Development 140, 1762-1773 (2013) doi:10.1242/dev.085357


# 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 posteriormost 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 posteriormost 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 Lufkin, 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/Arnt2 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; Löhr 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., 2005) 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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**Accepted 11 February 2013**

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; Ohyama 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 these 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 requirements for the specification of distinct MA neurons.

## MATERIALS AND METHODS

### Fish maintenance, plasmids, strains and genetics

Zebrafish maintenance and breeding were carried out under standard conditions at 28.5°C (Westerfield, 2000). To avoid pigmentation, embryos were incubated in 0.2 mM 1-phenyl-2-thiourea. Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at the desired time points. The *otpa*$^{m866}$ mutant allele and genotyping protocol have been described (Ryu et al., 2007). For the transgenic lines overexpressing full-length cDNAs of *otpa*, *sim1a*, *fezf2* or *foxb1.2*, the viral 2A peptide (Tang et al., 2009) was placed between the cDNA and EGFP or tdTomato and placed after the *hsp70* heat-shock promoter (Halloran et al., 2000) in a multicistronic gene expression cassette. The full-length cDNAs of *otpa*, *sim1a*, *fezf2* and *foxb1.2* were PCR amplified and cloned into the pCRII vector (Invitrogen) using the primers listed in supplementary material Table S1. A single G0 founder was used to create a stable F1 family. The following stable transgenic lines were established: *Tg(hsp70:fezf2-2A-EGFP)hd6*, *Tg(hsp70:otpa-2A-EGFP)hd7*, *Tg(hsp70:sim1a-2A-lyntdTomato)hd8* and *Tg(hsp70:foxb1.2-2A-EGFP)hd9*. Heat-shock treatments were performed at 34 hours post-fertilization (hpf) or 2.5 days post-fertilization (dpf) by adding 42°C egg water to the embryos and incubating them overnight at 37°C.

### Probes, *in situ* hybridization and immunohistochemistry

*In situ* hybridization probes for *otpa* and *otpb* (Ryu et al., 2007), *sim1a* (Löhr et al., 2009), *foxb1.2* (Thisse et al., 2001), *shh* (Krauss et al., 1993), *emx2* (Morita et al., 1995), *nkx2.1a* (Rohr and Concha, 2000), *wnt8b* (Kelly et al., 1995), *fgf8* (Reifers et al., 1998), *avpl* (*avp* – Zebrafish Information Network) (Eaton et al., 2008), *oxtl* (*oxt* – Zebrafish Information Network) (Eaton and Glasgow, 2006), *fgf3* (Maroon et al., 2002), *arx* (Miura et al., 1997), *dlx2a* (Akimenko et al., 1994), *mcm5* (Ryu et al., 2005), *pit1* (*pou1f1* – Zebrafish Information Network) (Nica et al., 2004), *prl*, *pomca* (Herzog et al., 2003) and *crabp1a* (Liu et al., 2005) have been described. Probes for *fezf2*, *lef1*, *lhx6*, *irx5a*, *gsx2*, *vip*, *uts1* and *nr5a1a* were PCR amplified using cDNA of 2-5 dpf embryos with the primers listed in supplementary material Table S2 and cloned into the pCRII vector. Whole-mount *in situ* hybridization and multicolor fluorescence *in situ* hybridization were performed as described (Hauptmann and Gerster, 1994; Lauter et al., 2011). Fluorescent immunohistochemistry was performed adapting an existent protocol (Pearson et al., 2011) using primary antibodies against Otpa (made by S.R.) and acetylated Tubulin (Sigma-Aldrich).

### Morpholino (MO), DNA injection and IWR-1 treatment

MOs (Gene Tools) are listed in supplementary material Table S3. The standard control, *otpb*, *sim1a*, *fezf2*, *fgf8* and *wnt8b* MOs were described previously (Fürthauer et al., 2001; Jeong et al., 2006; Löhr et al., 2009; Riley et al., 2004; Ryu et al., 2007). The efficiency of the *foxb1.2* MO was tested with the transient expression of a construct containing the *foxb1.2* MO binding site in frame with GFP. In MO-injected embryos, no GFP expression was detected. Both DNA and MOs were diluted in water containing 0.05% Phenol Red and injected into one-cell stage embryos. PhotoMorph caging strand (Gene Tools) was designed to bind and block the *fezf2* MO. Before injection, caging strand and MO were mixed in a molar ratio of 1.7:1 and hybridized at 65°C for 30 minutes. After hybridization the mixture was cooled, stored overnight at 4°C, and was injected at the one-cell stage. The caged MO solution and the injected embryos were kept in the dark until uncaging. Uncaging of the MO was performed at 24 or 48 hpf by transferring the embryos to glass dishes and exposing them to UV light at 312 nm using a UV table (Intas, Göttingen, Germany) for 30 minutes. The efficiency of caging and uncaging was confirmed by PCR analysis of cDNA derived from injected embryos using the primers listed in supplementary material Table S4. IWR-1 treatment was performed with 200 μM and 100 μM in DMSO for 12 hours prior to fixation and 50 μM in DMSO 24 hours prior to fixation at 2 dpf.

## 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 *otpa*$^{m866}$ mutant embryos injected with 4 ng *otpb* morpholino (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

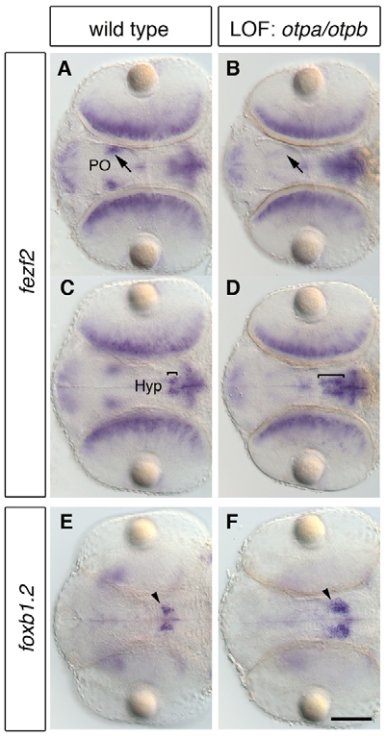


**Fig. 1. Otp regulates the expression of *fezf2* and *foxb1.2* in the putative mammillary area.** Whole-mount *in situ* hybridization (WISH) to detect the expression of *fezf2* and *foxb1.2* in 2-dpf wild-type (A,C,E) and *otpa*$^{m866}$ mutant zebrafish embryos injected with 4 ng *otpb* morpholino (MO) (B,D,F). (**A-D**) In embryos lacking Otp activity, the *fezf2* expression domain is strongly reduced in the PO (arrows) and expanded in the posterior part of the hypothalamus (brackets; C and D are more dorsal planes to A and B in the same embryo; *n*=11/11). (**E**,**F**) In embryos lacking Otp activity, *foxb1.2* expression is expanded in the putative mammillary area (MA) (arrowheads; *n*=17/17). Dorsal views are shown. PO, preoptic area; Hyp, hypothalamus; LOF, loss-of-function. Scale bar: 100 μm.

FOXB1 (Odenthal and Nüsslein-Volhard, 1998), was also expanded in this region (Fig. 1E,F).

To determine whether *foxb1.2* and *fezf2* are expressed in the posteriormost part of the hypothalamus, which could represent the mammillary area (MA), and not in the neighboring tuberal hypothalamus, we compared their expression domains with those of markers for the tuberal hypothalamus, including *pomca* (Song et al., 2003), *dlx2a* (Bulfone et al., 1993a; Bulfone et al., 1993b) and *nr5a1a* (supplementary material Fig. S1). In particular, nuclear receptor 5a 1 (NR5A1; also called steroidogenic factor 1 or SF-1) (Lala et al., 1992) is a specific marker of the tuberal region in the early developing mouse and later a marker of the ventromedial hypothalamus (VMH) (Allen Institute for Brain Science, 2012, http://developingmouse.brain-map.org) (Ikeda et al., 1995; Ikeda et al., 1994). Whereas *pomca*, *dlx2a* and *nr5a1a* are expressed adjacent to the adenohypophysis, further supporting their tuberal location in zebrafish, *foxb1.2* and *fezf2* were expressed in a distinct location that was more dorsal and posterior. Since FOXB1 is expressed in the MA in mouse (Labosky et al., 1997; Wehr et al., 1997) and both *foxb1.2* and *fezf2* are expressed in the posteriormost part of the zebrafish hypothalamus and not in the tuberal hypothalamus, we refer to this region as the putative MA.

Thus, our data show expression of *fezf2* and *foxb1.2* in the putative MA in zebrafish larvae and suggest that Otp negatively regulates *fezf2* and *foxb1.2* expression in this region.

## Fezf2 is important for early development of the posterior hypothalamus

To gain insight into the roles of Otp, Fezf2 and Foxb1.2 in posterior hypothalamus development, we first examined their early roles by performing co-expression analysis with hypothalamic markers and loss-of-function experiments at 1 dpf. We used *nkx2.1a* as a marker of the entire hypothalamus (Rohr et al., 2001) and *shh* and *emx2* for the anterior-dorsal and the posterior-ventral hypothalamus, respectively (Mathieu et al., 2002). At this stage, the differentiation between tuberal and mammillary hypothalamus might be difficult because the location of adenohypophysis/neurohypophysis is anterior to the hypothalamus and tuberal markers are not yet well expressed within the hypothalamus (supplementary material Figs S2, S3) (Herzog et al., 2003; Liu et al., 2005; Nica et al., 2004). We observed that both *foxb1.2* and *fezf2* were expressed within the posterior part of the *nkx2.1a*-positive hypothalamic domain where *fezf2* labels more ventral and *foxb1.2* more dorsal domains (Fig. 2A-A‴). The expression domains of *shh* and *fezf2* did not overlap, whereas those of *shh* and *foxb1.2* partially overlapped (Fig. 2B-B‴). *emx2* expression was entirely contained within the hypothalamic *fezf2* domain (Fig. 2C-C‴). By contrast, the hypothalamic *otpb* expression overlapped with *nkx2.1a*, *fezf2* and *foxb1.2*, but showed little overlap with *emx2* (Fig. 2D-E‴).

Because of its broad expression in the posterior hypothalamus, we next tested whether *fezf2* affects *emx2* expression. Embryos that were injected with *fezf2* MO (Jeong et al., 2006) showed a strong reduction in *emx2* expression in the posterior-ventral hypothalamus and loss of *otpa* and *otpb* (Blechman et al., 2007) expression in the posterior hypothalamus (Fig. 2F,G; supplementary material Fig. S4A-D). Hypothalamic *foxb1.2* expression was not reduced (Fig. 2H,I). In addition, the previously reported reduction of prethalamus was apparent in *fezf2* morphants (Jeong et al., 2007) (Fig. 2F-I; supplementary material Fig. S4E-H).

Fezf2 may regulate *emx2* directly or affect signaling pathways that control posterior hypothalamus regionalization. In particular, Fezf2 overexpression downregulates *wnt8b* in the prospective zona limitans intrathalamica (ZLI) and in the mid-hindbrain boundary (MHB) and *fgf8* expression in the MHB at the mid-somitogenesis stage (Jeong et al., 2007). We asked whether downregulation of *emx2* in *fezf2* morphants might be mediated by *wnt8b* or *fgf8*. *wnt8b* morphants showed reduction in hypothalamic *emx2* expression, whereas in *fgf8* morphants *emx2* was unchanged (supplementary material Fig. S5A-C,K). However, neither *nkx2.1a* nor *shh* expression was altered in these morphants (supplementary material Fig. S5D-I). Interestingly, in 1-dpf *fezf2* morphants, we observed clear expansion of *wnt8b* and *fgf8* expression in the posterior hypothalamus (Fig. 2J-M; supplementary material Fig. S6). Therefore, although Fezf2 regulates *wnt8b* expression in the posterior hypothalamus, its effect on *emx2* is likely to be independent of *wnt8b*. In fact, *wnt8b* expression at 1 dpf mostly does not overlap with that of *emx2* (supplementary material Fig. S7). In contrast to *fezf2* morphants, embryos that lack Otp or Foxb1.2 activity do not show apparent changes in *shh*, *emx2*, *wnt8b* or *fgf8* expression (supplementary material Fig. S8, Fig. S9A-G; data not shown). Hypothalamic *fgf3* expression was also unchanged in embryos that lack Otp, Fezf2 or Foxb1.2 activity (supplementary material Fig. S9H-L).

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.

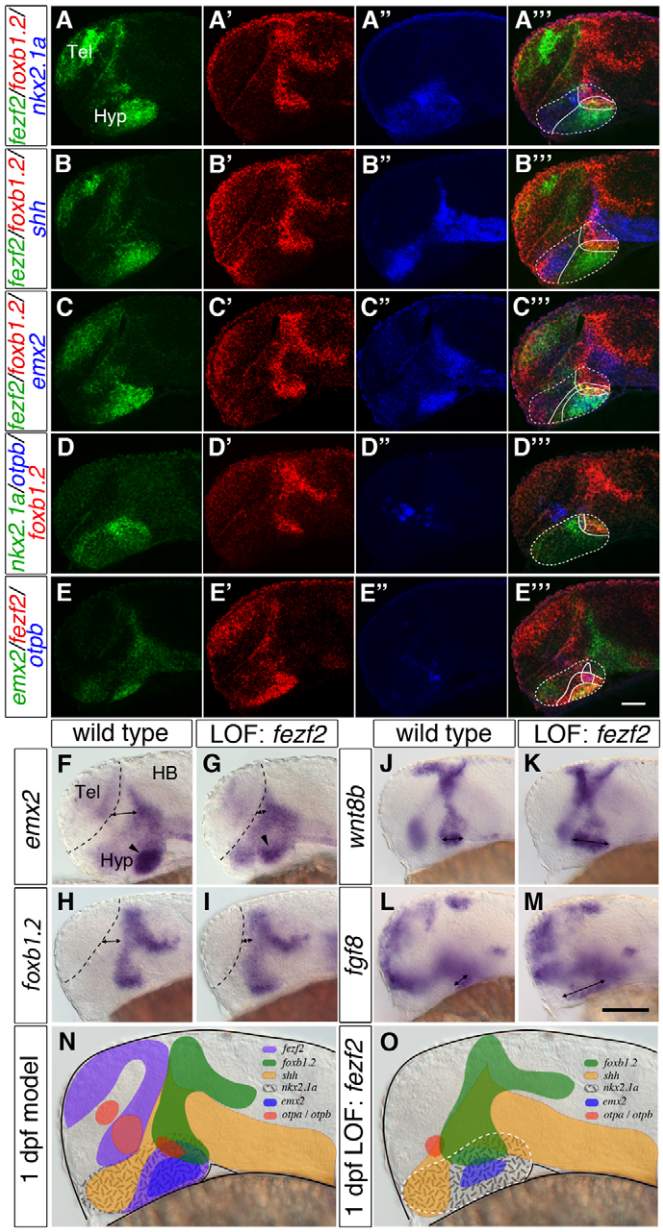


**Fig. 2. Fezf2 is important for early development of the posterior hypothalamus.** (**A-E‴**) Three-color fluorescent whole-mount *in situ* hybridization (FISH) of 1-dpf wild-type zebrafish embryos with probes for *nkx2.1a*, *shh*, *emx2*, *fezf2*, *foxb1.2* and *otpb*. Maximum projections of 10 µm confocal stacks are shown. (A-A‴) The hypothalamic expression domains of *fezf2* and *foxb1.2* are within the posterior part of the *nkx2.1a* expression domain. (B-B‴) *fezf2* expression in the posterior hypothalamus and *shh* expression in the anterior hypothalamus do not overlap, whereas *foxb1.2* is partially co-expressed with *fezf2* and *shh*. (C-C‴) *emx2* is expressed in the posterior-ventral hypothalamus within the *fezf2* domain, slightly overlapping with *foxb1.2*. (D-E‴) The posterior hypothalamic *otpb* domain is contained within the *nkx2.1a* and *foxb1.2* domain and is also *fezf2* positive. *emx2* in E is smaller than in C″, as E represents a more lateral plane than C″. The white dashed lines delineate the hypothalamus as defined by *nkx2.1a* expression; solid white lines outline the different expression domains. (**F-M**) WISH at 1 dpf on uninjected (F,H,J,L) and 4 ng *fezf2* MO-injected (G,I,K,M) embryos reveals that the *emx2* expression domain in the posterior-ventral hypothalamus is severely reduced in *fezf2* morphants (arrowheads in F,G), whereas the *foxb1.2* expression domain is not reduced (H,I). Reduction in prethalamus is apparent in *fezf2* morphants (double-headed arrows in F-I). The black dashed lines delineate the telencephalon-diencephalon border. (J-M) *fezf2* morphants show an expansion in the posterior hypothalamic domains of *wnt8b* and *fgf8* (double-headed arrows in J-M). (**N**,**O**) Summaries of co-expression of the transcription factors in wild type (N) and in *fezf2* morphants (O). Lateral views are shown. Tel, telencephalon; Hyp, hypothalamus; HB, hindbrain; LOF, loss-of-function. Scale bars: 60 µm in A-E‴; 100 µm in F-M.

## 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* (premammillary nucleus), *Lhx6* (tuberomammillary terminal), *Irx5* (supramammillary nucleus) and *Foxb1* (MB or mammillary nucleus). Strikingly, their zebrafish homologs *lef1*, *lhx6*, *irx5a* and *foxb1.2* are also expressed in distinct domains in the posterior hypothalamus (Fig. 3).

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 hypothalamus neuronal development (Lee et al., 2006; Marion et al., 2005) (Fig. 4D-J″). *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

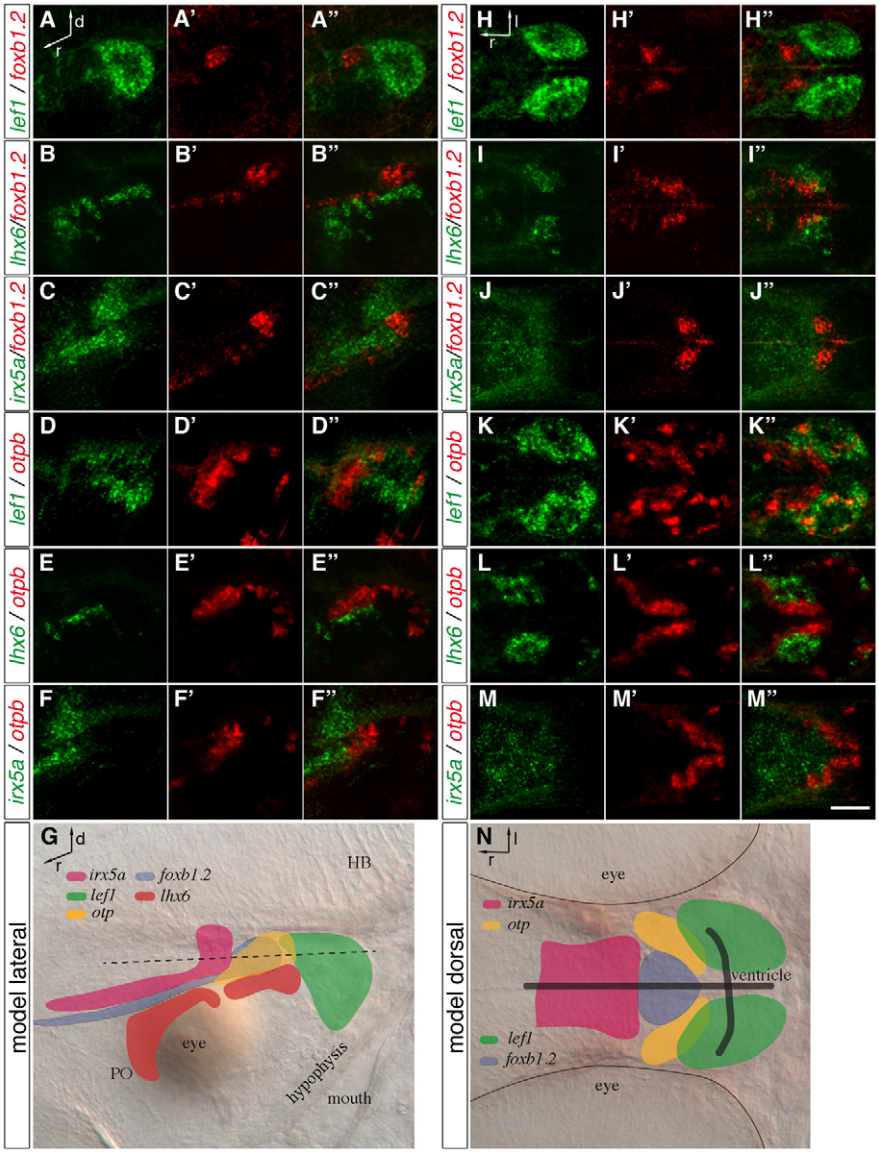


**Fig. 3. Distinct MA domains are present at 2 dpf.** (**A-F″,H-M″**) Two-color FISH of 2-dpf wild-type zebrafish embryos with probes detecting *foxb1.2*, *otpb*, *lef1*, *lhx6* and *irx5a* expression. Maximum projections of up to 40 μm confocal stacks are shown in lateral (A-F″) and dorsal (H-M″) view. (A-A″,D-D″,H-H″,K-K″) The *lef1* expression domain is positioned posterior to *foxb1.2* and *otpb*, slightly overlapping with *otpb* expression. (B-B″,E-E″,I-I″,L-L″) *lhx6* is expressed ventral and lateral to the *foxb1.2* and *otpb* domain. (C-C″,F-F″,J-J″,M-M″) *irx5a* expression is found anterior and dorsal to *foxb1.2* and *otpb* expression. (**G**,**N**) Representation of the transcription factor expression domains in lateral (G) and dorsal (N) view. The black dashed line (G) indicates the plane of the expression domains shown in the dorsal view. Black lines in N indicate the position of the ventricle. PO, preoptic area; HB, hindbrain; d, dorsal; l, lateral; r, rostral. Scale bar: 60 μm.

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

Thus, changing the levels of Otp, Fezf2 or Foxb1.2 leads to changes in the expression of the other transcription factors, whereas changes in Sim1a levels and interference with Wnt signaling do not. Otp, Fezf2 and 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 *fezf2*, this is particularly pertinent because we have shown that *fezf2* affects early development of the posterior hypothalamus at 1 dpf. In order to distinguish between early and late effects of *fezf2*, we designed a PhotoMorph (PM) (Tomasini et al., 2009) specific to *fezf2* MO sequences, which led to near complete caging of the *fezf2* MO and partial uncaging upon UV exposure at 1 dpf (supplementary material Fig. S15). The expansion of *foxb1.2* and *sim1a* was still apparent in the embryos injected with *fezf2* PM after uncaging, whereas the *otpa* reduction was not (supplementary material Fig. S16). This suggests the existence of

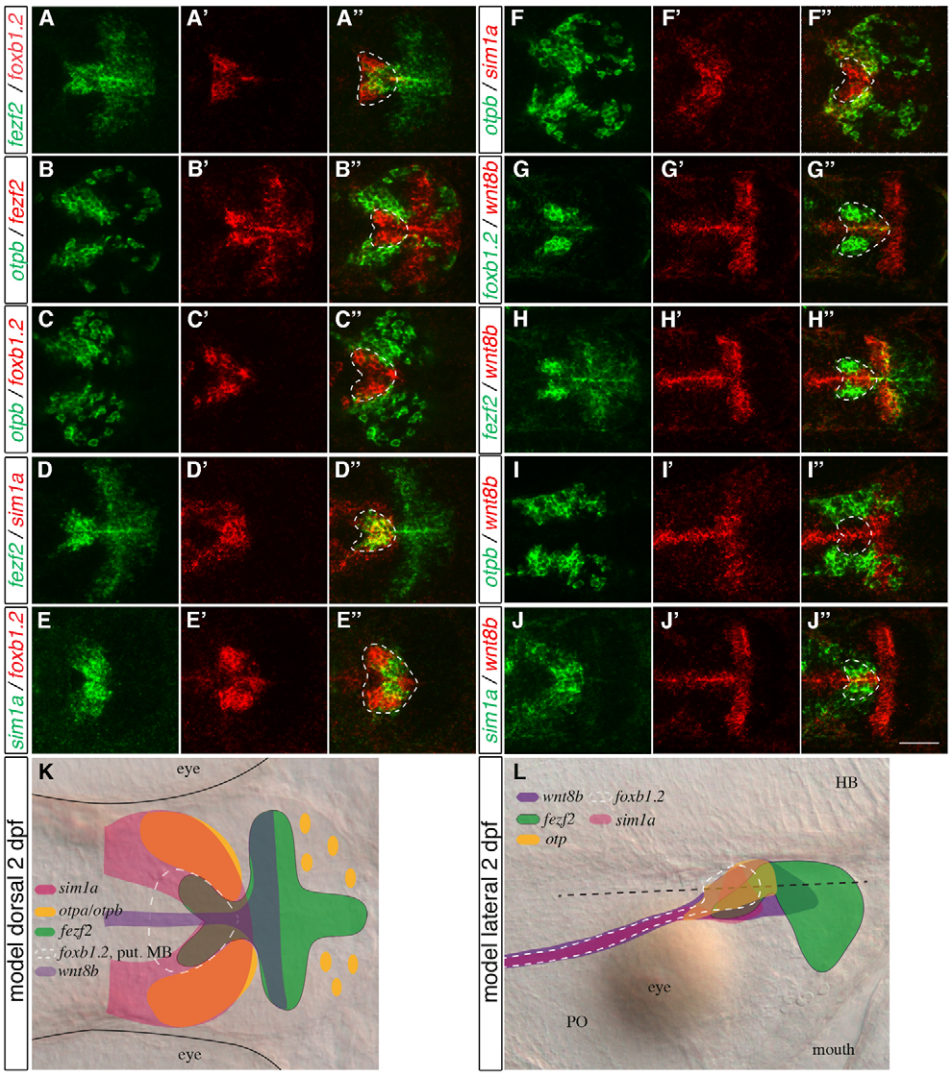


**Fig. 4. The expression of *foxb1.2*, *fezf2*, *otpb*, *sim1a* and *wnt8b* in the MA.** (**A-J″**) Two-color FISH of 2-dpf wild-type zebrafish embryos with probes for *otpb*, *sim1a*, *fezf2*, *foxb1.2* and *wnt8b* reveals their relative expression patterns in the MA. (A-A″) Strong *foxb1.2* expression marks the putative mammillary body (MB) where *fezf2* expression partially overlaps but extends further posterior. (B-C″) By contrast, *otpb* is expressed laterally and adjacent to *fezf2* (B-B″) and *foxb1.2* (C-C″). (D-D″) *fezf2* and *sim1a* are partially co-expressed in the putative MB. (E-E″) *sim1a* partially overlaps with *foxb1.2*. (F-F″) Lateral to the putative MB, *sim1a* is co-expressed with *otpb*. (G-J″) *wnt8b* is expressed in the midline and in a domain posterior to the putative MB (G-G″) and is partially co-expressed with *fezf2* (H-H″), *otpb* (I-I″) and *sim1a* (J-J″). (**K**) Summary of the relative expression domains of *foxb1.2*, *sim1a*, *otp*, *fezf2* and *wnt8b* in the dorsal view. The white dashed lines demarcate the putative MB. (**L**) Representation of the relative expression domains of *foxb1.2*, *sim1a*, *otp*, *fezf2* and *wnt8b* in the lateral view. Dorsal views of maximum projections of 10-15 µm confocal stacks are shown. Scale bar: 60 µm.

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 $otp^{+}$, $sim1a^{+}$, $fezf2^{-}$ and $foxb1.2^{-}$ and Uts1 cells are $otp^{-}$, $sim1a^{+}$, $fezf2^{+}$ and $foxb1.2^{+}$.

## 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-F″,H-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*

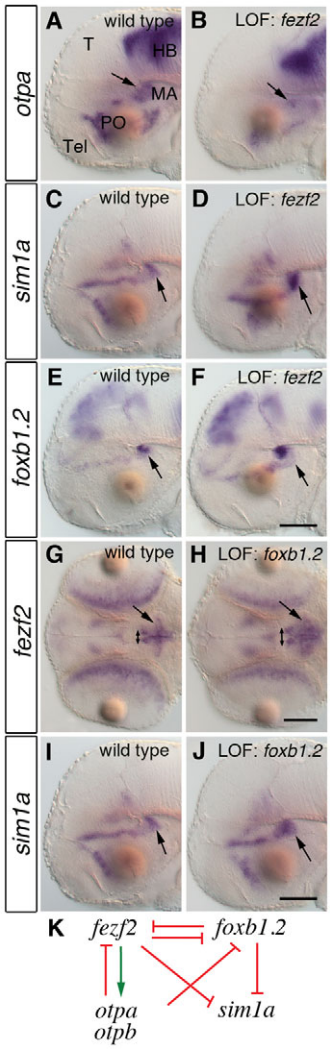


**Fig. 5. Genetic interactions among *fezf2*, *otp*, *sim1a* and *foxb1.2*.** (**A-J**) WISH of 2-dpf wild-type zebrafish embryos (A,C,E,G,I) and embryos injected with 4 ng *fezf2* MO (B,D,F) or 8 ng *foxb1.2* MO (H,J) to detect changes in the expression of *otpa*, *sim1a*, *foxb1.2* and *fezf2*. Lateral (A-F,I,J) and dorsal (G,H) views are shown. (A,B) The expression of *otpa* is reduced in the MA in *fezf2* morphants (B, arrows; *n*=11/14). (C,D) In the MA, *sim1a* is expanded in *fezf2* morphants (D, arrow; *n*=19/19). (E,F) The loss of Fezf2 activity results in an expansion of the *foxb1.2* domain (F, arrows; *n*=32/32). (G,H) The expression of *fezf2* in *foxb1.2* morphants is expanded in the anterior (double-headed arrows) and posterior MA (H, arrows; *n*=19/25). (I,J) Also the expression of *sim1a* in *foxb1.2* morphants is expanded (J, arrow; *n*=12/12). The same wild-type image is shown in C and I for the purposes of direct side-by-side comparison with morphants; the *in situs* for C/I, D and J were all conducted in parallel. (**K**) Summary of the observed genetic interactions. T, tectum; Tel, telencephalon; PO, preoptic area; MA, mammillary area; HB, hindbrain; LOF, loss-of-function. Scale bar: 100 μm.

(Fig. 7G,I,J,N; Fig. 6B-C″). However, the loss of Otp or Foxb1.2 activity resulted in an increase in Uts1-positive cells (Fig. 7G,H,L,N), which is likely to be due to the increase in *fezf2* in these embryos. In contrast to Vip-positive cells, a decrease in Uts1-positive cells was still detected in embryos that were injected with *fezf2* PM and uncaged at 2 dpf, suggesting an important role of Fezf2 for the specification of these neurons (Fig. 7G,K,N). Injection of 8 ng standard control MO led to no apparent changes in the expression of the investigated transcription factors or in the expression of *vip* and *uts1* (supplementary material Table S5).

To test whether the transcription factors expressed in Vip- or Uts1-positive cells are sufficient to promote their fates, we performed temporally controlled gain-of-function analyses. We generated transgenic zebrafish lines that overexpress *otpa*, *sim1a*, *foxb1.2* or *fezf2* cDNA. The heat-shock experiments were performed at 34 hpf for Vip or at 2.5 dpf for Uts1. Heat-shock treatments induced clear overexpression of the specific transcription factors (supplementary material Fig. S17). Since *vip* is co-expressed with *otp* and *sim1a* (Fig. 6F-F″,H-H″) but not with *fezf2* and *foxb1.2* (Fig. 6E-E″,G-G″), we hypothesized that overexpression of Otpa, Sim1a and/or a combination of both might lead to supernumerary Vip-positive cells. Indeed, we found more Vip-positive cells in embryos overexpressing Otpa, Sim1a or a combination of both compared with wild-type embryos that were heat shocked (Fig. 7O; supplementary material Fig. S18A-D). Notably, the overexpression of Fezf2 or Foxb1.2 did not lead to an increase in the number of Vip-positive cells, consistent with the fact that Vip-positive cells are *fezf2*- and *foxb1.2*-negative at this stage (Fig. 7O; supplementary material Fig. S18A,E,F).

Since *uts1* was co-expressed with *fezf2*, *sim1a* and *foxb1.2* (Fig. 6A-C″), we tested the effect of overexpression of these factors on the number of Uts1-positive cells. Although overexpression of Sim1a alone had no effect, overexpression of Fezf2, Sim1a plus Fezf2, or Foxb1.2 led to an increase in Uts1-positive cells (Fig. 7P; supplementary material Fig. S18G-K). The increase in cell number was small, but nonetheless significant and consistent across multiple experiments.

Thus, taken together, our loss-of-function and overexpression analyses demonstrate that Vip-positive neuron specification requires Otp and Sim1a, whereas Uts1-positive neurons require 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

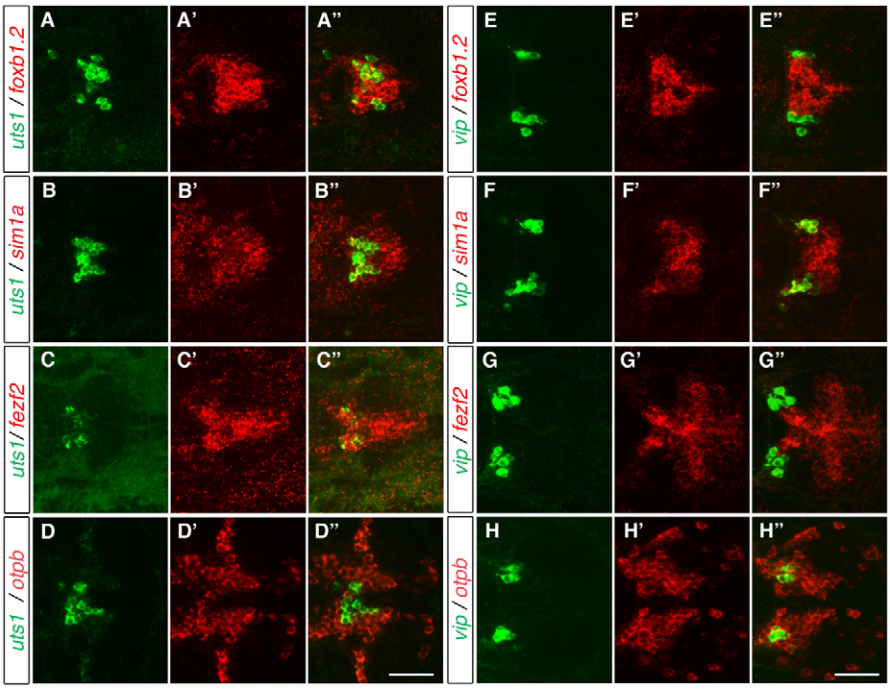


**Fig. 6. Vip- and Uts1-positive neurons develop within different MA domains.** (**A-H″**) Two-color FISH of 3-dpf (A-D″) and 2-dpf (E-H″) wild-type zebrafish embryos to detect the relative expression of *vip*, *uts1*, *otpb*, *sim1a*, *fezf2* and *foxb1.2* in the MA. *uts1* is co-expressed with *foxb1.2* (A-A″), *sim1a* (B-B″) and *fezf2* (C-C″) but not with *otpb* (D-D″). By contrast, *vip* is not co-expressed with *foxb1.2* (E-E″) and *fezf2* (G-G″), whereas it is co-expressed with *sim1a* (F-F″) and *otpb* (H-H″). Maximum projections of 10-15 μm confocal stacks in the dorsal view are shown. Scale bar: 60 μm.

contrast, although Fezf2 is expressed early in the hypothalamus, its function in the posterior hypothalamus was not known. We showed that *fezf2* expression is limited to the posterior half of the *nkx2.1a* domain at 1 dpf, perfectly abutting but not overlapping with the anterior *shh* domain, suggesting a role for Fezf2 in the posterior hypothalamus. Indeed, our results show that, in *fezf2* morphants, the posterior hypothalamic regional marker *emx2* was severely reduced.

Since Wnt signaling influences regionalization within the hypothalamic subdomains, promoting posterior fate at the expense of anterior fate, we tested whether the effect of Fezf2 on *emx2* could be mediated by Wnt signaling. It has been shown that the expression of exogenous *wnt8b* in the presumptive hypothalamus results in the expansion of *emx2* (Kapsimali et al., 2004). Consistent with this overexpression phenotype, we observed a strong reduction of *emx2* in *wnt8b* morphants. However, our results show that although *fezf2* regulates *wnt8b* and *emx2* expression, its effect on *emx2* cannot be via *wnt8b* because we observed the expansion and not the reduction of *wnt8b* in *fezf2* morphants. Previous studies have shown that *wnt8b* acts upstream of *fezf2* in the neural plate (Hashimoto et al., 2000) as well as in controlling DA cell number (Russek-Blum et al., 2008). Our results reveal that Fezf2 can in turn regulate *wnt8b* expression, revealing a novel feedback control by Fezf2. Nodal signaling and inhibition of Hedgehog signaling are important for establishing the posterior-ventral hypothalamus (Mathieu et al., 2002). *shh* expression is indeed reduced in *fezf2* morphants (Jeong et al., 2007). Therefore, it is possible that the loss of *emx2* expression that we observe in *fezf2* morphants might involve early alterations of Hedgehog signaling.

## 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 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; Vosko 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.

## 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

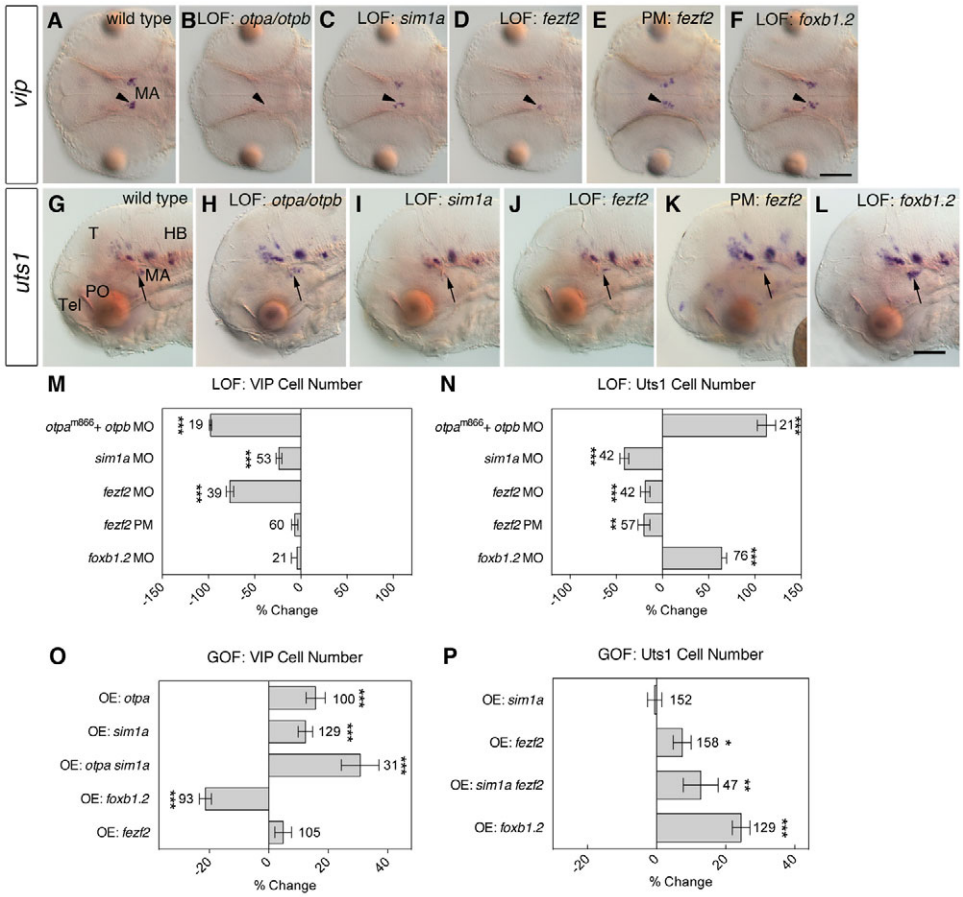


**Fig. 7. Transcription factor requirements and sufficiency for Vip- and Uts1-positive neuron specification.** (**A-L**) *vip* and *uts1* WISH at 2 dpf (A-F) and 3 dpf (G-L) in wild-type zebrafish embryos (A,G), *otpa*$^{m866}$ mutant embryos injected with 4 ng *otpb* MO (B,H) and wild-type embryos injected with 1 ng *sim1a* MO (C,I), 4 ng *fezf2* MO (D,J), 4 ng *fezf2* MO caged with PhotoMorph (PM) until 1 or 2 dpf (E,K) and 8 ng *foxb1.2* MO (F,L). Dorsal (A-F) and lateral (G-L) views are shown. (A-F) *vip* expression in the MA (arrowheads) is reduced in embryos with loss of Otp (B; *n*=19/19), Sim1a (C; *n*=30/53) and Fezf2 (D; *n*=37/39) activity. (E) Embryos with *fezf2* PM uncaged at 1 dpf (*n*=60/60) and *foxb1.2* morphants (F; *n*=20/20) show no changes in *vip* expression. (G-L) The expression of *uts1* in the MA (arrows) is upregulated in embryos that lack Otp function (H; *n*=21/21) whereas it is downregulated in *sim1a* (I; *n*=29/42) and in *fezf2* (J; *n*=17/42) morphants. (K) Uncaging of the *fezf2* PM at 2 dpf resulted in a reduction of *uts1* expression (*n*=32/57). (L) The loss of Foxb1.2 function leads to an expansion of the *uts1*-positive cells (*n*=58/76). (**M-P**) Percentage change in the number of Vip-positive (M,O) and Uts1-positive (N,P) neurons in loss-of-function embryos compared with uninjected wild-type embryos (M,N) and in transgenic embryos overexpressing cDNAs for *otpa*, *sim1a*, *fezf2* or *foxb1.2* compared with heat shocked wild-type embryos (O,P). Cell number changes were compared with the theoretical median cell number of wild-type embryos and analyzed using the Wilcoxon signed rank test. At 2 dpf, the number of Vip-positive cells in wild-type embryos has median and mean (±s.e.m.) values of 14 and 14.6±0.36, respectively. (M) The loss of Otp, Sim1a and Fezf2 function leads to a reduction in VIP cell number in the MA compared with wild-type embryos, whereas the uncaged *fezf2* PM and the *foxb1.2* morphants show no difference compared with the wild type (*P*=0.0812 and *P*=0.7423, respectively). (N) The number of Uts1-positive cells in wild-type embryos has median and mean values of 8 and 8.5±0.22, respectively. Embryos lacking Otp and Foxb1.2 activity have an increased number of Uts1-positive cells, whereas the loss of Sim1a, Fezf2 and the uncaged *fezf2* PM leads to a reduction. (O,P) Heat shocked wild-type embryos have median and mean values of 13 and 12.8±0.17 Vip-positive cells, respectively. (O) Overexpression of Otpa and Sim1a leads to a higher number of Vip-positive cells. Combined overexpression of Otpa and Sim1a also leads to a higher number of Vip-positive cells, whereas overexpression of Foxb1.2 results in a lower number of Vip-positive cells. Overexpression of Fezf2 led to no change in the number of Vip-positive cells (*P*=0.2170). (P) The number of Uts1-positive cells in heat shocked wild-type embryos has median and mean values of 9 and 8.8±0.19, respectively. Overexpression of Fezf2, a combination of Fezf2 and Sim1a, and Foxb1.2 leads to a higher number of Uts1-positive cells at 3 dpf in the MA compared with the wild type, whereas overexpression of Sim1a showed no change in the number of Uts1-positive cells (*P*=0.5645). **P*<0.05, ***P*<0.01, ****P*<0.001; error bars indicate s.e.m. The number of embryos analyzed is shown alongside each bar. T, tectum; Tel, telencephalon; PO, preoptic area; MA, mammillary area; HB, hindbrain; LOF, loss-of-function; GOF, gain-of-function; PM, PhotoMorph. Scale bar: 100 μm.

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*, *foxb1.2* and *sim1a* 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 redeployed later to generate different neuronal subtypes. Second, common transcription factor sets are used in distinct hypothalamic regions in a different regulatory context.

**Acknowledgements**
We thank Gonzalo Alvarez-Bolado for helpful discussions and comments on the manuscript; Jose Arturo Guiterrez-Triana, Colette Maurer, Ingrid Lohmann and Detlev Arendt for critical reading of the manuscript; Matthias Carl, Laure Bally-Cuif, Wolfgang Driever and Hans-Martin Pogoda for providing probes or MOs; Günes Ozhan-Kizil and Rodrigo De Marco for helpful advice; Regina Singer for technical assistance; and Gabi Shoeman, Regina Singer and Angelika Schoell for expert fish care.

**Funding**
This work was supported by the Max Planck Society.

**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**
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.085357/-/DC1

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