Specification of posterior hypothalamic neurons requires coordinated activities of Fezf2, Otp, Sim1a and Foxb1.2

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SUMMARY
The hypothalamus is a key integrative center in the brain that consists of diverse cell types required for a variety of functions including homeostasis, reproduction, stress response, social and cognitive behavior. Despite our knowledge of several transcription factors crucial for hypothalamic development, it is not known how the wide diversity of neuron types in the hypothalamus is produced. In particular, almost nothing is known about the mechanisms that specify neurons in the posteroiormost part of the hypothalamus, the mammillary area. Here, we investigated the specification of two distinct neuron types in the mammillary area that produce the hypothalamic hormones Vasoactive intestinal peptide (Vip) and Urotensin 1 (Uts1). We show that Vip- and Uts1-positive neurons develop in distinct domains in the mammillary area defined by the differential expression of the transcription factors Fezf2, Otp, Sim1a and Foxb1.2. Coordinated activities of these factors are crucial for the establishment of the mammillary area subdomains and the specification of Vip- and Uts1-positive neurons. In addition, Fezf2 is important for early development of the posterior hypothalamus. Thus, our study provides the first molecular anatomical map of the posterior hypothalamus in zebrafish and identifies, for the first time, molecular requirements underlying the specification of distinct posterior hypothalamic neuron types.

KEY WORDS: Hypothalamus, Neuronal specification, Zebrafish, Mammillary area, Fezf2, Vasoactive intestinal peptide, Urotensin 1

INTRODUCTION
The hypothalamus is an evolutionarily ancient integrative center that plays a pivotal role in the survival and propagation of vertebrate species. It orchestrates complex adaptive behaviors that regulate numerous functions including stress responses, food intake, thermoregulation, fluid homeostasis and reproductive behavior. Reflecting the diversity of its function, the organization of the hypothalamus is highly complex with various cell types and fiber pathways tightly packed into a small space. Based on Nissl-stained tissues, the hypothalamus can be broadly divided into four major areas in the anteroposterior direction (preoptic, anterior, tuberal and mammillary) (Saper, 2004; Simerly, 2004; Swanson, 1987). More than a dozen nuclei with distinct functions and different cell types are interspersed within the different hypothalamic regions. Although a large number of studies have assigned distinct physiological functions to them, the mechanisms by which the different hypothalamic areas and neurons develop are poorly understood. In particular, very little is known about the development of the neurons in the posteroiormost part of the hypothalamus, which is referred to as the mammillary area (MA). One of the most prominent structures of the MA is the mammillary body (MB; also called the mammillary nucleus), which forms connections with the hippocampus, tegmentum and anterior thalamus and plays an important role in memory both in humans and in rodents (Vann, 2010; Vann and Aggleton, 2004). The MB is a large neuronal group comprising two nuclei, termed the lateral and medial mammillary nuclei (Swanson, 1987; Vann, 2010), each with distinct connections and different electrophysiological properties (Radyushkin et al., 2005; Vann, 2005; Vann, 2010; Vann and Aggleton, 2004). In addition to the MB, other nuclei found within the MA are the tuberomammillary nucleus, the supramammillary and the premammillary area, which have diverse functions such as defense behavior, anxiety, arousal, sleep, memory and feeding (Aguiar and Guimarães, 2011; Canteras et al., 2008; Haas and Panula, 2003; Pan and McNaughton, 2004).

Several transcription factors that control the specification of hypothalamic neurons have been identified. Genetic analyses in mouse revealed factors that control the development of neuroendocrine cells in the rostral hypothalamus including SIM1, SIM2, BRN2 (POU3F2), ARNT2 and OTP (Goshu et al., 2004; Michaud et al., 2000; Michaud et al., 1998; Nakai et al., 1995; Schonemann et al., 1995; Wang and Luftin, 2000). The function of hypothalamic regulators seems to have been highly conserved during evolution and zebrafish orthologs have similar roles to their mouse counterparts (Machluf et al., 2011). For example, zebrafish Otp and Sim1/Arn1 are required for the development and function of neuroendocrine cells as well as for the development of diencephalic dopaminergic (DA) neurons (Amir-Zilberstein et al., 2012; Blechman et al., 2007; Del Giacco et al., 2006; Eaton and Glasgow, 2006; Eaton and Glasgow, 2007; Eaton et al., 2008; Lühr et al., 2009; Ryu et al., 2007). Zebrafish otpb is regulated by the zinc-finger protein Fezf2 (Blechman et al., 2007), which was originally identified as a factor upregulated by overexpression of Dkk1 in zebrafish (Hashimoto et al., 2000). Fezf2 is expressed early on in the developing forebrain and controls regionalization of the diencephalon in both zebrafish and mouse (Hirata et al., 2006; Jeong et al., 2007; Jeong et al., 2006; Scholpp and Lumsden, 2010; Shimizu and Hibi, 2009). Later in development, Fezf2 is important for the development of certain forebrain neurons in mouse (Chen et al., 2005a; Chen et al., 2005b; Hirata et al., 2004; Komuta et al., 2007; Molyneaux et al., 2003) and for the development of DA, Oxytocin-like (Oxtl), GABAergic and serotonergic neurons in zebrafish (Blechman et al., 2007; Guo et al., 1999; Jeong et al., 2006; Levkowitz et al., 2003; Yang et al., 2012).
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By contrast, very little is known about the mechanism that controls the development of distinct neurons in the posterior hypothalamus. Several studies have elucidated important roles for Shh, BMP, Wnt, FGF and Nodal signaling in proper patterning of the posterior hypothalamus and the development of the hypothalamic progenitors (Alvarez-Bolado et al., 2012; Kapsimali et al., 2004; Lee et al., 2006; Manning et al., 2006; Mathieu et al., 2002; Ohiyama et al., 2008; Pearson et al., 2011; Tsai et al., 2011; Wang et al., 2012). However, downstream factors that contribute to posterior hypothalamic neuronal specification are not well understood. In mouse mutants for the winged helix-loop-helix protein FOXB1 the MB does not develop properly, suggesting a crucial role for this transcription factor (Alvarez-Bolado et al., 2000; Labosky et al., 1997; Wehr et al., 1997). More recent studies revealed additional transcription factors, including SIM1, PAX6 and PITX2, that are involved in late aspects of MB differentiation, such as the development of the mammillothalamic tract (Marion et al., 2005; Skidmore et al., 2012; Szabó et al., 2011). However, only a few regulators of MA neuron development are currently known. Interestingly, many of the known hypothalamic regulators are broadly expressed in the hypothalamus, suggesting that they play roles in the formation of multiple cell types. For example, although to date Otp function has been mainly analyzed in the neuroendocrine hypothalamus, it is expressed in other hypothalamic regions including the retrochiasmatic region, ventral tuberal region and the MA in mouse (Shimogori et al., 2010; Simeone et al., 1994) and in the posterior hypothalamus in zebrafish (Blechman et al., 2007; Ryu et al., 2007). Similarly, Fezf2 is found in both the anterior and posterior hypothalamus in zebrafish (Levkowitz et al., 2003), yet its role in the posterior hypothalamus is unknown.

Here we demonstrate crucial roles of Fezf2, Otp, Sim1a and Foxb1.2 in the development of distinct MA neuron types. First, we show that Fezf2 is required for early development of the posterior hypothalamus. At a stage when neuronal specification takes place, Fezf2, Otp, Foxb1.2 and Sim1a form distinct subdomains within the MA. We show that neuron types producing two hypothalamic hormones, Vasoactive intestinal peptide (Vip) and Urotensin 1 (Uts1), develop in different subdomains. Using loss-of-function and temporally controlled gain-of-function studies, we demonstrate that VIP neuron specification requires Otp and Sim1a, whereas Uts1-positive neurons require Fezf2, Sim1a and Foxb1.2. Thus, our study provides the first molecular map of the posterior hypothalamus in zebrafish and identifies, for the first time, transcription factor controls multiple aspects of hypothalamic neuronal development. In order to identify potential regulators of posterior hypothalamic neuronal development, we began our study with Otp, as this transcription factor controls multiple aspects of hypothalamic neuronal development and is expressed broadly in the hypothalamus.

RESULTS

Otp regulates the expression of fezf2 and foxb1.2 in the putative mammillary area

In order to identify potential regulators of posterior hypothalamic neuronal development, we began our study with Otp, as this transcription factor controls multiple aspects of hypothalamic neuronal development and is expressed broadly in the hypothalamus. To find factors that might mediate Otp function, we performed an in situ hybridization-based screen in zebrafish embryos lacking Otp activity. More than 80 transcription factors expressed in the hypothalamus were selected from the zebrafish expression database at the Zebrafish Information Network (www.zfin.org) and tested in 2-dpf optha<sup>−/−</sup> mutant embryos injected with 4 ng othb morphinol (MO) (Ryu et al., 2007).

Two transcription factors showed changes in expression in the diencephalon of embryos lacking Otp activity. Expression of the zinc-finger transcription factor fezf2 was reduced in the preoptic area (PO) and expanded in the posterior part of the hypothalamus (Fig. 1A-D). The expression domain of the winged helix-loop-helix transcription factor foxb1.2, which encodes the zebrafish ortholog of...
Our results thus show that, at 1 dpf, Fezf2 is required for maintaining emx2 expression and limiting the extent of hypothalamic wnt8b and fgf8 expression in the posterior hypothalamus.
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Molecular anatomical map of the mammillary area

In order to investigate neuronal development in the posterior hypothalamus, it is necessary to first define its anatomical features. Since the anatomy of the posterior hypothalamus has not been characterized in detail in larval zebrafish, we first identified putative MA nuclei, taking advantage of the recently reported mouse hypothalamus atlas (Shimogori et al., 2010). Markers that define different MA nuclei in rodents are Lef1 (preammamillary nucleus), Lhx6 (tuberomammillary terminal), Irx5 (supramammillary nucleus) and Foxb1 (MB or mammillary nucleus). Strikingly, their zebrafish homologs (MB or mammillary nucleus) and Foxb1 (MB or mammillary nucleus) are also expressed in distinct domains in the posterior hypothalamus (Fig. 2).

To gain insight into the roles of Otp, Fezf2 and Foxb1.2 in the neuronal development of the MA in zebrafish, we first determined in which MA domains they are found at 2 dpf, when many neuron types in the posterior hypothalamus are specified. This we achieved first by comparing foxb1.2 and otpb expression relative to that of lef1, lhx6 and irx5a (Fig. 3A-M*). A schematic map of their relative domains was drawn taking into consideration the location of landmarks in combination with DIC images (Fig. 3G,N; supplementary material Figs S10, S11). Next, we compared the relative expression of foxb1.2, otpb and fezf2 (Fig. 4A-C*). Similar analysis was performed for sim1a and wnt8b given their reported roles in posterior hypothalamic neuronal development (Lee et al., 2006; Marion et al., 2005) (Fig. 4D-F*). Fezf2 expression overlapped in the posterior extent of the foxb1.2 domain but mostly formed a separate domain (Fig. 4A-A*). The otpb and otpa domains in the anterior part of the posterior hypothalamus were lateral to the fezf2 and foxb1.2 domains (Fig. 4B-C*, supplementary material Fig. S12). The sim1a domain overlapped with the fezf2 and foxb1.2 domains, but extended more laterally, overlapping with otpb (Fig. 4D-F*). Wnt8b was expressed along the midline and in a band posterior to the foxb1.2 and sim1a domains, partially overlapping with fezf2 and otpb expression (Fig. 4G-J*). Thus, strong foxb1.2 expression demarcates a distinct domain within the posterior hypothalamus with contributions by sim1a and fezf2 expression.

This spatial relationship of the transcription factors was further confirmed in the lateral view (supplementary material Fig. S13). Schematic maps of the expression domains in the dorsal and lateral view are shown (Fig. 4K,L). To corroborate the identity of the foxb1.2-expressing domain as the MB, we sought to visualize potential mammillothalamic tracts. Axon bundles in this region were visible (supplementary material Fig. S14), but the precise target area definition requires more refined tracing techniques. Nevertheless, given the conservation of foxb1.2 expression we will refer to this region as the putative MB.

In summary, expression of fezf2, otpa, otpb and sim1a was found in domains including and surrounding the putative MB, with fezf2 and wnt8b marking the posterior, otpa/otpb in the lateral and posterior, and sim1a labeling anterior domains. Thus, the expression analysis of otp, fezf2, sim1a, wnt8b and foxb1.2 revealed the
existence of unexpectedly complex and distinct subdomains in the region surrounding the putative MB.

**Fezf2, Foxb1.2 and Otp play crucial roles in the establishment of the MB domains**

What is the mechanism responsible for generating these distinct transcription factor domains surrounding the putative MB? The largely non-overlapping expression of \( \text{otp} \) and \( \text{foxb1.2} \), or of \( \text{otp} \) and \( \text{fezf2} \), could result from the negative regulation of \( \text{foxb1.2} \) and \( \text{fezf2} \) by \( \text{otp} \) (Fig. 1). To explore other regulatory interactions that could contribute to this subregionalization, we analyzed the phenotypes of \( \text{fezf2} \), \( \text{foxb1.2} \) and \( \text{sim1a} \) (Löhr et al., 2009) morphants. At 2 dpf, \( \text{fezf2} \) morphants showed a reduction in \( \text{otpa} \) and \( \text{otpb} \) (Blechman et al., 2007) expression in the region surrounding the putative MB, but a clear expansion of \( \text{sim1a} \) and \( \text{otpb} \) (Fig. 5A-F). In \( \text{foxb1.2} \) morphants, \( \text{sim1a} \) and \( \text{fezf2} \) expression was expanded (Fig. 5G-J). By contrast, in \( \text{sim1a} \) morphants we observed no detectable changes in the expression of \( \text{otpa}, \text{otph}, \text{foxb1.2} \) and \( \text{fezf2} \) (data not shown). Similarly, embryos with reduced \( \text{Otp} \) activity showed no changes in \( \text{sim1a} \) expression (data not shown). To test the effect of \( \text{Wnt8b} \) we treated embryos at 1 dpf with \( \text{IWR-1} \) in order to disrupt \( \text{Wnt} \) signaling without affecting early patterning (Chen et al., 2009; Moro et al., 2012). \( \text{IWR-1} \)-treated embryos showed no changes in the expression of \( \text{fezf2}, \text{foxb1.2}, \text{sim1a} \) and \( \text{otp} \) (data not shown).

Thus, changing the levels of \( \text{Otp}, \text{Fezf2} \) or \( \text{Foxb1.2} \) leads to changes in the expression of the other transcription factors, whereas changes in \( \text{Sim1a} \) levels and interference with \( \text{Wnt} \) signaling do not. \( \text{Otp}, \text{Fezf2} \) and \( \text{Foxb1.2} \) might directly regulate each other’s expression domains or levels. Alternatively, it is possible that the changes that we observe are due to their effects on other transcription factors or signaling pathways. In case of \( \text{fezf2} \), this is particularly pertinent because we have shown that \( \text{fezf2} \) affects early development of the posterior hypothalamus at 1 dpf. In order to distinguish between early and late effects of \( \text{fezf2} \), we designed a PhotoMorph (PM) (Tomasini et al., 2009) specific to \( \text{fezf2} \) MO sequences, which led to near complete caging of the \( \text{fezf2} \) MO and partial uncaging upon UV exposure at 1 dpf (supplementary material Fig. S15). The expansion of \( \text{foxb1.2} \) and \( \text{sim1a} \) was still apparent in the embryos injected with \( \text{fezf2} \) PM after uncaging, whereas the \( \text{otpa} \) reduction was not (supplementary material Fig. S16). This suggests the existence of
stage-specific roles of fezf2 and an Otp-independent interaction between fezf2 and foxb1.2 or fezf2 and sim1a.

Thus, taken together our results show that fezf2, foxb1.2 and otp play an important role in the establishment of the MB domains. Our data are also consistent with the possibility that fezf2 might directly regulate foxb1.2 and sim1a in this process.

**Vip- and Uts1-positive neurons develop within different MB domains**

To test whether the distinct domains surrounding the putative MB give rise to different neuron types, we next cloned and examined the expression patterns of 30 genes encoding different hypothalamic neuropeptides (our unpublished data). We found that Uts1-positive neurons were generated in the foxb1.2-expressing putative MB (Fig. 6A-A’), whereas Vip-positive neurons were generated in the adjacent foxb1.2-free region (Fig. 6E-E’). Uts1-positive neurons were generated in a domain that is also positive for sim1a and fezf2, whereas the expression domains of uts1 and otpb were mostly exclusive (Fig. 6B-D’). By contrast, vip expression overlapped with those of otpb and sim1a, but there was no significant overlap with fezf2 expression (Fig. 6F-H”). Although fezf2 and foxb1.2 were expressed in largely non-overlapping regions, Uts1-positive cells developed in a small area where they overlapped. These results show that different transcription factor domains in and around the putative MB give rise to distinct neuronal types, where VIP cells are otp+ sim1a+ fezf2+ foxb1.2+ and Uts1 cells are otp+ sim1a+ fezf2+ foxb1.2+.

**Transcription factor requirements for Vip- and Uts1-positive neuron specification**

To test whether transcription factors co-expressed with Vip- or Uts1-positive neurons are indeed necessary for their development, we performed loss-of-function analyses. Reduction of Otp or Sim1a led to fewer Vip-positive cells in the MA, consistent with the fact that Vip-positive neurons express both otpb and sim1a (Fig. 7A-C,M; Fig. 6F-H”). Interestingly, although fezf2 is not co-expressed with vip at this stage, fezf2 morphants exhibited fewer Vip-positive cells (Fig. 7A,D,M; Fig. 6G-G”). However, this effect is likely to be due to an early function of fezf2 because in embryos that were injected with fezf2 PM and uncaged at 1 dpf no such reduction is seen (Fig. 7A,E,M). The number of Vip-positive cells did not change in foxb1.2 morphants, consistent with the fact that foxb1.2 is not expressed in these cells (Fig. 7A,F,M; Fig. 6E-E”).

The loss of Sim1a or Fezf2 led to a reduction in Uts1-positive neurons, which normally express both sim1a and fezf2.
Development 140 (8)

The expression of Sim1a and Foxb1.2.

Although a few transcription factors have been identified that are required for MA development, almost nothing was known about how the MA neurons are specified. In this study, we identified two important hypothalamic hormones, Vip and Uts1. We show that these two neuron types are specified through coordinated activities among the transcription factors Otp, Fezf2, Sim1a and Foxb1.2.

DISCUSSION

Despite its functional importance, the mechanism underlying the development of the posteriormost part of the hypothalamus, the MA, is poorly understood. In this study we provided the first molecular map of the MA in zebrafish and showed that in zebrafish embryos the MA consists of domains with strong conservation of marker genes expressed in the mammalian MA. Our analysis also revealed the existence of distinct subdomains that overlap and surround one of the MA nuclei, the MB (or mammillary nucleus). Although a few transcription factors have been identified that are required for MA development, almost nothing was known about how the MA neurons are specified. In this study, we identified molecular requirements for the specification of neurons that produce two important hypothalamic hormones, Vip and Uts1. We show that these two neuron types are specified through coordinated activities among the transcription factors Otp, Fezf2, Sim1a and Foxb1.2, which define distinct subdomains of the MB. Our results also highlight the important dual roles of Fezf2 for both early development of the posterior hypothalamus and late specification of Uts1 neurons.

Fezf2 is required for early development of the posterior hypothalamus

Fezf2 is a key transcription factor controlling multiple aspects of forebrain development. One of the highly conserved functions of Fezf2 is the regulation of forebrain regionalization by maintaining anterior forebrain fate (Jeong et al., 2007; Scholpp and Lumsden, 2010; Staudt and Houart, 2007; Toro and Varga, 2007). By
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Distinct roles of Fezf2 in Vip- and Uts1-positive neuron specification

In this study, we showed that the numbers of both Vip- and Uts1-positive neurons are reduced in fezf2 morphants, demonstrating that Fezf2 is required for their development. What might be the mechanism by which Fezf2 controls Vip- and Uts1-positive neuron development? In Vip-positive neurons, Fezf2 present in neighboring cells might non-cell-autonomously regulate Vip-positive neuron development. Such a non-cell-autonomous effect of Fezf2 has been related to corticotropin-releasing hormone (CRH) and consist of UCN1 (or simply UCN), UCN2 and UCN3 (Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001; Vaughan et al., 1995). UCN1 is broadly distributed in the brain and in the periphery and has been implicated in a wide range of functions including appetite suppression, immunomodulation and stress adaptation (Oki and Sasano, 2004). Although UCN1 is not normally expressed in the MA (according to the mouse expression atlas, http://www.brain-map.org), in colchicine-treated rats it is detected in the lateral mammillary and supramammillary nuclei and posterior hypothalamic area, suggesting that these are sites of low levels of Ucn1 expression in mammals (Bittencourt et al., 1999). In adult zebrafish, uts1 expression is found within the corpus mamillare (Alderman and Bernier, 2007).

Similar to Uts1, Vip is expressed broadly and has pleiotropic regulatory functions. In the central nervous system, Vip expression is found in the suprachiasmatic nucleus and the paraventricular nucleus of the hypothalamus, where it influences circadian clocks and pituitary adrenocorticotropic hormone secretion, respectively (Ceccatelli et al., 1989; Hökfelt et al., 1987; Nussdorfer and Malendowicz, 1998; Voiskov et al., 2007). Although Vip immunoreactivity and Vip binding sites have been found in the mammillary nuclei (Saper, 2000; Sarrieau et al., 1994), VIP expression itself in the MA is not reported in the mouse expression atlas, suggesting that the expression of Vip in this region in zebrafish might not be an evolutionarily conserved feature.

**Evolutionary conservation of the Vip- and Uts1-positive neurons in zebrafish**

To the best of our knowledge, this is the first study to identify transcription factor requirements for Uts1- and Vip-positive neuron development. Uts1 is the teleost ortholog of mammalian urocortin 1 (UCN1) (Vaughan et al., 1995). The urocortins are structurally similar to corticotropin-releasing hormone (CRH) and consist of UCN1 (or simply UCN), UCN2 and UCN3 (Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001; Vaughan et al., 1995). UCN1 is broadly distributed in the brain and in the periphery and has been implicated in a wide range of functions including appetite suppression, immunomodulation and stress adaptation (Oki and Sasano, 2004). Although UCN1 is not normally expressed in the MA (according to the mouse expression atlas, http://www.brain-map.org), in colchicine-treated rats it is detected in the lateral mammillary and supramammillary nuclei and posterior hypothalamic area, suggesting that these are sites of low levels of Ucn1 expression in mammals (Bittencourt et al., 1999). In adult zebrafish, uts1 expression is found within the corpus mamillare (Alderman and Bernier, 2007).

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**Distinct roles of Fezf2 in Vip- and Uts1-positive neuron specification**

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proposed for 5-HT- and DA-positive neurons, as Fezf2 is not expressed in these cells either (Levkowitz et al., 2003). Alternatively, Fezf2 might affect the early development of VIP progenitor cells. We consider the latter possibility to be more likely because Fezf2 is expressed broadly in the posterior hypothalamus at early stages and downregulates \( otp \) expression. In fact, our \( fezf2 \) PM experiments show that the effect of Fezf2 on \( vip \) as well as on \( otp \) takes place before 1 dpf. Further, Fezf2 overexpression at 34 hpf had no effect on the number of Vip-positive cells. Since Fezf2 overexpression was not spatially restricted, a non-cell-autonomous effect of Fezf2 should have been apparent in such manipulations.

In contrast to Vip, Fezf2 is expressed in Uts1-positive cells. Fezf2 loss-of-function led to Uts1 reduction and Fezf2 overexpression led to an increase in cell number. Here we believe it likely that Fezf2
directly regulates late aspects of Uts1 specification. In Fezf2 overexpression embryos we did not observe changes in the expression of several hypothalamic transcription factors (data not shown), making it unlikely that Fezf2 overexpression leads to gross patterning defects. The relatively mild effect of Fezf2 overexpression on Uts1-positive cell number is likely to be due to the fact that heat shock was performed at 2.5 dpf. Much of the hypothalamic differentiation program has already been initiated at this stage, making it difficult to induce significant changes. Furthermore, the expression level of Fezf2 in our overexpression experiments is likely to be significantly lower than that in the Fezf2 overexpression system reported previously, which uses the Gal4-UAS system for strong transgene induction (Jeong et al., 2007). That we nonetheless detected an effect in the number of Uts1-positive cells suggests that these cells might be exquisitely sensitive to the level of Fezf2.

The coordinated activities of a common transcription factor set generate hypothalamic neuronal diversity

Several mechanisms have been suggested to explain how great neuronal diversity can be achieved with a small number of common factors. For example, in the developing spinal cord, feed-forward regulatory loops segregate the fate of motoneurons and interneurons although they are specified by related LIM complexes (Lee et al., 2008). In the *Drosophila* central nervous system, a temporal cascade of transcription factors limits progenitor competence over time, and can act in conjunction with subtemporal genes to create diverse cell types (Baumgardt et al., 2009). By contrast, although several transcription factors have been identified as being involved in hypothalamic neuronal development, it is not understood how these factors interact to generate different neuron types. Our study identified that interplay among *fezf2*, *otp*, *foxh1.2* and *sim1* is crucial to define different domains within the MA and generate distinct neuronal subtypes. Strikingly, Otp and Sim1a are transcription factors that regulate the development of the anterior hypothalamic nucleus, such as the paraventricular nucleus in mouse, as well as the homologous PO in zebrafish. Here we showed that Otp regulates *fezf2* differently in the PO and the MA, thereby identifying one possible mechanism by which Otp exerts different functions in distinct hypothalamic regions.

Our findings provide important mechanistic insight into the generation of neuronal diversity in the hypothalamus. First, early regulators such as Fezf2 are redployed later to generate different neuronal subtypes. Second, common transcription factor sets are used in distinct hypothalamic regions in a different regulatory context.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

S.R. conceived the project. S.R. and A.W. designed the research. A.W. performed the research. S.R. and A.W. analyzed the data. S.R. and A.W. wrote the paper.

Supplementary material

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