been studied in mouse models using both gene disruption and transgenic approaches. One example of GHRH reduction has been identified using targeted disruption of *Gsh1*, a homeobox gene identified as a direct transcriptional activator of *Ghrh* (Mutsuga *et al.*, 2001). Targeted disruption of *Gsh1* leads to the complete absence of *Ghrh* expression resulting in severe attenuation of growth and an associated decrease in overall pituitary size (Li *et al.*, 1996). The haematopoietic transcription factor Ikaros is also expressed in GHRH neurons and is required for *Ghrh* expression (Ezzat *et al.*, 2006). In contrast, GHRH over-expression in a mouse model harboring the human GHRH gene coupled to the murine metallothionein I promoter (Hammer *et al.*, 1985) results in massive pituitary hyperplasia and an overabundance of somatotropes (Kineman *et al.*, 2001; Mayo *et al.*, 1988). These transgenic mice also exhibit pituitary tumors, albeit with incomplete penetrance, indicating that sustained elevated GHRH exposure predisposes somatotropes to neoplastic transformation.

7.5. SS

SS acts as an inhibitor of GH and TSH secretion. The inhibition of GH by SS appears to be independent of GHRH, although the precise mechanism remains unknown. GH secretion stimulates somatostatinergic neurons in the PeVN to secrete SS from the nerve terminals located at the ME into the hypothalamo-hypophysial portal circulation for delivery to the AP (Chihara *et al.*, 1981). SS neurons that project into the ME are located within the rostral PeVN and the PVN. They first appear at 12.5 dpc in the rat (Markakis and Swanson, 1997). To date, transcription factors that specifically regulate the differentiation of hypothalamic SS neurons have not been identified, although it is possible that similar pathways to those that control SS neuron

differentiation in other parts of the brain (e.g. the cerebral cortex) may be employed (Du *et al.*, 2008).

7.6. TRH

TRH-synthesizing neurons exert multiple, species-dependent hypophysiotropic activities. However, for the purpose of this review, we will focus on the effects of TRH on TSH. Anatomically, the TRH neuroendocrine cells are situated in the hypothalamic PVN. TRH stimulates the secretion of TSH from the anterior pituitary thereby initiating thyroid hormone synthesis and release from the thyroid gland (Engel and Gershengorn, 2007; Nikrodhanond *et al.*, 2006). TRH, identified by mRNA expression of the biosynthetic precursor pre-pro-TRH, was initially localized within the rat lateral hypothalamus at 13.5 dpc, and in the presumptive PVN at 15.5 dpc (Burgunder and Taylor, 1989). Immunohistochemical analysis of the TRH peptide revealed the first TRH-immunoreactive perikarya at 16.5 dpc as well as 17.5 dpc within the presumptive PVN (Okamura *et al.*, 1991). There are four populations of TRH neurons (appearing at different developmental stages in the rat): (1) lateral hypothalamus (14.5 dpc); (2) VMN (15.5 dpc); (3) PVN (16.5 dpc); and (4) the POA (17.5 dpc). Thus, the differentiation and development of these neuronal populations will differ. Additionally, the identity and origin of the cues that direct TRH neuronal differentiation are poorly understood. However, it has been shown that brain derived neurotrophic factor (BDNF) effects TRH neuronal differentiation by tropomyosin-related kinase B receptors during early development (Huang and Reichardt, 2001). BDNF also regulates the expression of pre-pro-TRH throughout development and into postnatal life in the rat (Ubieta *et al.*, 2007).

7.7. CRH

CRH-synthesizing neurons are the principal hypothalamic regulators of the glucocorticoid axis and, like the TRH-synthesizing neurons, are closely situated in the hypothalamic PVN. Immunohistochemical analysis in rat embryos show CRH expression as early as 15.5 dpc, with immunopositive fibers seen at 16.5 dpc in the ME (Daikoku *et al.*, 1984). *Crh* mRNA expression studies have also identified CRH expressing cells from 16.5 dpc (Grino *et al.*, 1989). Given that most CRH neurons are

born at around 13.5 dpc (Markakis and Swanson, 1997), it appears that approximately 3 days is required for CRH neuron differentiation. While this process is poorly understood, one protein that has been shown to be required for generating CRH neurons is the homeodomain transcription factor OTP (Acampora *et al.*, 1999). As discussed above, *Otp* is expressed in the developing PVN, SON, aPeVN and ARC and mutants lack CRH, as well as TRH and SS neurons (Acampora *et al.*, 1999).

7.8. Other hypothalamic releasing hormones

In addition to the well-characterized hypothalamic releasing/inhibiting hormones, described above, there are several other hypothalamic specific factors that play a role during hypothalamic neuron differentiation and development and impact on pituitary function. For simplicity, we will not examine the other hypothalamic releasing/inhibiting hormones in this review because our purpose is to provide an in-depth review that covers hypophysiotropic factors that have a direct impact in AP function. However, of the well-characterized hypothalamic releasing/inhibiting hormones it is worth briefly mentioning kisspeptins. Kisspeptins, a family of peptides that activate G-protein coupled receptors (GPCR), are strongly implicated in puberty onset as well as in the regulation of the hypothalamo–pituitary gonadal axis in mammals (Mikkelsen and Simonneaux, 2008). By directly stimulating GnRH release and subsequent LH release (Messenger *et al.*, 2005), achieved through a GPCR (KISS1R), kisspeptins prepare entry into puberty and the pre-ovulatory LH surge. Kisspeptin neurons located in discrete regions of the hypothalamus make close appositions with GnRH. However, the distribution of neurons varies between species.

8. Summary and future perspectives

The past decade has witnessed significant progress in the identification of genetic determinants that control hypothalamic development. Although the full cast of characters is yet to be identified, it is clear that distinct sets of transcription factors play a role in the differentiation of hypothalamic progenitor cells into neurons and the commitment of subsets of neurons into cells that secrete hypophysiotropic factors. These factors provide an important framework for further functional studies that may lead to the generation of a transcriptional code for hypothalamic development. This process will likely be informed by parallel studies of other brain regions where

knowledge of neuronal subtype specification and differentiation is further advanced. While parallel studies will provide useful intellectual synergy, it will also be necessary to focus on the discovery of novel hypophysiotropic cell molecules and pathways. This will be facilitated by recent advances in molecular and cellular biology including the identification of hypothalamic transcription factor gene targets using ChIP sequencing analysis, directed differentiation of ES cells into hypothalamic neuronal fates (Wataya *et al.*, 2008) and characterization of novel mouse models using N-ethyl-N-nitrosourea (ENU) mutagenesis. Together, these approaches will help address critical issues such as the role of morphogens in establishing regional identity in the hypothalamic primordium, the timing and mechanism of parvicellular neuronal subtype specification, and the composition of the genetic program controlling terminal differentiation. As the role of new hypothalamic genes is deciphered it may become possible to detect patterns that will lead to a clearer understanding of brain development and evolution of the neuroendocrine system. This information will also advance our understanding of the molecular pathogenesis of hypothalamic dysfunction in humans and, perhaps, lead to improved therapies for related disorders.

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Figures and Tables

Figure 1. Illustration of the organization of hypothalamic nuclei, in the murine brain. **A.**

A lateral view of the organization of the hypothalamic nuclei. The hypothalamus is organized into distinct zones containing tight clusters of cell bodies. **B.** Representation of the neuroendocrine hypophysiotropic factors and their neuronal projections through the median eminence (ME) and into the adenohypophysis (anterior pituitary). PVN: paraventricular nucleus; POA: preoptic area; AH: anterior hypothalamus; SCN: supra-chiasmatic nucleus; SON: supra-optic nucleus; DMN: dorsal-medial nucleus; VMN: Ventro-medial nucleus; ARC: arcuate nucleus; GH: growth hormone; ACTH: adrenocorticotropin hormone; TSH: thyroid stimulating hormone; FSH: follicle stimulating hormone; LH: luteinizing hormone; PRL: prolactin. GnRH: gonadotropin –releasing hormone; GHRH: GH-releasing hormone; GnIH: gonadotropin –inhibiting hormone; DA: dopamine.

Figure 2. SOX3 is expressed in the developing murine hypothalamus. **A.** Nissl stain of the

hypothalamus (Hyp) in the sagittal orientation. **B.** Neighboring section showing SOX3 expression throughout the hypothalamic neuroepithelium (HNe and VMN), medial preoptic area (MPOA), median eminence (ME) and paraventricular nucleus (PVN). 3rd V: third ventricle, OC: optic chiasm, OR: optic recess, OT: optic tract, P: pons, PG: pituitary gland, SPH: sphenoid cartilage, Th: thalamus.

Table 1. Differentiation of hypothalamic parvicellular neurons in rat, mouse, human

and chicken. *Birth-dating studies have not been examined. #Hypothalamic neurons may arise earlier during development; no known earlier stages have been investigated. MPON: medial preoptic nucleus; DMN: dorsal-medial nucleus; ARC: arcuate nucleus; PVN: paraventricular nucleus; PeVN: periventricular nucleus; g.w: gestation week; e: embryonic day; N.D: not determined.

Figure 1.

Figure 2.

Table 1.

Hypothalamic Factor	Location of Neuronal Nuclei	Action on pituitary cell(s)	Neuronal Appearance Date			
			Rat	Mouse	Human	Chicken
GnRH	MPON	Stimulates gonadotropes	e11 (Markakis & Swanson, 1997)	e10.5 (Wray et al, 1989)	5.5 g.w. (Verney et al, 1996) 6 g.w. (Schwanzel-Fukuda et al., 1996)	e4.5 (Sullivan & Silverman, 1993)
GnIH	DMN (in rodent species)	Inhibits gonadotropes	adult stage* (Kriegsfeld et al, 2006)	adult stage* (Kriegsfeld et al, 2006)	N.D.	adult stage* (Dockray et al., 1983)
DA	ARC	Inhibits lactotropes	e11 (Markakis & Swanson, 1997) e12 (Balan et al, 1996) e13.5 (Daikoku et al, 1986)	e10.5 (Son et al, 1996)	4.5 g.w. (Verney et al, 1991 & 1995 ; Zecevic & Verney, 1995 ; Verney et al, 1996)	e5 (Ohyaama et al, 2005)
GHRH	ARC	Stimulates somatotropes	e11 (Markakis & Swanson, 1997)	N.D.	29 g.w. (Bloch et al, 1984)	N.D.
SS	PeVN	Inhibits somatotropes	e12.5 (Daikoku, 1983) e12 (Markakis and Swanson, 1997)	e17 [#] (Gross & Longer, 1979)	10 g.w. (Aubert et al, 1977)	e14 [#] (Geris et al, 1998)
CRH	PVN	Stimulates corticotropes	e12 (Markakis & Swanson, 1997)	e13.5 (Keegan et al., 1994)	16 g.w. (Bugnon et al, 1982)	e15 (Jósza et al, 1986)
TRH	PVN	Stimulates thyrotropes	e12 (Markakis and Swanson, 1997)	e13 (Faivre-Bauman et al, 1978)	8 g.w. [#] (Winters et al, 1974)	e5.5 (Thommes et al, 1985)