Dissertation

submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by

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born in:	Karl-Marx-Stadt, today Chemnitz
Oral examination:	9th November 2012

Molecular Mechanism of Hypothalamus Development in Zebrafish

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Für Boris

Wahr ist an einer Geschichte immer nur das, was der Zuhörer glaubt.

Hermann Hesse

Abstract

The hypothalamus is a key integrative center in the brain consisting of diverse cell types that differ in morphology and the neuropeptides they produce. These neuropeptides are required for the pivotal functions of the hypothalamus including homeostasis, reproduction, cognitive behavior and stress response. Despite the knowledge of several transcription factors important for the hypothalamic neuronal development, little is known about how these transcription factors act in concert to generate the amazing diversity of neuron types present in the hypothalamus. In this study, I focus on the development of two hypothalamic areas in zebrafish, the preoptic area and the posterior hypothalamus. The preoptic area plays an important role in homeostasis and stress response whereas the posterior hypothalamus is important for cognitive behavior in mammals. Consistent with their different functions, these two areas are composed of distinct neuron types. However, the mechanism that contributes to this difference remains largely unknown. In this thesis, I characterize the roles of four transcription factors, Fezf2, Otp, Sim1a and Foxb1.2, for the development of the preoptic area and posterior hypothalamus. I show that early in development Fezf2 is important for the regionalization of the posterior hypothalamus, while later Fezf2 interacts with Otp, Sim1a and Foxb1.2 to specify distinct neuron types. Interestingly, these regulatory interactions change during development and vary between the anterior and the posterior hypothalamus, contributing to the formation of regional differences in these areas. Further, these interactions generate different domains in the posterior hypothalamus and contribute to the specification of distinct neuron types such as those that produce vasoactive intestinal peptide (VIP) and urotensin 1 (Uts1). In summary, this study provides the first regulatory and molecular map of the posterior hypothalamus and demonstrates that the specification of preoptic and posterior hypothalamic neurons require the dynamic regulatory interactions of Fezf2 together with Otp, Sim1a and Foxb1.2.

Kurzzusammenfassung

Der Hypothalamus ist das integrative Hauptkontrollzentrum im Gehirn, bestehend aus verschiedenen Zelltypen, welche sich in ihrer Morphologie und den Neuropeptiden die sie produzieren, unterscheiden. Diese Neuropeptide werden für die essentiellen Funktionen des Hypothalamus, wie Homöostase, Reproduktion, kognitives Verhalten und Stress Antwort benötigt. Trotz der Kenntnis von einigen Transkriptionsfaktoren, welche für die neuronale Entwicklung des Hypothalamus wichtig sind, ist es immer noch kaum verstanden, wie Transkriptionsfaktoren zusammenarbeiten um die erstaunliche Vielfalt an Neuronen im Hypothalamus zu bilden. In dieser Arbeit konzentriere ich mich auf die Entwicklung zweier Regionen des Hypothalamus im Zebrafisch, die präoptische Region und der posteriore Hypothalamus. Die präoptische Region hat eine wichtige Aufgabe bei der Aufrechterhaltung der Homöostase und der Stress Antwort, wohingegen der posteriore Hypothalamus beim kognitiven Verhalten in Säugetieren eine Rolle spielt. Entsprechend ihrer vielzähligen Aufgaben sind diese zwei Regionen aus verschiedenen Neuronentypen aufgebaut. Der Mechanismus der zu dieser Diversität führt, ist jedoch kaum verstanden. In dieser Doktorarbeit charakterisiere ich die Aufgabe von vier Transkriptionsfaktoren, Fezf2, Otp, Sim1a und Foxb1.2, für die Entwicklung der präoptischen Region und des posterioren Hypothalamus. Ich zeige, dass in der frühen Entwicklung Fezf2 wichtig für die Regionalisierung des posterioren Hypothalamus ist, während Fezf2 später mit Otp, Sim1a und Foxb1.2 interagiert, um bestimmte Neuronentypen zu generieren. Interessanterweise ändern sich diese regulatorischen Interaktionen während der Entwicklung und variieren zwischen anterioren und posterioren Hypothalamus. Das trägt dazu bei regionale Unterschiede in diesen Arealen zu etablieren. Zudem gestallten diese Interaktionen verschiedene Domänen im posterioren Hypothalamus, wo sie zur Festlegung von speziellen Neuronentypen beitragen, welche zum Beispiel vasoaktives intestinales Peptid (VIP) und Urotensin 1 (Uts1) produzieren. Zusammenfassend bietet diese Doktorarbeit die erste regulatorische und molekulare Karte des posterioren Hypothalamus. Außerdem zeigt sie, dass die Entwicklung von präoptischen und posterior hypothalamischen Neuronen die dynamische regulatorische Interaktion von Fezf2 zusammen mit Otp, Sim1a und Foxb1.2 benötigt.

Parts of this thesis are published:

Andrea Wolf and Soojin Ryu
Molecular mechanism of posterior hypothalamic neuron development
Development, in revision

Andrea Wolf and Soojin Ryu
Molecular mechanism of the preoptic neuron specification
Manuscript in preparation

Parts of this thesis were presented:

Andrea Wolf and Soojin Ryu
Transcriptional regulatory networks for the development of two different hypothalamic regions in zebrafish
Poster, 7th European Zebrafish Meeting, Edinburgh, July 2011

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1 Introduction

The central nervous system (CNS) is a highly complex structure composed of billions of neurons, oligodendrocytes and astrocytes. Despite a large amount of studies addressing the development of the CNS, the mechanism for the generation of the different neuronal cell types is not fully understood. In vertebrates a single factor is not sufficient to induce a distinct neuronal fate but a combination of factors is necessary. These factors are mostly transcription factors (TF) that act together in a temporally and spatially restricted regulatory code (Cayouette et al., 2006; Guillemot, 2007; Shirasaki and Pfaff, 2002).

1.1 Transcription factors in cell type specification

Transcription factors are proteins that bind to specific DNA sequences within the enhancer region of a gene acting as an activator or a repressor of transcription (Latchman, 1997). The enhancer regions of genes contain several binding sites for TFs, also called response elements, which are able to respond to developmental cues or to regulatory signals. Transcription factors have specific DNA-binding domains and are classified on the basis of these domains such as homeodomain (HD) TF, zinc-finger TF or basic helix-loop-helix (bHLH) TF. Since approximately 8% of the vertebrate genome codes for transcription factors (Babu et al., 2004), the combinatorial action of a subset of the transcription factors is thought to be sufficient for the unique regulation of every gene in the genome. However, how these TFs form a temporally and spatially restricted regulatory code for cell type specification still remains elusive. In addition, transcription factor often interact with other proteins (other transcription factors or co-regulatory proteins), changing binding specificity and therefore changing their function. This fact could results in a re-deployment of early regulators to become a part of a subtype-specific combinatorial code (Baumgardt et al., 2007; Lee et al., 2008; Shirasaki and Pfaff, 2002). Broadly, there are two different types of TFs: regulators that are constitutively active nuclear factors in every cell and at every time point and those that are highly regulated. Constitutively active regulators have important facilitating roles in the transcription of genes such as structural proteins or ubiquitous metabolic enzymes. Further, these TFs also participate in the formation of enhanceosomes together with TFs that are highly regulated (Brivanlou and Darnell, 2002). These regulatory TFs are classified into two broad categories: the first class are developmental or cell-type specific TFs and the second

class are signal-dependent TFs that are inactive until the presence of an appropriate intra- or extracellular signals (Brivanlou and Darnell, 2002). For this thesis the cell-type specific transcription factors are of particular interest since these TFs are important for the formation of distinct neuron types.

How neuron types in the cortex, the retina or within the spinal cord are specified has been extensively studied over the last decades. The spinal cord is so far the most extensively studied area of neuron subtype specification. In the ventral neural tube of the spinal cord at least five different subtypes of neurons are present. Each of them expresses a unique combination of transcription factors and emerges in a precise spatial order from the proliferating progenitor cells along the dorsoventral axis (Shirasaki and Pfaff, 2002). The dorsoventral patterning of the spinal cord is achieved through a secreted gradient of Sonic hedgehog (Shh). Therefore, different Shh concentration leads to the formation of a distinct neuronal cell type, which is dependent on the distance from the Shh source. This graded Shh signaling leads to the expression of the so called class I and class II proteins based on whether their expression is repressed or induced by Shh. Cross-repressive interactions between class I and class II proteins (HD and bHLH proteins) that abut a common progenitor domain lead to the establishment of individual progenitor domains (Briscoe and Novitch, 2008; Shirasaki and Pfaff, 2002). Each of the progenitor domains gives rise to specific neuronal subtypes indicating that neuronal subtype specification involves the combinatorial use of a limited number of TFs (Shirasaki and Pfaff, 2002).

Other mechanisms have been suggested to explain how the great neuronal diversity can be achieved by a small number of common factors. In the vertebrate retina, HD proteins determine the laminar position of the retinal cells while bHLH proteins like Ath5 determine their time of birth. In the retina the time of birth is tightly correlated with the neuron type that is produced (Guillemot, 2007). The lineage and the number of cell divisions of the progenitor cell is important to determine which set of TFs are expressed by the progenitor and therefore also by the daughter cell. In addition, the orientation of cell divisions is used to define whether the progenitor cell undergoes a symmetric or an asymmetric cell division. To ensure that the right amount of retinal subtypes will be established, terminal differentiated neuronal subtypes provide a feedback inhibition signal (Cayouette et al., 2006). Also cerebral cortex neurons are born on a predictable schedule depending on timing mechanisms achieved by cell-intrinsic and environmental factors. Similar to retinal cells, the multipotent progenitor cells in the cortex undergo repeated asymmetric cell divisions to generate neuronal subtypes in their normal *in vivo* order. Once a progenitor generates neurons of a particular layer, they can not generate earlier born neurons. This timing mechanism is encoded within the progenitor cells by distinct TF expression, generated by a division pattern, which leads to the generation of characteristic neuronal lineage trees (Shen et al., 2006). The patterning of the cortex

takes place by gradients of extracellular signaling molecules, like Shh, that comes from instructive cell populations also known as local organizers. This graded signaling of morphogens leads to a distinct expression of TF that specify the neocortical progenitors (Molyneaux et al., 2007; Scholpp and Lumsden, 2010).

In contrast to spinal cord, retina and cortex our understanding of the mechanisms by which hypothalamic neurons are specified is limited.

1.2 Development of the hypothalamus

The hypothalamus is an evolutionary ancient key integrative center in the brain pivotal for the survival and the propagation of the vertebrate species. It orchestrates complex adaptive behaviors that regulate multiple physiological functions including stress response, food intake, thermoregulation, fluid homeostasis and reproductive behavior. The hypothalamus can be grossly divided in the anteroposterior extent into four major areas, the preoptic, the anterior, the tuberal and the mammillary area. In addition, it can be divided in the mediolateral direction into the periventricular, the medial and the lateral region (Saper, 2004; Simerly, 2004; Swanson, 1987; Szarek et al., 2010). More than a dozen hypothalamic nuclei with specific functions and different cell types are intermingled within different hypothalamic regions. In the lateral hypothalamus, for example, the lateral hypothalamic area (LHA) can be found. A lesion of this area leads to a reduction in food intake (Jain et al., 1999). Another nucleus of the lateral hypothalamus is the supraoptic nucleus (SON), which is implicated in fluid homeostasis and lactation (Swanson, 1987). The medial region of the hypothalamus contains the ventromedial nucleus (VMH), which is most commonly associated with satiety or sexual behavior (Saper, 2004). A special nucleus of the medial region of the hypothalamus is the mammillary body (MB) associated with spatial and working memory and implicated in neurodegenerative disorders such as the Korsakoff syndrome (Beracochea, 2005; Radyushkin et al., 2005). Notably the periventricular region of the hypothalamus contains most of the well-delineated nuclei of the hypothalamus including the arcuate nucleus (ARC), which is important for feeding behavior and autonomic nervous system control and the suprachiasmatic nucleus (SCN) responsible for controlling endogenous circadian rhythms. The paraventricular nucleus (PVN) within the periventricular region of the hypothalamus is a particularly good example for the fact that each nucleus typically has multiple functions: The PVN is important for stress response, maternal behavior, lactation and fluid homeostasis.

Critical for the diverse function of the hypothalamus are the cells that secrete neurohormones located throughout the entire hypothalamus. These neurosecretory cells can be divided into two groups: the magnocellular neurosecretory cells and the

parvocellular neurosecretory cells. The magnocellular neurosecretory system directly secretes the neurohormones arginine-vasopressin (AVP) and oxytocin (OXT) into the posterior pituitary. The neurohormones AVP and OXT are expressed in the PVN and in the SON. In contrast, the parvocellular neurosecretory system controls the release of neurohormones into the anterior pituitary through the median eminence. Currently there are seven parvocellular neurosecretory neuronal subtypes known (Szarek et al., 2010): thyrotropin-releasing hormone (TRH, located within the PVN), corticotropin-releasing hormone (CRH, located within the PVN), growth hormone-releasing hormone (GHRH, located within the ARC), somatostatin (SS, located within the anterior periventricular nucleus), gonadotropin-releasing hormone (GnRH, located within the ARC), dopamine (DA, located within the ARC) and the gonadotropin-inhibiting hormone (GnIH, located within the dorsomedial nucleus).

1.2.1 The early patterning of the diencephalon

1.2.1.1 The neural plate

Studies in mouse, chicken and fish revealed that the patterning of the neural plate and the formation of the diencephalon is highly conserved among vertebrates (Blackshaw et al., 2010; Cavodeassi and Houart, 2012; Scholpp and Lumsden, 2010; Toro and Varga, 2007; Wilson and Houart, 2004). Although the forebrain is a highly complex structure, it derives from a relatively simple sheet of neuroepithelial cells, the neural plate. In zebrafish within the neural plate the anterior-posterior (A-P) axis are determined by caudalizing factors, which are secreted by the germ ring that moves during epiboly towards the vegetal pole. These caudalizing factors are mainly Wnt, but include also Fgf and Bmp, that form global gradients by diffusion and also by the movement of the germ ring (Wilson and Houart, 2004). A source of antagonists of the caudalizing factors is the prechordal plate that migrates rostrally beneath the neural plate. Due to the gradients of the caudalizing factors, domains with distinct TF expressions are formed within the neural plate. In the anterior neural plate, *pitx3* is expressed defining an area important for adenohypophysis placode specification (Toro and Varga, 2007). Another important TF expression domain within the neural plate is the domain of the Drosophila odd-paired like homolog *zic1*. Fate map studies showed that Zic1-positive cells are retinal precursors that form the eye field (Toro and Varga, 2007). A factor that abuts the *zic1* expression domain is the forkhead homolog *foxb1.2*. *foxb1.2* is exclusively expressed posterior to the eye field by diencephalic precursor cells. Interestingly, the prospective hypothalamus is demarcated by a median protrusion of this *foxb1.2* expression domain (Staudt and Houart, 2007; Toro and Varga, 2007). After gastrulation these prospective hypothalamic cells move anterior within the neural plate displacing the more dorsal forebrain tissue laterally.

This cell movement depends on Nodal signaling (Varga et al., 1999) and is important for the formation of left and right eyes from the single *zic1* expression domain (Toro and Varga, 2007; Wilson and Houart, 2004). Importantly, by the end of gastrulation these hypothalamic precursors also start to express Hedgehog (Hh), which in concert with nodal signaling is important for the hypothalamus specification (Mathieu et al., 2002).

1.2.1.2 The patterning of the diencephalon

Subsequent to the initial regionalization of the neural plate, local cell populations like the floorplate or the isthmic organizer are established to build local sources of signals within the developing forebrain. By the end of gastrulation these local organizers lead to an initial regional patterning by inducing genes that start to subdivide the neural plate into discrete areas displaying the features of the mature CNS (Wilson and Houart, 2004). One of the first local organizers established during the CNS patterning is the mid-hindbrain boundary (MHB). This local organizer secretes posteriorizing signals like Wnt, which is important for the A-P patterning of the diencephalon (Cavodeassi and Houart, 2012; Sylvester et al., 2010). In addition, the anterior neural ridge expresses a Wnt antagonist Six3 leading to a region of low graded Wnt activity within the forebrain. Six3 is repressed by enhanced Wnt activity and vice versa, Six3 activity represses the transcription of Wnt genes (Cavodeassi and Houart, 2012; Wilson and Houart, 2004). Within this region of graded Wnt activity, other transcription factor expression domains are established. In the anterior region of low Wnt activity close to Six3, the expression of the zinc finger protein Fezf2 is established whereas in the more posterior region of higher Wnt activity the expression of the iroquois homeobox protein Irx3 and of the orthodenticle homolog Otx is initiated (Scholpp and Lumsden, 2010; Wilson and Houart, 2004). The Fezf2 domain directly abuts the Otx domain whereas there is a small gap between the Fezf2 and the Irx3 domain. The repressive interaction of these factors define a sharp boundary where within the Fezf2 and Irx3 interspace the zona limitans intrathalamica (ZLI) is established (Blackshaw et al., 2010; Hirata et al., 2006; Jeong et al., 2007; Scholpp and Lumsden, 2010; Staudt and Houart, 2007; Wilson and Houart, 2004). Within the ZLI, is an organizing cell population called mid-diencephalic organizer (MDO), whose signals are crucial for the establishment of the entire thalamus. After the correct positioning of the ZLI the expression of Shh is induced. The Shh expression therefore expands progressively from the basal plate to the roof plate (Scholpp and Lumsden, 2010) maintained by canonical Wnt signaling (Mattes et al., 2012). This progressive maturation from ventral to dorsal is mediated by the expression of retinoic acid in the roof plate that blocks Shh expression. The fully mature MDO expresses genes of the Hedgehog family and other secreted signaling molecules in all vertebrates but the

significant organizing function is mediated by Shh itself (Scholpp and Lumsden, 2010). Following the exposure to Shh the prethalamus and the thalamus acquire their distinct fates. Shh signaling also results in the zonation of proneural gene expression leading to the rostral and the caudal thalamus and to the specification of thalamic neuronal subtypes (Blackshaw et al., 2010; Scholpp and Lumsden, 2010).

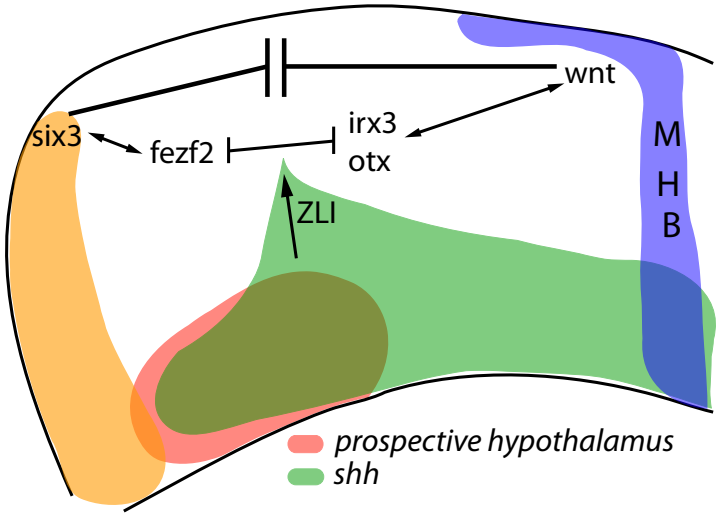

Fig. 1.1: The patterning of the diencephalon
A scheme that summarizes the interaction of different signaling pathways to establish the ZLI and the prospective hypothalamus in zebrafish. The Wnt source is shown in blue while the Six3 source is shown in orange. Adapted from Sylvester et al. (2010). A lateral view is shown and anterior is to the left.

In contrast to the thalamus, the patterning of the hypothalamus is much less understood. Apart from the establishment of the ZLI an additional function of Shh is the dorsal-ventral (D-V) patterning of the prospective forebrain in concert with BMP signaling. BMP, which is expressed in the roof plate, opposes the Shh action that is expressed in the floor plate establishing a graded D-V axis (Cavodeassi and Houart, 2012). All prospective hypothalamic cells are Shh-positive and through the opposing interaction of Shh with BMP ventral forebrain cells are guided towards a hypothalamic rather than a floorplate fate (Dale et al., 1997). Additionally, there are also locally derived BMPs mediating the downregulation of Shh expression in the posterior-ventral hypothalamus (Manning et al., 2006). This downregulation of Shh induces the expression of Fgf and of the empty spiracles homeobox protein Emx2, which is a marker for the posterior-ventral (PV) hypothalamus, while the continuous Shh expression demarcates the anterior-dorsal (AD) hypothalamus (Mathieu et al., 2002; Wilson and Houart, 2004). The function of Fgf signaling within the hypothalamus is not completely understood. However, there is a study showing that Fgf signaling from the hypothalamic floor regulates the proliferation

of progenitors in the tuberal hypothalamus (Manning et al., 2006). Complementing the Shh-BMP signaling, a further pathway important for the ventral CNS midline cells to adopt hypothalamic instead of floorplate identity is the Wnt/Axin/β-catenin pathway. In this pathway Axin1 acts as a repressor of Wnt signaling to induce hypothalamic fate while it additionally promotes Nodal signaling in the PV hypothalamus (Kapsimali et al., 2004). Nodal signaling is required for the hypothalamic precursor cells to adopt PV hypothalamic fate. In contrast, Shh signaling is essential for the development of the AD hypothalamus. Cells that do not receive signals from either signaling pathway fail to become hypothalamic cells (Mathieu et al., 2002). After adopting a hypothalamic fate, canonical Wnt signaling is required for the neurogenesis in the posterior hypothalamus. This is mediated through the lymphoid enhancer factor Lef1 that is required for the expression of proneural and neural genes in that area (Lee et al., 2006). Interestingly Fezf2, which is strongly expressed in the hypothalamus by late somitogenesis is part of the Wnt/Lef1 pathway (Hashimoto et al., 2000; Jeong et al., 2007; Russek-Blum et al., 2008). This and the role of Fezf2 on DA neuron development imply a role of Fezf2 in promoting the development of hypothalamus (Jeong et al., 2007). Although the above mentioned signaling pathways are known to be important for the establishment of the hypothalamic cell fate, how distinct domains or nuclei and their neuronal cell types in hypothalamus form is still poorly understood.

1.2.2 Transcriptional regulation of the hypothalamus development

1.2.2.1 The homeodomain transcription factor Otp and the bHLH-PAS transcription factors Sim1/Arnt2

Genetic analyses in mouse have begun to identify transcription factors that play key roles in the formation of hypothalamic nuclei. The homeodomain transcription factor Orthopedia (OTP) and the heterodimeric complex formed by the bHLH-PAS transcription factors SIM1 and ARNT2 are required for development of virtually all magno- and parvocellular neurons of the PVN and SON, as well as neurons in the anterior periventricular nucleus (aPV) (Michaud et al., 2000). Both OTP-/- and SIM1-/- mutant mouse embryos lack AVP, OXT, CRH and TRH neurons in the PVN, SON and aPV while only OTP -/- mice lack SS neurons in the ARC (Acampora et al., 1999; Caqueret et al., 2005; Michaud et al., 1998; Szarek et al., 2010; Wang and Lufkin, 2000). Furthermore, OTP-/- mice lack a small population of hypothalamic dopamine (DA) neurons present near the central periaquiductal gray (belonging to the so-called A11 DA group) (Ryu et al., 2007). Notably, OTP-/- mice show reduced cell proliferation resulting

in hypocellular PVN and SON. Additionally, they show abnormal cell migration and defects in the differentiation of neuroendocrine progenitors (Acampora et al., 1999; Wang and Lufkin, 2000).

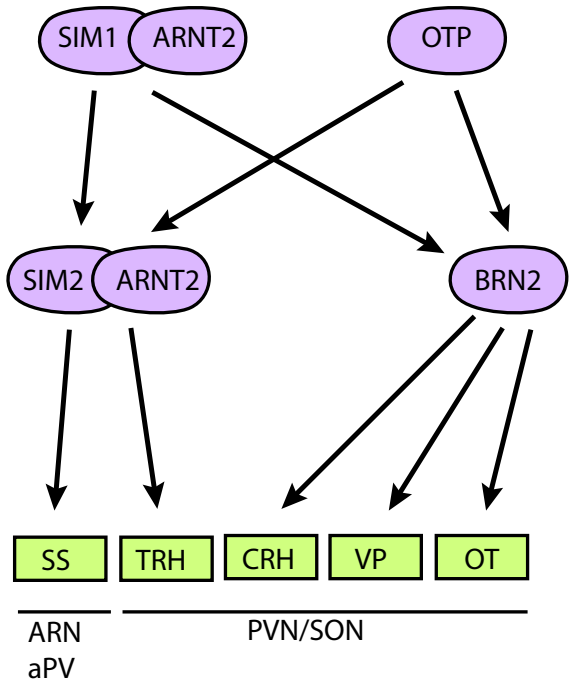

Fig. 1.2: Transcriptional regulation of hypothalamic neuron types in mouse
A scheme summarizing the role of OTP, SIM1, ARNT2, SIM2, BRN2 on the development of distinct neuronal subtypes in mouse. The arrows do not indicate direct interactions but genetic interactions. Modified from Caqueret et al. (2005).

Reflecting their parallel action, *Otp* and *Sim1* show an identical expression pattern within the hypothalamus and the amygdala (Wang and Lufkin, 2000). Accordingly, SIM1-/- mice exhibit hypocellular PVN and SON. However in contrast to Otp, SIM1/ARNT2 act during the late differentiation stage of the postmitotic hypothalamic progenitors (Michaud et al., 1998). Notably, SIM1+/- mice become hyperphagic and obese revealing a gene dosage effect of SIM1. These heterozygous SIM1 mutants have fewer AVP-, OXT- and TRH-positive neurons while there was no effect on CRH and SS neurons (Duplan et al., 2009; Xi et al., 2012). SIM1/ARNT2 and OTP act upstream of both the POU domain transcription factor BRN2 and the close homolog of SIM1, SIM2 to control the development of PVN, SON and a part of aPV neurons. Both Brn2 and Sim2 expression are diminished in Otp and Sim1 mutant mice. BRN2 mutant mice do not show defects in the initial hypothalamic development nor in the migration of progenitors but the mutants display defects in the maturation of the neurosecretory neurons CRH, AVP and OXT (Nakai et al., 1995; Schonemann et al., 1995). SIM2 mutant mice show a

reduction in SS and TRH neurons in the PVN and aPV. Since SIM1 is acting upstream of SIM2 and is a close homolog to SIM2, SIM1 can partially compensate for the loss of SIM2. Additionally, it is likely that ARNT2 is a heterodimerization partner of SIM2 as well (Goshu et al., 2004).

Interestingly, the function of the hypothalamic regulators appears to be highly conserved during evolution. In zebrafish, recent analyses show that Otp and Sim1/Arnt2 are required for the development of oxytocin-like (Oxtl), arginine-vasopressin-like (Avpl), as well as CRH and SS neurons in the neurosecretory preoptic area (NPO) (Blechman et al., 2007; Eaton and Glasgow, 2006, 2007; Eaton et al., 2008; Hill et al., 2009; Löhr et al., 2009; Ryu et al., 2007). Additionally, similar to mouse, Otp (this thesis) as well as Sim1 (Löhr et al., 2009) are required for the development of hypothalamic TRH neurons. Moreover, Brn2 (Pou3f2) expression in the NPO is downregulated in zebrafish embryos lacking either Sim1a/Arnt2 or Otp function (Löhr et al., 2009). Interestingly, studies in zebrafish first revealed the importance of Otp and Sim1 for the formation of DA neurons (Blechman et al., 2007; Borodovsky et al., 2009; Löhr et al., 2009; Ryu et al., 2007). This Otp dependency has been shown to be the case for A11 DA neurons in mouse as well (Ryu et al., 2007).

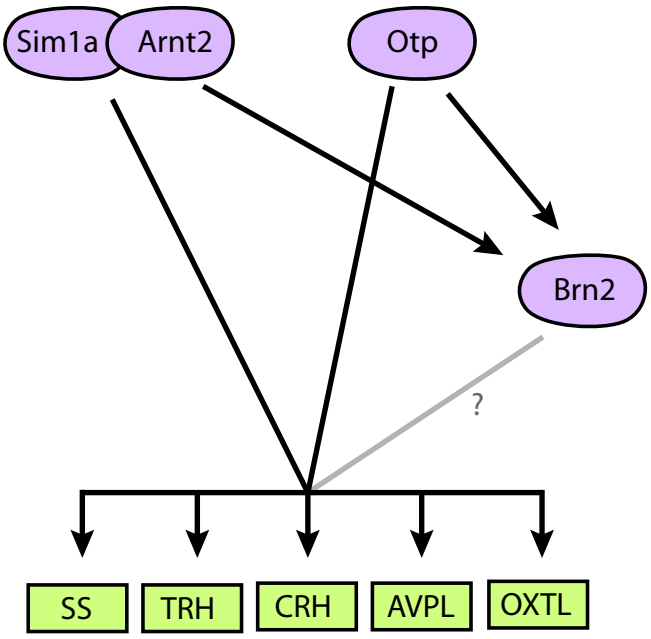

Fig. 1.3: Transcriptional regulation of hypothalamic neuron types in zebrafish

The scheme summarizes the requirement of Otp, Sim1a, Arnt2 and Brn2 for the formation of distinct hypothalamic neuron types. Black arrows show genetic but no direct interactions while the gray bar suggests a potential interaction.

The zebrafish was also the fist model organism where upstream regulators of Otp and Sim1 activity were found. For Sim1, so far, only one upstream regulator has been discovered. The bHLH protein Olig2 that is known to be one of the first factors expressed in neural progenitors activates *sim1a* expression in a specific progenitor population important for the formation of A11 related DA neurons (Borodovsky et al., 2009).

Recent studies revealed factors that regulate Otp. Nodal in concert with Fgf8 and Shh modulates *otp* expression in the NPO while Nodal but not Fgf8 and Shh regulates *otp* expression within the posterior tuberculum (PT) (Del Giacco et al., 2006). Further, the homeobox gene Prox1, a homolog to the Drosophila prospero, acts upstream of *otp* to specify DA neurons in the PT (Pistocchi et al., 2008). Two additional factors acting upstream of *otp* are the zinc finger-containing transcription factor Fezf2 and the G-protein-coupled receptor Pac1. While Fezf2 is a transcriptional activator for *otp*, Pac1 regulates *otp* on the post-transcriptional level to specify DA and serotonergic neurons in the PT and Oxtl neurons in the NPO (Blechman et al., 2007).

1.2.2.2 The zinc finger protein Fezf2

The forebrain embryonic zinc finger-like protein Fezf2 (also known as Fezl) contains six C2H2-type zinc fingers and an engrailed-homolog repressor motif (Shimizu and Hibi, 2009). Fezf2, which was originally discovered as a gene that is positively regulated by the over-expression of the Wnt inhibitor Dickkopf 1 (Dkk1) (Hashimoto et al., 2000), was known to control monoaminergic neuron development in a non-cell autonomous manner in zebrafish (Guo et al., 1999; Levkowitz et al., 2003). In addition, Fezf2 controls the expression of the bHLH protein Neurogenin 1 (Ngn1), which is also a determinant of PT DA neurons in zebrafish (Jeong et al., 2006). Fezf2 plays a role in establishing regional subdivisions within the diencephalon and in promoting the development of the hypothalamus and the telencephalon has been found (Jeong et al., 2007) (also see paragraph 1.2.1.2). More recently, two more downstream targets of Fezf2 the TF eomesa or Tbr2 and the Lim homeobox protein Lhx2 have been found (Chen et al., 2011).

The function of Fezf2 appears to be highly conserved across species. In mouse, it is expressed in the thalamic eminence, the prethalamus, the hypothalamus and the dorsal telencephalon, which later becomes confined to the deep layers of the cortex (Shimizu and Hibi, 2009). Like in zebrafish (Jeong et al., 2007) FEZF2 controls the rostro-caudal patterning of the diencephalon in mouse, since FEZF2 -/- mice show a complete loss of the prethalamus and defects in the formation of the ZLI. In addition, it represses the caudal diencephalic fate and establishes the prethalamic fate (Hirata et al., 2006; Shimizu and Hibi, 2009). Although *Fezf2* is expressed in the developing mouse hypothalamus (Hirata et al., 2004) it remains unknown whether it plays a role in hypothalamic neuron

development in mammals. Another function of FEZF2 in mouse is its importance for the birth, specification and axon growth of corticospinal motorneurons and other subcerebral projection neurons. A lack of FEZF2 activity leads to a fate change of those neurons since no cell death or abnormal migration was detected (Chen et al., 2005a,b; Molyneaux et al., 2005; Shimizu and Hibi, 2009). Unlike the zebrafish mutant FEZF2 mutant mice do not show defects in hypothalamic monoaminergic neurons suggesting that this function is not conserved between mouse and zebrafish (Hirata et al., 2004).

1.2.2.3 Additional transcription factors involved in the hypothalamic development

Another TF that appears to be crucial for the correct development of the hypothalamus is the thyroid specific-binding protein T/ebp also known as Nkx2.1. In mouse *Nkx2.1* is expressed in lung progenitors, heart progenitors, the thyroids and a restricted area in the ventral forebrain (Kimura et al., 1996; Pabst et al., 2000). NKX2.1 -/- mice, which die at birth, display in addition to the defects in the formation of the lung, heart and the thyroid also defects in the ventral forebrain: from the septal area anteriorly to the MB of the hypothalamus posteriorly (Kimura et al., 1996). A more detailed analysis showed that NKX2.1 mutants have largely normal PVN, SON and SCN while the aPV might be slightly smaller. The ventromedial hypothalamus (VMH) and the dorsomedial nucleus are reduced whereas the ARC and the whole mammillary area (MA) containing the MB, the premammillary nucleus (PM), the supramammillary area (SuM) as well as the tuberomammillary nucleus (TM) are absent (Kimura et al., 1996; Szarek et al., 2010). Additionally, the pituitary does not form (Kimura et al., 1996). In zebrafish two *nkx2.1* genes exist, *nkx2.1a* that is exclusively expressed in the ventral diencephalon and *nkx2.1b* that is primarily expressed in the ventral telencephalon. Since the ventral diencephalic expression domain of *nkx2.1a* is exclusively found in the hypothalamus, this expression is used as a marker to demarcate the zebrafish hypothalamus (Rohr et al., 2001). Further, Nkx2.1a can be detected in the thyroid of zebrafish as well (Elsalini et al., 2003) indicating an evolutionary conserved role of Nkx2.1a between zebrafish and mammals.

For the development of the VMH, in addition to NKX2.1 the orphan nuclear hormone receptor steroidogenic factor 1 (SF1) is important. SF1 is involved in the development of several neuroendocrine systems, since SF1 -/- mice have no gonads, adrenals and an impaired function of pituitary gonadotrophs (Caqueret et al., 2005). SF1 is specifically expressed in the VMH and is important for several phases of its development. Initially it is involved in the survival and migration of the VMH neurons but not for an appropriate VMH cell number. Later in development it is required for the aggregation

and condensation of the VMH and the terminal differentiation (Caqueret et al., 2005; Szarek et al., 2010). In zebrafish Sf1 is called nuclear receptor subfamily 5, group A, member 2 (Nr5a2). The expression of the SF1 homolog *nr5a2* is restricted within a distinct hypothalamic domain implicating a conserved function for this factor as well (Kurrasch et al., 2007). Another protein implicated in the VMH and ARC development is the bHLH transcription factor Mash1 or Ascl1, which plays a role in neurogenesis and subtype specification of several regions in the CNS. MASH1 -/- mice display an underdevelopment of the VMH and the ARC caused by neurogenic failure and an increase in apoptosis (McNay et al., 2006). Additionally, MASH1 is required for the expression of the homeobox gene Gsh1, which is important for the formation of GHRH expressing neurons in the ARC (Szarek et al., 2010). In addition to MASH1, the homeobox genes Hmx2 and Hmx3 are required for the expression of Gsh1 and therefore for the formation of GHRH neurons within the ARC but not within the VMH (Wang et al., 2004). The sine oculis homeobox homolog 3 protein (Six3) is required for the formation of the SCN assessed by using SIX3 -/- mice (VanDunk et al., 2011). For the development of the MB the only transcription factor found so far is the forkhead box B protein Foxb1. Mice lacking Foxb1 fail to develop a proper MB (Wehr et al., 1997).

Although several transcription factors important for the formation of distinct nuclei in the hypothalamus have been identified the mechanism by which these factors define distinct hypothalamic nuclei still remains to be determined. Currently all identified transcription factors affect the development of several neuronal types therefore it still needs to be determined how distinct neuronal fates are specified.

1.3 The hypothalamus anatomy and function

1.3.1 The paraventricular nucleus

The PVN is one of the best studied nuclei of the hypothalamus. Already nearly 100 years ago a role of the PVN in energy homeostasis has been suggested (Cleveland and Davis, 1936). Monkeys, dogs, cats and rats with distinct hypothalamic lesions that involved the PVN display an extremely rapid developing obesity caused by a highly enhanced food intake (Brobeck, 1946). Around 40 years later more defined knife cut studies and electrolytic lesions could show that it was indeed the PVN, or afferents and efferents in the vicinity of this nucleus, that are important for normal regulation of food ingestion (Leibowitz et al., 1981). In addition to its function in controlling normal eating and drinking behavior, the PVN is also one of the most important parts of the brain to control the stress response. It is a major part of the hypothalamic-pituitary-adrenal gland (HPA) axis by controlling the release of adrenocorticotropic hormone (ACTH) through

its CRH neurons. It also controls the release of neuroendocrine hormones both from the anterior and the posterior part of the pituitary glands (Löhr and Hammerschmidt, 2011). By far the largest proportion of parvocellular neurosecretory neurons projecting to the median eminence can be found in the PVN consisting of CRH, TRH, SS, DA and GHRH neurons (Swanson, 1987). Together with the SON it also forms the magnocellular neurosecretory system where OXT and AVP are released into the bloodstream through the posterior pituitary. These two hormones are important for parturition and lactation and for controlling diuresis and blood pressure respectively (Gainer and Wray, 1994). In addition to its magno- and parvocellular neurosecretory neuron portion, there is a third type of neurons in the PVN, which forms the descending division (Fink, 2000). These neurons send descending projections to the brain stem and the spinal cord, especially to the regions important for nociceptive information, autonomic reflexes and somatic motor behavioral responses. The afferent control of the PVN is highly complex and includes more than 50 different sources of neural input (Fink, 2000). Most of the neural inputs appear to innervate more than one cell type within the PVN. Inputs that relay sensory information come from distinct regions of the hindbrain while cognitive influences come from the bed nuclei of the stria terminalis. Also many other neurons from other nuclei within the hypothalamus send projections to the PVN such as the neurons in the SCN (circadian rhythm). Apart from the neural inputs, the PVN might be also influenced by hormonal inputs since several neurons in the PVN express hormone receptors, which may change plasma membrane electrical properties and by this the gene expression in PVN neurons (Fink, 2000).

In zebrafish the homologues structure to the PVN and SON is the neurosecretory preoptic area (NPO) (Forlano and Cone, 2007; Peter, 1977). It was found due to its unique composition of neuronal subtypes that are typical for the PVN/SON. From 2 days post fertilization (dpf) onwards there is an *otp* and *sim1a* positive region in the anterior diencephalic area that contains Avpl, Oxtl, CRH and more ventral TRH and SS neurons (Blechman et al., 2007; Löhr et al., 2009; Ryu et al., 2007). Since this is the only region in the zebrafish hypothalamus that is Oxtl- and Avpl-positive it was concluded that the NPO is the homologous structure to both the PVN and the SON. Since the Oxtl, Avpl and CRH neurons project axons to the pituitary gland and have extensive axonal projections throughout the teleost brain and to the spinal cord, these neurons are likely to exhibit similar functions as their mammalian counterpart (Goossens et al., 1977; Pepels et al., 2002; Saito et al., 2004; Yulis et al., 1986).

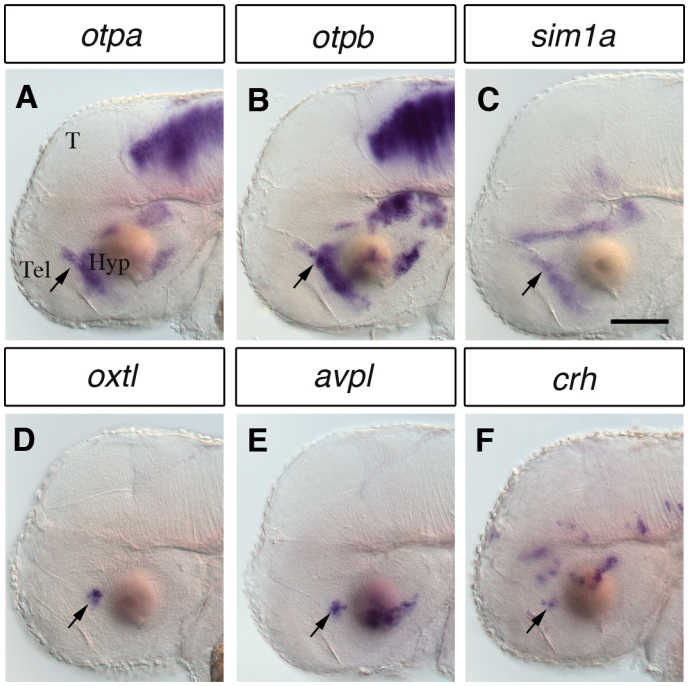

Fig. 1.4: The NPO is the homologous structure to the PVN/SON
(A-F) Whole mount in situ hybridization (WISH) with probes for *otpa* (A), *otpb* (B), *sim1a* (C), *oxtl* (D), *avpl* (E) and *crh* (F) to detect their conserved expression within the PVN/SON homologues structure, the NPO. Lateral views are shown. The arrows point to the NPO. Abbreviations used: telencephalon (Tel), hypothalamus (Hyp), tectum (T). The scale bar represents 200 µm.

1.3.2 The mammillary area

The posterior most region of the hypothalamus the mammillary area (MA) or corpus mamillare is an evolutionary highly conserved hypothalamic area, whose existence is even discussed in lamprey where anatomists interpreted the caudal part of the dorsal hypothalamic nucleus as a primordial corpus mamillare (Nieuwenhuys et al., 1998). In mammals the most prominent structure of the MA is the mammillary body (MB), which is comprised of two or three nuclei depending on anatomist: the medial, the lateral and the dorsal premammillary nucleus (Alvarez-Bolado et al., 2000a; Canteras and Swanson, 1992; Swanson, 1987; Vann, 2010). The medial mammillary nucleus is the largest of the three nuclei, which can be divided further into the pars lateralis, the pars medials and the pars basalis (Vann, 2010). Both the medial as well as the lateral mammillary nucleus are different in terms of their cell morphology while within each nucleus there appears to be only one cell type (Allen and Hopkins, 1988; Veazey et al., 1982) of mainly projection neurons (Takeuchi et al., 1985) and no apparent interneurons (Veazey et al., 1982). The medial and the lateral mammillary nuclei have connections to the same structures. While the main inputs originate from the hippocampus and from the tegmentum (tegmental

nuclei of Gudden), their main outputs are to the thalamus (anterior thalamic nucleus) and to the tegmentum (tegmental nuclei of Gudden) (Vann, 2010). The principal mammillary tract (pm) is the major axon bundle from the MB that sprouts at a specific point forming the mammillotegmental (mtg) and the mammillothalamic tract (mth)(Szabo et al., 2011). Since the lateral and the medial mammillary nuclei are connected with the same structures but different subregions of those structures they form two parallel systems (Vann and Aggleton, 2004). In contrast, the dorsal premammillary nucleus (PMd) projects to the precommissural nucleus, the periaquiductal gray and the thalamus while it receives input from parts of the hypothalamus, the perifornical region and some telencephalic regions (Canteras et al., 2001).

Beside the MB the MA consists of the supramammillary area (SuM) and the tuberomammillary nucleus (TM) both of which innervate the MB nuclei (Vann, 2010). The TM axons project widely throughout the CNS from the cerebral cortex, amygdala to the posterior pituitary and the spinal cord (Haas and Panula, 2003; Saper, 2000) while the supramammillary area projects to the entire cerebral cortex (Saper, 2000), the thalamus, to the amygdala and to other parts of the hypothalamus (Pan and McNaughton, 2004).

In humans only very few cases of accidental MB lesions are known. These patients suffered from slight anterograde amnesia, impaired recognition memory and a delayed recall. More severe symptoms can be detected in patients suffering from Korsakoff's syndrome. This syndrome is most frequent in alcoholics and the patients suffer from degeneration of the MB and other brain regions (Beracochea, 2005; Vann and Aggleton, 2004). Behavioral lesion studies in mammals led to the assignment of specific functions to distinct domains within the MA. These studies showed that the MB is not a typical hypothalamic nucleus that controls endocrine function or homeostasis but rather cognitive processes. MB lesions disrupt spatial working memory, object recognition and scene discrimination, which can also be affected by lesions of the mammillothalamic tract although to a smaller extent (Vann, 2010; Winter et al., 2011). Animals with MB lesions are also less anxious or less emotionally aroused (Vann, 2010). Lesions mainly specific to the SUM area showed its involvement in cognitive processing, fear conditioning and passive avoidance (Pan and McNaughton, 2004). Since lesion studies usually include a larger area than a distinct nucleus, electrophysiological studies have been carried out to define functions of the MA. Although the distinct mammillary body nuclei appear to consist of only one cell type, physiological studies showed that neurons in the medial mammillary nucleus are electrophysiologically different whereas lateral mammillary nuclei neurons are electrophysiologically homogeneous (Vann, 2010). These studies additionally showed that the lateral mammillary nucleus is part of the head-direction system containing head-direction cells and angular velocity cells (Blair et al., 1999, 1998; Stackman and Taube, 1998) connected with other head-direction

regions of the brain forming (Bassett et al., 2007). In contrast, the medial mammillary nucleus is situated within the "theta-related" system. It contains no head-direction cells but cells responsive to angular head velocity, which fire differentially for clockwise and counterclockwise movements (Sharp and Turner-Williams, 2005). In addition, nearly all cells of the medial mammillary nucleus modulate their firing rate at a frequency of theta showing a strong correlation with the CA1 field of the hippocampus (Vann, 2010). It has been suggested that spatial working memory is theta-dependent (Givens, 1995). Therefore, it is likely that the medial and the lateral mammillary nucleus are not only electrophysiological but also functionally distinct.

Neurophysiological studies on the supramammillary area showed that it is also part of the "theta-related" system, although even large lesions of the SuM led only to a slight reduction in theta frequency (Pan and McNaughton, 2004). The function of the tuberomammillary area was investigated after the discovery that the TM is the sole seat of the histaminergic neurons with their wide projections in the brain (Haas and Panula, 2003). Studies in mouse lacking histamine or with injected histamine as well as neurophysiological studies revealed that the TM is involved in arousal, sleep, memory as well as feeding (Haas and Panula, 2003). In zebrafish it was found that the histamine system is essential for the development of a functional hypocretin system as well as for the rapid and adequate behavioral responses of zebrafish larvae to changes in the environment (Sundvik et al., 2011). For the function of the PMd, cFos staining and chemical lesion initially suggested that it is involved in the expression of fear responses (Canteras et al., 2001). Later pharmacological blockade of the PMd showed that the NMDA/NO pathway plays an important role in the processing of predatory threats mediated by the PMd (Aguiar and Guimaraes, 2011; Canteras et al., 2008).

An impressive amount of work has been put into the investigation of the function and the neurophysiology of the mammillary area but so far the development of the MA is surprisingly poorly understood. The only transcription factor known to play a role in MB development is the winged helix-loop-helix protein FOXB1. It was shown that mouse mutants for FOXB1 do not develop a proper MB (Wehr et al., 1997). In addition, FOXB1 is essential for the formation of the mammillothalamic tract (Alvarez-Bolado et al., 2000b) and it is expressed in migrating cells (Alvarez-Bolado et al., 2000a; Zhao et al., 2008). Consistent with recent findings, FOXB1 mutant mice display impairment in spatial and working memory mainly through the ablation of the medial mammillary nucleus as well as the mammillothalamic tract (Radyushkin et al., 2005). More recent studies revealed additional factors that are involved in later aspects of MB differentiation contributing to the formation of the mammillothalamic tract like the bHLH transcription factor SIM1 (Marion et al., 2005), the paired box protein PAX6 (Szabo et al., 2011) and the paired-like homeodomain transcription factor PITX2 (Skidmore et al., 2012).

However, apart from the above mentioned transcription factors no additional regulators of MA development have been identified yet.

Furthermore, only limited studies about neuropeptides expressed within the MA have been published. In adult zebrafish it has been shown that urotensin1 (Uts1) the teleost ortholog of the mammalian urocortin1 (UCN1) (Vaughan et al., 1995) is homogeneously distributed within the corpus mamillare (Alderman and Bernier, 2007). In mammals UCN1 is widely distributed in the brain and also in the periphery having important physiological functions in various tissues. In the central nervous system it appears to modulate energy and water homeostasis and stress adaptation, while in the periphery it is implicated in immunomodulation and cardioprotection (Oki and Sasano, 2004). UCN1 is widely expressed in the CNS as its expression was first detected in the Edinger-Westphal nucleus, the lateral superior olive of the midbrain, the supraoptic nucleus of the hypothalamus as well as in the spinal chord (Oki and Sasano, 2004). Later also other places of UCN1 expression have been detected. Interestingly, in colchicine treated rats, UCN1 expression was detected in the lateral mammillary nucleus, the supramammillary area as well as in the posterior hypothalamic area displaying low-level expression of UCN1 (Bittencourt et al., 1999). Although the function of UCN1 in the MA is not known the conserved expression in zebrafish suggests an evolutionary conserved function, which still needs to be elucidated. Another neuropeptide known to be expressed within the MA is VIP. VIP-ir as well as VIP binding sites have been found in the MA (Saper, 2000; Sarrieau et al., 1994). The function of VIP in this area of the hypothalamus is poorly understood. Additionally, VIP is broadly expressed in the body and has pleiotropic regulatory effects. In the CNS the strongest VIP expression can be found in the suprachiasmatic nucleus of the hypothalamus where it is involved in the generation and synchronization of the circadian oscillatory clocks (Vosko et al., 2007) and in the PVN where it is co-expressed with CRH (Ceccatelli et al., 1989; Hokfelt et al., 1987).

2 Aim of the thesis

The hypothalamus is a key integrative center in the brain, which is composed of several nuclei that consist of multiple neuronal subtypes. Although a great amount of work led to the assignment of specific functions to distinct nuclei, the development of these nuclei and the neurons that are present within them, remains largely unknown. So far, several transcription factors important for the specification of hypothalamic neuron types have been identified using genetic analyses in rodents and in zebrafish. These regulators include OTP, SIM1, ARNT2, BRN2, SIM2 and FOXB1.2 (Eaton and Glasgow, 2006, 2007; Eaton et al., 2008; Goshu et al., 2004; Löhr et al., 2009; Michaud et al., 2000, 1998; Nakai et al., 1995; Ryu et al., 2007; Schonemann et al., 1995; Wang and Lufkin, 2000; Wehr et al., 1997). Although the function of hypothalamic regulators appears to be highly conserved from mammals to fish (Machluf et al., 2011), how these factors specify distinct neuron types and nuclei is poorly understood. Additionally, many of the so far identified transcription factors are broadly expressed, suggesting that these regulators might play a role in the development of several neuron types in the hypothalamus. The overall goal of my thesis was to gain insight into the transcriptional mechanism that specify distinct nuclei and neuron types focusing on the neurosecretory preoptic area (NPO) and the posterior most region of the hypothalamus the mammillary area (MA).

3 Results

3.1 Regulatory interactions of transcription factors, crucial for the development of the preoptic area and the posterior hypothalamus

3.1.1 The identification of transcription factors regulated by Otp

It is known that Otp plays an important role in the development of neuroendocrine neurons (Blechman et al., 2007; Eaton and Glasgow, 2007; Eaton et al., 2008; Ryu et al., 2007). However, its expression is found in multiple hypothalamic regions, including the retrochiasmatic region, the ventral tuberal region and the mammillary area (Shimogori et al., 2010; Simeone et al., 1994). Also in zebrafish *otpa* and *otpb* have additional expression domains in the posterior and in the ventral hypothalamus (Ryu et al., 2007) suggesting an evolutionary conserved function of Otp in this region. To find novel regulators of hypothalamic neuron type specification, I hypothesized that such factors may act downstream of broad hypothalamic regulators such as Otp. To this end I carried out an in situ hybridization-based screen analyzing transcription factors in zebrafish embryos lacking Otp activity. I selected from the zebrafish expression database (www.zfin.org)(Bradford et al., 2011) more than 80 different transcription factors (see also Material and Methods chapter 6 in section 6.1.12) expressed in Otp-positive hypothalamic domains and analyzed their expression in 2 dpf *otpa*^{m866} mutant embryos injected with 4 ng of *otpb* morpholino (MO) (Ryu et al., 2007). Using this approach I was able to find three transcription factors, which showed changes in their expression in the embryos lacking Otp activity.

One of these factors is the zinc-finger transcription factor Fezf2. Although known to act upstream of Otp (Blechman et al., 2007), its expression was reduced in the NPO (Fig. 3.1A,B, n=11/11), while it was expanded in the posterior hypothalamus (Fig. 3.1C,D, n=11/11). The second factor affected by the loss of Otp function is the winged helix-loop-helix transcription factor Foxb1.2, the zebrafish ortholog of mouse FOXB1 (Odenthal and Nüsslein-Volhard, 1998). Similar to *fezf2*, the *foxb1.2* expression domain was expanded in the posterior hypothalamus (Fig. 3.1E,F, n=17/17).

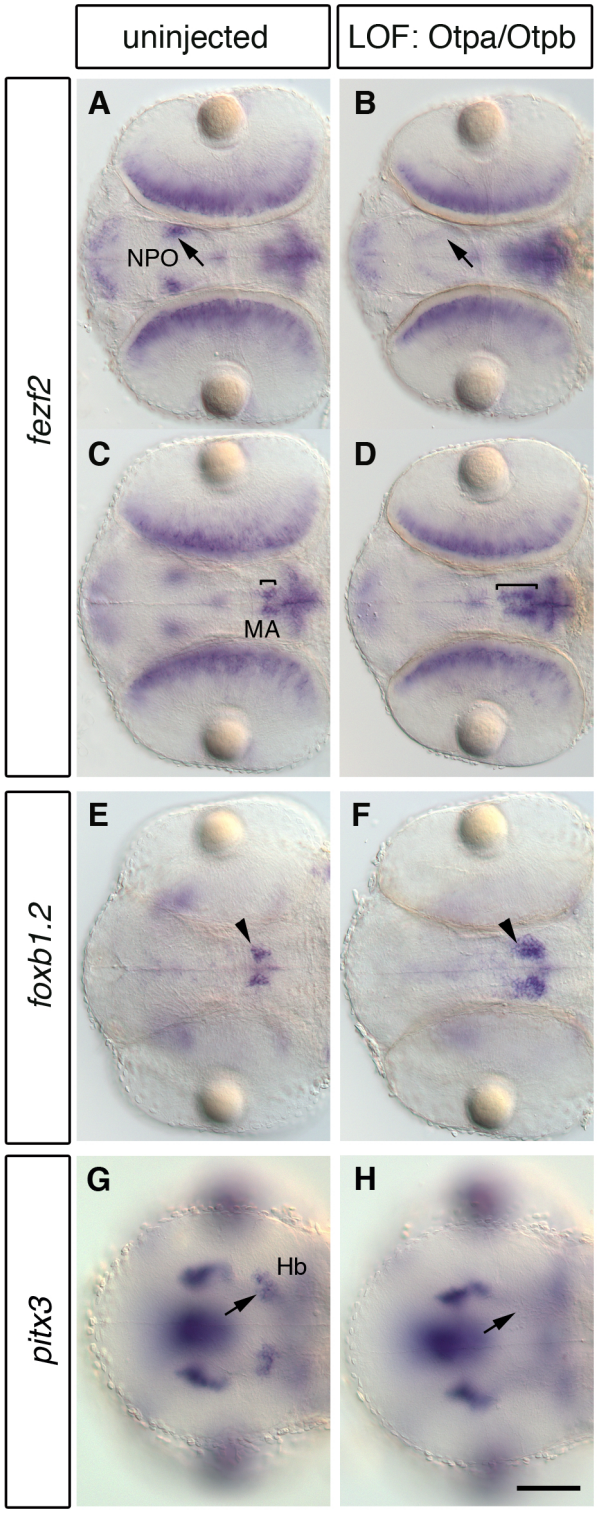

Fig. 3.1: Otp regulates the expression of *fezf2*, *foxb1.2* in the diencephalon and of *pitx3* in the hindbrain

(A-H) Whole-mount in situ hybridization (WISH) to detect the expression of *fezf2* (A-D), *foxb1.2* (E,F) and *pitx3* (G,H) in 2 dpf wild-type (A,C,E) and *otpa*^{m866} mutant embryos injected with 4 ng *otpb* morpholino (MO). (A-D) In embryos lacking Otp activity, the *fezf2* expression domain is strongly reduced in the NPO (arrows in A and B) and expanded

in the MA (bars in C and D, C and D represent 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 MA (arrowheads, n=17/17). (G,H) Loss of Otp function results in the loss of the hindbrain (Hb) expression domain of *pitx3* (arrows, n=4/4). Dorsal views are shown and the scale bar represents 200 µm.

FOXB1 in mouse is specifically expressed in the mammillary body (MB) where it is necessary for a proper development of this hypothalamic region (Wehr et al., 1997). Foxb1.2 in zebrafish displays one highly specific expression domain in the hypothalamus of 2 dpf embryos: in the posterior most part of the hypothalamus, where also Otp can be detected. In rodents, Otp was found to be expressed in the mammillary area (Shimogori et al., 2010). Based on the conserved expression of these factors in the posterior hypothalamus I refer to the region surrounding the Foxb1.2-positive domain as the mammillary area (MA) in zebrafish. The third transcription factor was *pitx3*, whose hindbrain expression domain was lost (Fig. 3.1G,H, n=4/4). Since the focus of my thesis is hypothalamus development, the regulatory interaction between *otp-pitx3* was not further characterized in the course of this thesis.

The development of the MA in zebrafish has not been studied so far, nevertheless these results show a conserved expression of *foxb1.2* in the MA of zebrafish and identify Foxb1.2 and Fezf2 as potentially involved in the formation of the MA downstream of Otp.

3.1.2 The expression domains of *fezf2* and *foxb1.2* delineate subregions within the posterior hypothalamus

Given the fact that Fezf2 as well as Foxb1.2 are important for the patterning of the diencephalon (Jeong et al., 2007; Scholpp and Lumsden, 2010; Staudt and Houart, 2007; Toro and Varga, 2007), I asked next whether these two factors also play a role in the patterning of the hypothalamus. To answer this question I first examined the expression of *fezf2* and *foxb1.2* in comparison to the known markers of the zebrafish hypothalamus. At 1 dpf, *nkx2.1a* is a marker for the whole hypothalamus (Rohr et al., 2001), whereas *shh* and *emx2* delineate the anterior-dorsal and the posterior-ventral hypothalamus, respectively (Mathieu et al., 2002). Using three-color whole-mount fluorescence *in situ* hybridization (FISH), I observed that *fezf2* as well as *foxb1.2* were expressed within the *nkx2.1a* positive posterior hypothalamic area, where *fezf2* labeled more ventral and *foxb1.2* more dorsal domains. The posterior hypothalamic domains of *fezf2* and *foxb1.2* overlapped in a small area (Fig. 3.2A-A"'). The expression of *shh* and *fezf2* directly abutted each other but did not overlap, while the expression domains of *shh* and *foxb1.2* partially overlapped in the more posterior and dorsal extent (Fig. 3.2B-B"'). The

expression of the posterior-dorsal hypothalamic marker *emx2* was completely contained within the hypothalamic *fezf2* domain, but smaller. *foxb1.2* slightly overlapped with the expression of *emx2* in its most ventral extent (Fig. 3.2C-C"'). A summary of the hypothalamic markers co-expressed with *fezf2* and *foxb1.2* is shown in Fig. 3.2D.

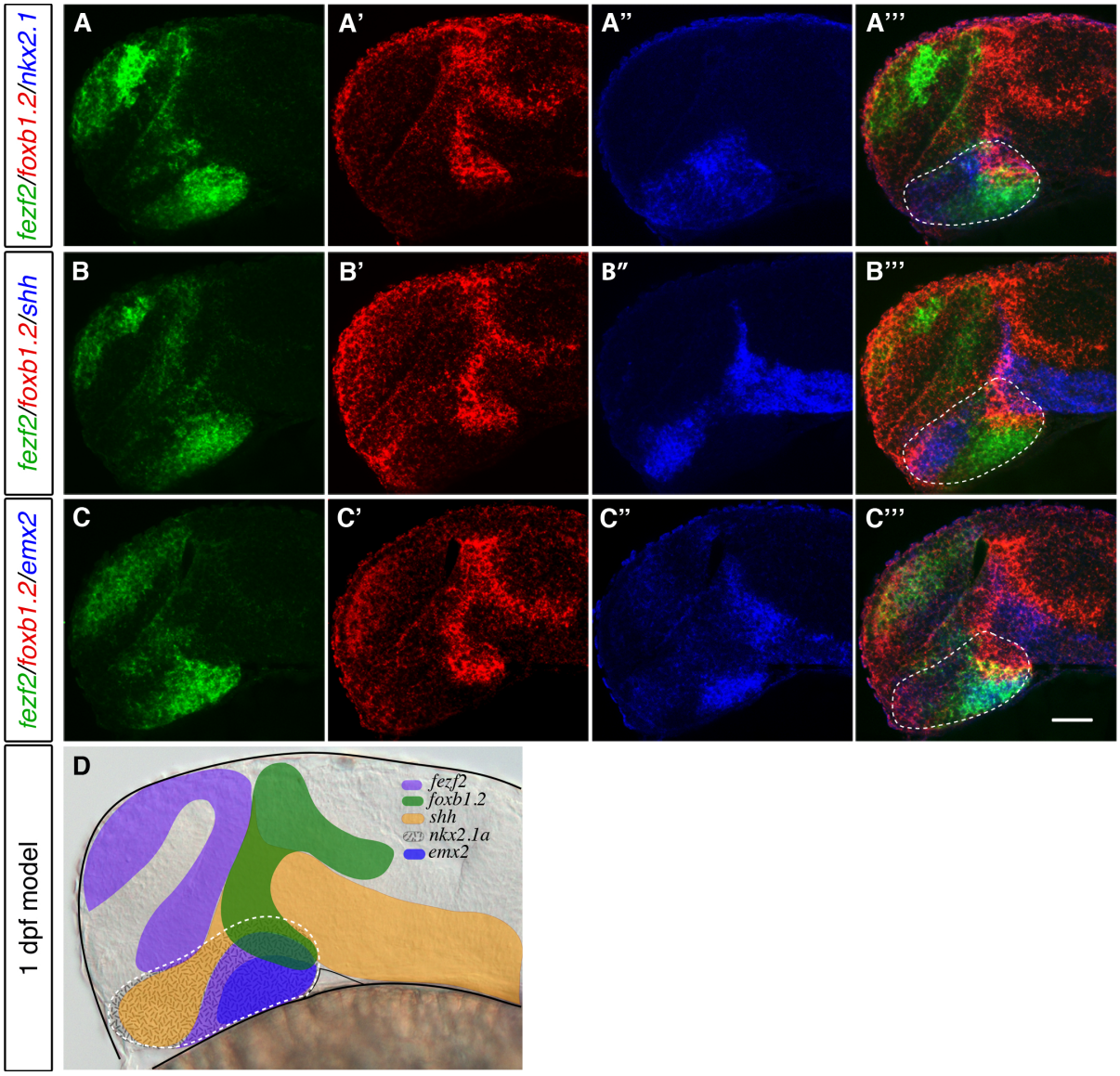

Fig. 3.2: *fezf2* and *foxb1.2* mark subregions within the posterior hypothalamus

(A-C"') Three-color fluorescent whole-mount in situ hybridization (FISH) of 1 dpf wild-type embryos with probes for *nkx2.1a*, *shh*, *emx2*, *fezf2* and *foxb1.2* revealed that *fezf2* and *foxb1.2* are expressed in the posterior hypothalamus. A maximum projection of 10 µm in the lateral view is shown. (A-A"') The hypothalamic expression of *fezf2* and *foxb1.2* is located within the posterior part of the *nkx2.1a* expression domain. (B-B"') The expression domains of *fezf2* in the posterior hypothalamus and *shh* in the anterior hypothalamus are non-overlapping, whereas *foxb1.2* is partially co-expressed with *fezf2*

and *shh* in the posterior-dorsal extent. (C-C"') *emx2* is expressed in the posterior-ventral hypothalamus within the *fezf2* domain, while *emx2* and *foxb1.2* expression slightly overlap. (D) A model summarizing the relative expression patterns of *fezf2*, *foxb1.2*, *shh*, *nkx2.1a*, and *emx2*. For simplicity, only the hypothalamic *emx2* expression is shown. The white dashed lines in A"', B"', C"' and D delineate the hypothalamus and the length of the scale bar is 60 µm.

These results demonstrate that the zebrafish hypothalamus at 1 dpf can be divided into more subregions than what was known before. The hypothalamic *fezf2* expression delineates mainly the posterior hypothalamus, whereas *foxb1.2* is a marker for the posterior-dorsal and *emx2* is a marker for the posterior-ventral hypothalamus.

3.1.3 Fezf2 is involved in the regionalization of the posterior hypothalamus

To directly test the role of Fezf2 and Foxb1.2 in the patterning of the hypothalamus I used a *fezf2* splice morpholino (MO) (Jeong et al., 2006) and a *foxb1.2* ATG-MO generate embryos with reduced Fezf2 and Foxb1.2 function. Neither the loss of Fezf2 (Fig. 3.3A,B, n=27) nor of Foxb1.2 (Fig. 3.3A,C, n=23) function led to a change in *nkx2.1a* expression. As already shown in previous studies (Jeong et al., 2007) in *fezf2* morphants the expression domain of *shh* along the ZLI moved towards anterior into the original prethalamic *fezf2* expression domain (double-headed arrows) and the anterior-dorsal hypothalamic area was reduced (Fig. 3.3D,E, n=23/23, double-headed arrows). The reduction of Foxb1.2 function led to no apparent change in *shh* expression (Fig. 3.3D,F, n=27). Interestingly, in *fezf2* morphants also the ZLI domain of *emx2* was shifted anteriorwards (double-headed arrow), whereas the domain labeling the posterior-ventral hypothalamus was highly reduced (Fig. 3.3G,H, n=34/43, arrowheads). Foxb1.2 morphants show no change in *emx2* expression (Fig. 3.3G,I, n=22). So far my results show that Fezf2 is important for the regionalization of the hypothalamus by regulating the expression of *emx2*. Since the *emx2* domain in the posterior ventral hypothalamus at 1 dpf is contained within the *fezf2* domain, I hypothesized that *emx2* might be regulated by *fezf2* later in development and that it might delineate a specific posterior hypothalamic domain at 2 dpf. To test this, I performed a loss-of-function analysis. The WISH with probes for *emx2*, *foxb1.2* and *fezf2* showed that indeed, *emx2* is downregulated in loss of Fezf2 function embryos (Fig. 3.3J,K, arrowheads, n=21/21), while reduced Foxb1.2 activity led to no apparent change in *emx2* expression (Fig. 3.3J,L, n=28/28). In addition, the loss of Otp function results in no change of *emx2* expression (data not shown).

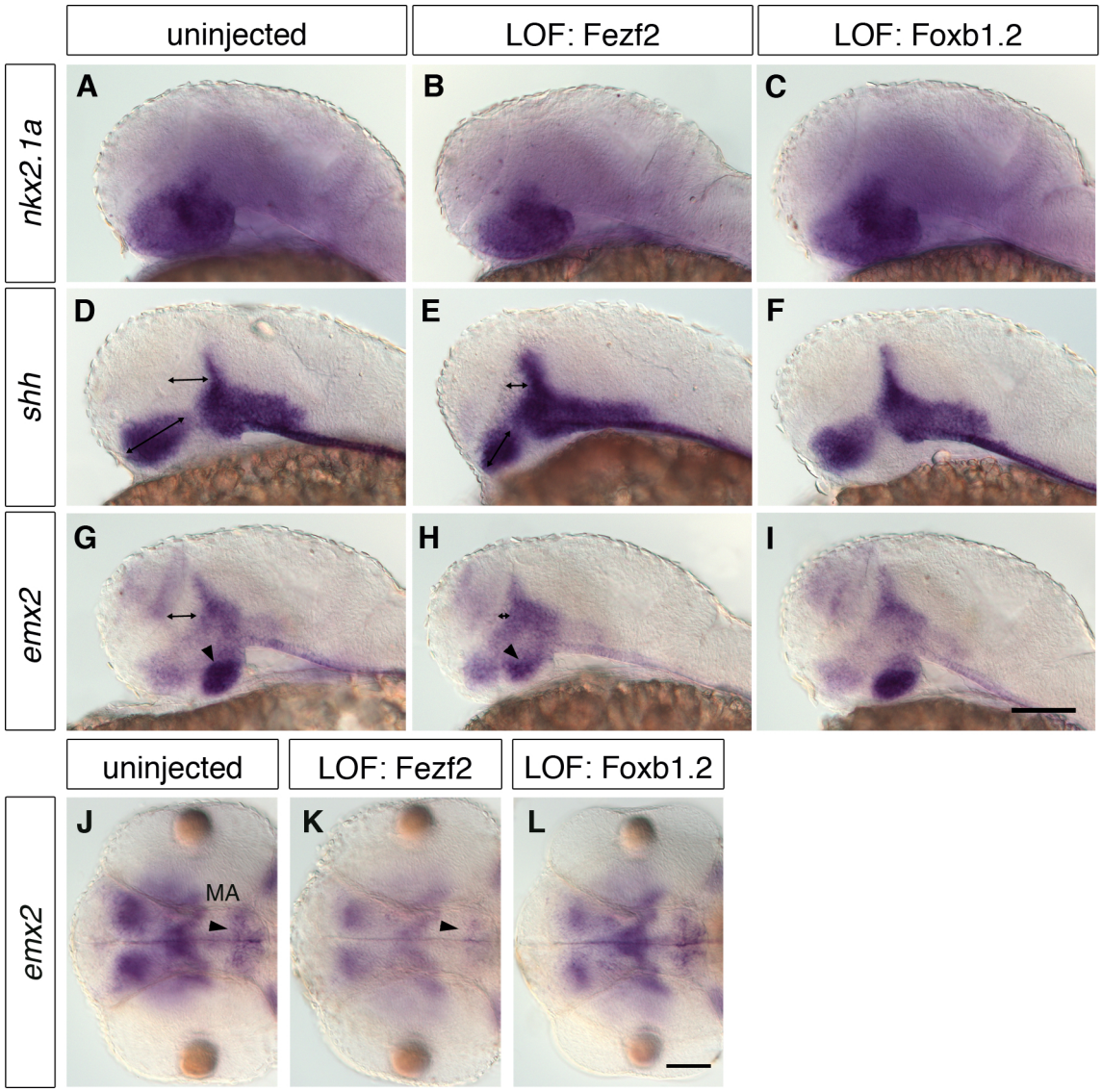

Fig. 3.3: Fezf2 plays a role in posterior hypothalamus formation
(A-L) WISH of wild-type embryos (A,D,G) and embryos injected with either 4ng *fezf2* MO (B,E,H) or 8 ng *foxb1.2* MO (C,F,I) to detect changes in the expression of *nkx2.1a* (A-C), *shh* (D-F) and *emx2* (G-L) at 1 dpf (A-I) and 2 dpf (J-L). Lateral views are shown in (A-I) and dorsal views are shown in (J-L). (A-C) The loss of Fezf2 (n=27) as well as the loss of Foxb1.2 (n=23) function leads to no change in *nkx2.1a* expression. (D,E) The expression of *shh* in the ZLI is shifted to the anterior and reduced in the anterior-dorsal hypothalamus in loss of Fezf2 function (double-headed arrows, n=23/23) while the loss of Foxb1.2 has no apparent effect (D,F, n=27). (G,H) *fezf2* morphants also show a shifted *emx2* expression domain along the ZLI (double-headed arrows) and a reduced posterior-ventral hypothalamic *emx2* domain (arrowheads, n=34/43). *foxb1.2* morphants show no change in *emx2* expression (G,I, n=22). (J-L) The loss of Fezf2 function results in a reduction of *emx2* expression in the posterior MA (n=21/21), while the loss of Foxb1.2 leads to no apparent change (n=28/28). The scale bar represents 200 µm.

My results indicate that *emx2* is expressed within the posterior *fezf2*-positive MA domain, regulated independently of Foxb1.2 and Otp. The positive regulation of Fezf2 on *emx2* expression suggests a co-expression of *fezf2* and *emx2* at 2 dpf.

Thus, similar to what has been reported in mouse (Wehr et al., 1997) Foxb1.2 does not affect hypothalamus patterning. In contrast, Fezf2 affects hypothalamus patterning at 1 dpf by regulating the expression of *emx2* in the posterior-ventral hypothalamus and through a non-cell autonomous effect on hypothalamic *shh* expression. Although Fezf2 affects the hypothalamus patterning it appears not to change the hypothalamic identity, because the expression of *nkx2.1a* is not altered.

Since Fezf2 plays a role in hypothalamus patterning and *foxb1.2* is expressed within the posterior-dorsal hypothalamus the question arises whether these two factors regulate each other. To answer this question, I performed WISH on *fezf2* and *foxb1.2* morphants to detect changes in the expression of *foxb1.2* and *fezf2*. While the loss of Foxb1.2 function had no apparent effect on the expression of *fezf2* (Fig. 3.4A,B, n=18), the loss of Fezf2 function led to a shift in the *foxb1.2* domain anteriorwards along the ZLI domain and a slight expansion in the posterior-dorsal hypothalamus (Fig. 3.4C,D, n=44/44 double-headed arrows). This shift and expansion of *foxb1.2* in the posterior-dorsal hypothalamus in *fezf2* morphants implies a negative regulation of Fezf2 on *foxb1.2* and shows the important role of Fezf2 for the patterning of the hypothalamus at 1 dpf.

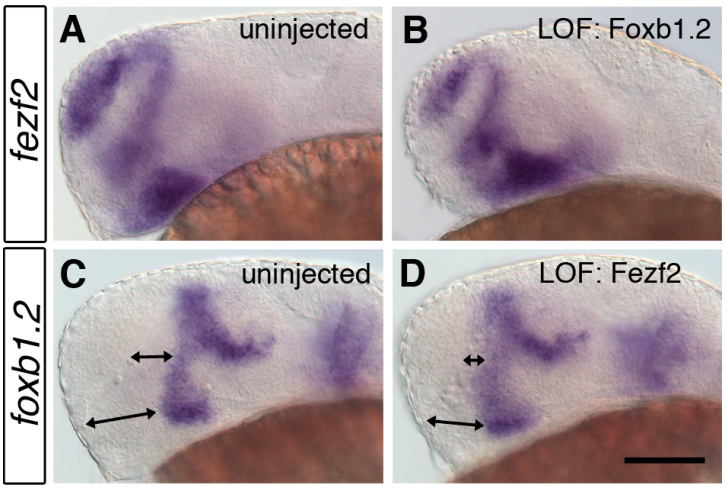

Fig. 3.4: Fezf2 negatively regulates *foxb1.2* at 1 dpf
(A-D) WISH of 1 dpf wild-type embryos (A,C) and embryos injected with 10 ng *foxb1.2* MO (B) and 4 ng *fezf2* MO (D) to detect the expression of *fezf2* (A,B) and *foxb1.2* (C,D). Lateral views are shown. (A,B) *foxb1.2* morphants show no effect on the expression of *fezf2* (n=18). (C,D) The loss of Fezf2 function results in a shifted and expanded expression of *foxb1.2* along the ZLI domain and in the posterior-dorsal hypothalamus (double-headed arrows, n=44/44). The scale bar represents 200 µm.

3.1.4 Fezf2 controls the expression of regulators for neuron type specification at 1 dpf

In zebrafish *otpa*/*otpb* and *sim1a* are involved in the specification of various neurosecretory neuronal subtypes in the diencephalon (Eaton and Glasgow, 2006, 2007; Eaton et al., 2008; Löhr et al., 2009; Ryu et al., 2007). Although the expression of *sim1a* already starts at the 2-somites stage (around 11 hpf) and *otp* starts to be expressed at the 14-somites stage (around 16 hpf), these two transcription factors appear not to be involved in the patterning of the hypothalamus, because the loss of these regulators has no effect on the expression of *fezf2* and *foxb1.2* in the hypothalamus (Fig. 3.5A-F, n=1, n=16, n=2, n=25) and also not on *shh* and *nkx2.1a* expression (Eaton and Glasgow, 2006, 2007).

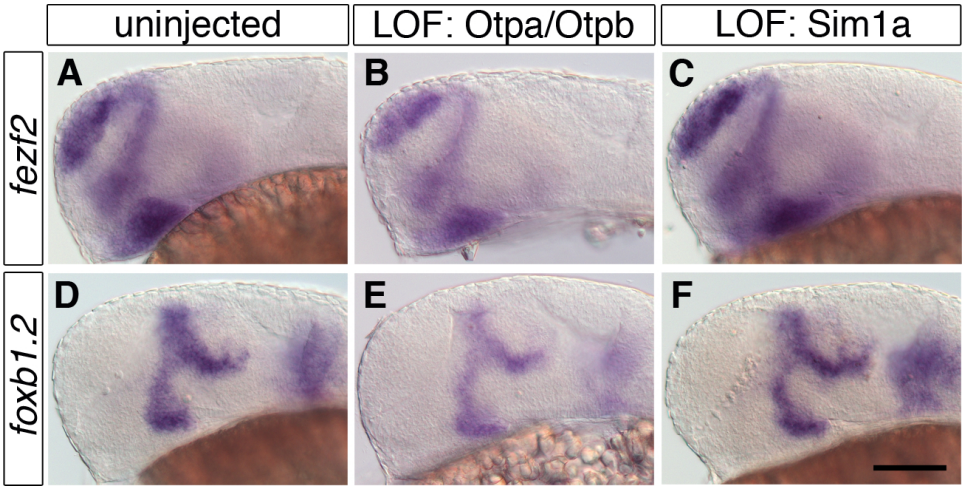

Fig. 3.5: Otp and Sim1a do not affect hypothalamus patterning
(A-F) Probes for *fezf2* (A-C) and *foxb1.2* (D-E) detected with WISH in 1 dpf wild-type embryos (A,D), *otpa*^{m866} mutant embryos injected with 4 ng *otpb* MO and embryos injected with 1 ng *sim1a* MO. (A-C) The expression of *fezf2* does not change upon loss of Otp (n=1) and loss of Sim1a function (n=16). Additionally, the expression of *foxb1.2* is not changed in embryos with reduced Otp (n=2) and Sim1a activity (n=25). The scale bar represents 200 µm.

In contrast, it is known that Fezf2 directly regulates the expression of *otpb* (Blechman et al., 2007) and that Fezf2 and Foxb1.2 are regulators important for diencephalic patterning (Jeong et al., 2007; Scholpp and Lumsden, 2010; Staudt and Houart, 2007; Toro and Varga, 2007). Therefore, it is likely that Fezf2 and Foxb1.2 may regulate *otpa*, *otpb* or *sim1a* expression. To validate this hypothesis I performed a WISH with probes for *otpa*, *otpb* and *sim1a* on 1 dpf wild-type embryos and embryos lacking either Fezf2 or Foxb1.2 function. Indeed, similar to what has been reported for *otpb* at 52

hpf (Blechman et al., 2007) the expression of *otpa* and *otpb* at 1 dpf was severely downregulated in *fezf2* morphants (Fig. 3.6A,B,D,E, n=27/29, n=24/24). This effect was observed in the telencephalic *otp* expression domain as well as in the anterior (arrows) and the posterior (arrowheads) diencephalic expression domains. Similarly, in loss of Fezf2 function embryos the expression of *sim1a* was reduced in the telencephalic expression domain as well as in the anterior (arrows) and posterior (arrowheads) diencephalic expression domain but not in the more posterior domains (Fig. 3.6G,H, n=21/21). The loss of Foxb1.2 function did not result in changes in the expression of *otpa*, *otpb* or *sim1a* (Fig. 3.6A,C,D,F,G,I, n=11, n=23, n=22).

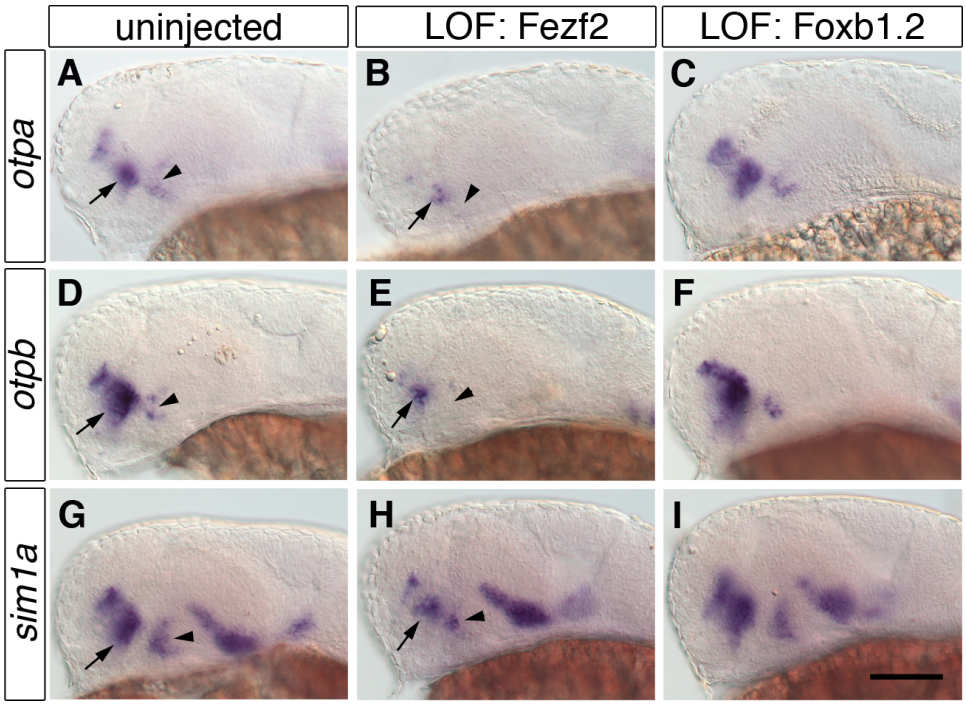

Fig. 3.6: Fezf2 regulates the expression of *otp* and *sim1a* at 1 dpf
(A-I) 1 dpf wild-type embryos and embryos injected with 4 ng *fezf2* and 8 ng *foxb1.2* MO subjected to WISH to detect changes in the expression of *otpa*, *otpb* and *sim1a*. Lateral views are shown. (A-C) The loss of Fezf2 function results in a reduction of *otpa* expression (anterior diencephalon arrows, posterior diencephalon arrowheads, n=27/29), while the loss of Foxb1.2 function shows no apparent effect on *otpa* (n=11). (D-F) Similar to *otpa*, *fezf2* morphants show a reduction of *otpb* expression in the telencephalic domain and the anterior (arrows) as well as posterior (arrowheads) diencephalic expression domain (n=24/24). *foxb1.2* morphants show no change in *otpb* expression (n=23). (G-I) *sim1a* expression in embryos with reduced Fezf2 function is reduced in its telencephalic, anterior (arrows) and posterior (arrowheads) diencephalic expression domain (n=21/21), while *sim1a* expression is not affected in the posterior most domains. A reduction in Foxb1.2 function results in no change of *sim1a* expression (N=22). The scale bar has a length of 200 µm.

Together these results show that Fezf2, in addition to its function in hypothalamus patterning, regulates *otp* and *sim1a* that are known to be important for neuron type specification. In contrast, Foxb1.2 is not important for the regulation *otp* and *sim1a* expression and also not for hypothalamus patterning, although its expression delineates a specific hypothalamic region.

In conclusion, my results revealed that Fezf2 is a key transcription factor in hypothalamus development. This conclusion is based on several results: Fezf2 positively regulates the expression of *emx2* in the posterior-ventral hypothalamus (Fig. 3.3), *otpa* (Fig. 3.6), *otpb* (Blechman et al., 2007) and *sim1a* (Fig. 3.6) and negatively regulates *foxb1.2* and *shh* in the posterior-dorsal and anterior-dorsal hypothalamus. In addition to its function within the hypothalamus, Fezf2 negatively controls the expression of *shh* (Jeong et al., 2007), *emx2* (Fig. 3.3) and *foxb1.2* (Fig. 3.4) in the prethalamus abutting the area of the ZLI. Figure 3.7 provides a summary of the observed genetic interactions of Fezf2 within the diencephalon at 1 dpf.

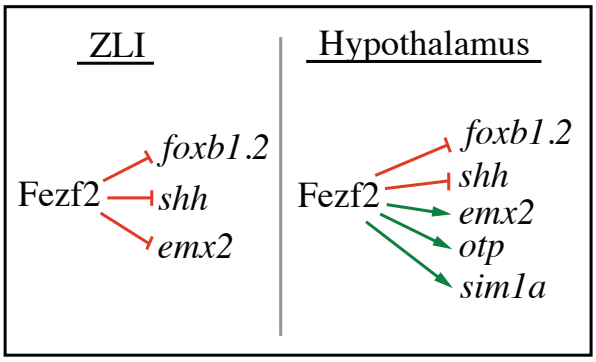

Fig. 3.7: Fezf2 is a key transcription factor for diencephalic development at 1 dpf

A scheme summarizing the observed genetic interactions of Fezf2 on *emx2*, *otp* and *sim1a* in the hypothalamus and on *shh* and *emx2* in the ZLI at 1 dpf. Green arrows specify positive regulation while red bars indicate negative regulation.

3.1.5 The interactions between Fezf2 and Foxb1.2 change over time and the regulatory interactions of Fezf2 vary between anterior and posterior hypothalamus

Since I found that Fezf2 is a key transcription factor for hypothalamus development at 1 dpf, I asked whether later in development Fezf2 still plays a major role in the specification of distinct regions or neuronal subtypes in the hypothalamus. Fezf2 controls the development of monoaminergic neurons in a non-cell autonomous manner (Guo et al.,

1999; Levkowitz et al., 2003) and dopaminergic cell fate by controlling the expression of Ngn1 (Jeong et al., 2006) or Otpb (Blechman et al., 2007). However, so far the role of Fezf2 in the specification of neurons in the preoptic and the posterior hypothalamus is not known. From mouse it is known that FOXB1 and SIM1/SIM2 play a role in the development and specification of the mammillary body (Marion et al., 2005; Wehr et al., 1997) and I showed that Otp regulates the expression of both *fezf2* in the PO and the MA and *foxb1.2* in the MA in 2 dpf old zebrafish embryos (Fig. 3.1). Additionally, Otp and Sim1a play a major role in the specification of neuroendocrine neurons in the NPO of zebrafish (Eaton and Glasgow, 2006, 2007; Eaton et al., 2008; Löhr et al., 2009; Ryu et al., 2007). Based on these findings I hypothesized that Fezf2, Foxb1.2, Otp and Sim1a play a role in the formation of the MA in zebrafish and tested the genetic interaction between *fezf2*, *foxb1.2*, *otpa*/*otpb* and *sim1a* in a loss-of-function analysis at 2 dpf.

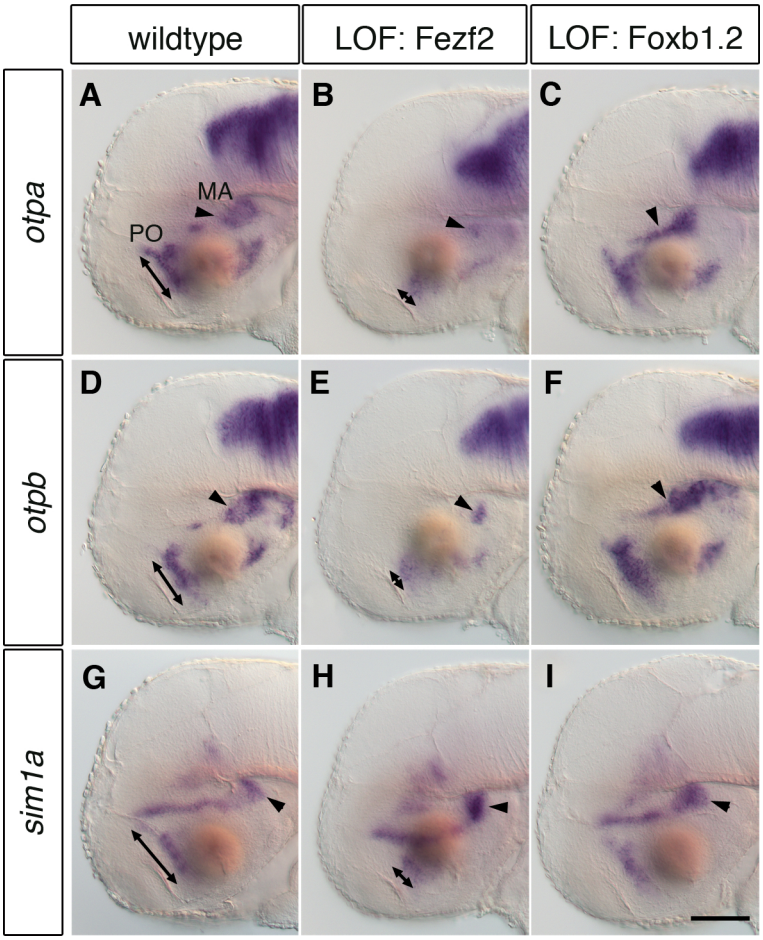

Fig. 3.8: Fezf2 and Foxb1.2 regulate the expression of *otp* and *sim1a* differentially

(A-I) WISH on 2 dpf wild-type (A,D,G) embryos and embryos injected with 4 ng *fezf2* MO (B,E,H) and 10 ng *foxb1.2* MO (C,F,I) to detect alterations in the expression of *otpa* (A-C), *otpb* (D-F) and *sim1a* (G-I). In the lateral views that are shown, double-headed

arrows display the extent of the PO expression domain, while arrowheads point to the MA. (A,B,D,E) Loss of Fezf2 function results in a reduction of *otpa* (n=11/14) and *otpb* (n=14/17) expression, while (A,C,D,F) loss of Foxb1.2 function results in a slight expansion of *otpa* (n=13/22) and *otpb* (n=7/22) MA domain to the anterior. (G,H) The expression of *sim1a* in *fezf2* morphants is reduced in the PO and expanded in the MA (n=19/19). *foxb1.2* morphants show a slight expansion of *sim1a* in the MA, while *sim1a* in the PO is not affected (n=12/12). The scale bar represents 200 µm.

Similar to what has been reported for *otpb* (Blechman et al., 2007) and to what I showed for 1 dpf old embryos (Fig. 3.6), the loss of Fezf2 function results in a severe reduction of *otpa* (Fig. 3.8A,B, n=11/14) and *otpb* (Fig. 3.8D,E, n=14/17) expression at 2 dpf both in the PO (double-headed arrows) and in the MA (arrowheads). The reduction of Fezf2 activity at 1 dpf resulted in a reduction of *sim1a* expression (Fig. 3.6). Interestingly at 2 dpf (Fig. 3.8G,H), *sim1a* expression in the PO was reduced (double-headed arrow), while it was expanded in the MA (arrowheads, n=19/19). The loss of Foxb1.2 function, in contrast, led to a slight expansion of the MA *otpa* (Fig. 3.8A,C, arrowheads, n=13/22) and *otpb* (Fig. 3.8D,F, arrowheads, n=7/22) domain anteriorwards. Also *sim1a* is slightly expanded in the MA (arrowheads) in *foxb1.2* morphants and not affected in the PO (Fig. 3.8G,I, n=12/12).

These genetic interactions show that Fezf2 positively regulates *otp*, while its interaction with *sim1a* changes over time and varies between the anterior and the posterior hypothalamus. Foxb1.2 although an important marker for the MA, affects the expression of *otp* and *sim1a* only slightly.

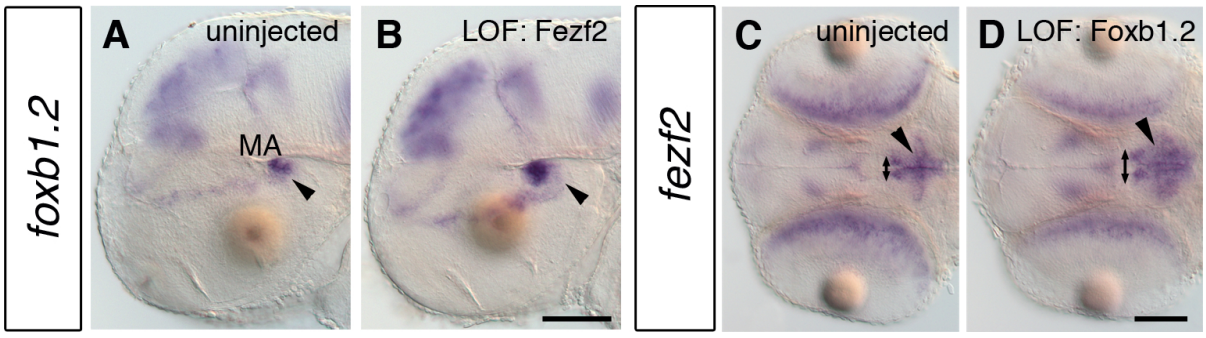

Fig. 3.9: *fezf2* and *foxb1.2* negatively regulate each other
(A-D) Wild-type (A,C), 4 ng *fezf2* MO (B) and 10 ng *foxb1.2* MO (D) injected 2 dpf embryos subjected to WISH to reveal cross-regulatory interactions between *foxb1.2* (A,B) and *fezf2* (C,D). Lateral views are shown in A, B and dorsal views are shown in C, D. (A,B) *fezf2* morphants display an expanded *foxb1.2* expression in the MA (n=32/32). (C,D) The expression of *fezf2* in *foxb1.2* morphants is expanded in the anterior (double-headed arrows) and posterior (arrowheads) MA (n=19/25). The scale bars show 200 µm.

Next I asked, whether there is a genetic interaction between *fezf2* and *foxb1.2* at 2 dpf, because in 1 dpf old embryos Fezf2 negatively regulates the expression of *foxb1.2*. Loss-of-function analysis of WISH embryos that were injected with *fezf2* or *foxb1.2* MO revealed that indeed there are genetic interactions between these two factors. Loss of Fezf2 function resulted in an expanded *foxb1.2* expression domain in the MA (Fig. 3.9A,B, arrowheads, n=32/32) while a reduction of Foxb1.2 activity led to an expansion of the anterior (double-headed arrows) and posterior MA (arrowheads) *fezf2* expression domain (Fig. 3.9C,D, n=19/25). The expansion of *foxb1.2* and *fezf2* in the MA of *fezf2* and *foxb1.2* morphants respectively, indicates the existence of cross-repressive genetic interactions between these two factors.

It has been suggested that Otp and Sim1a act in a parallel pathway to specify neuronal fates (Löhr et al., 2009). If this is true, Otp and Sim1a should not interact genetically. Further, the loss of Sim1a function should lead to similar phenotypes as the loss of Otp.

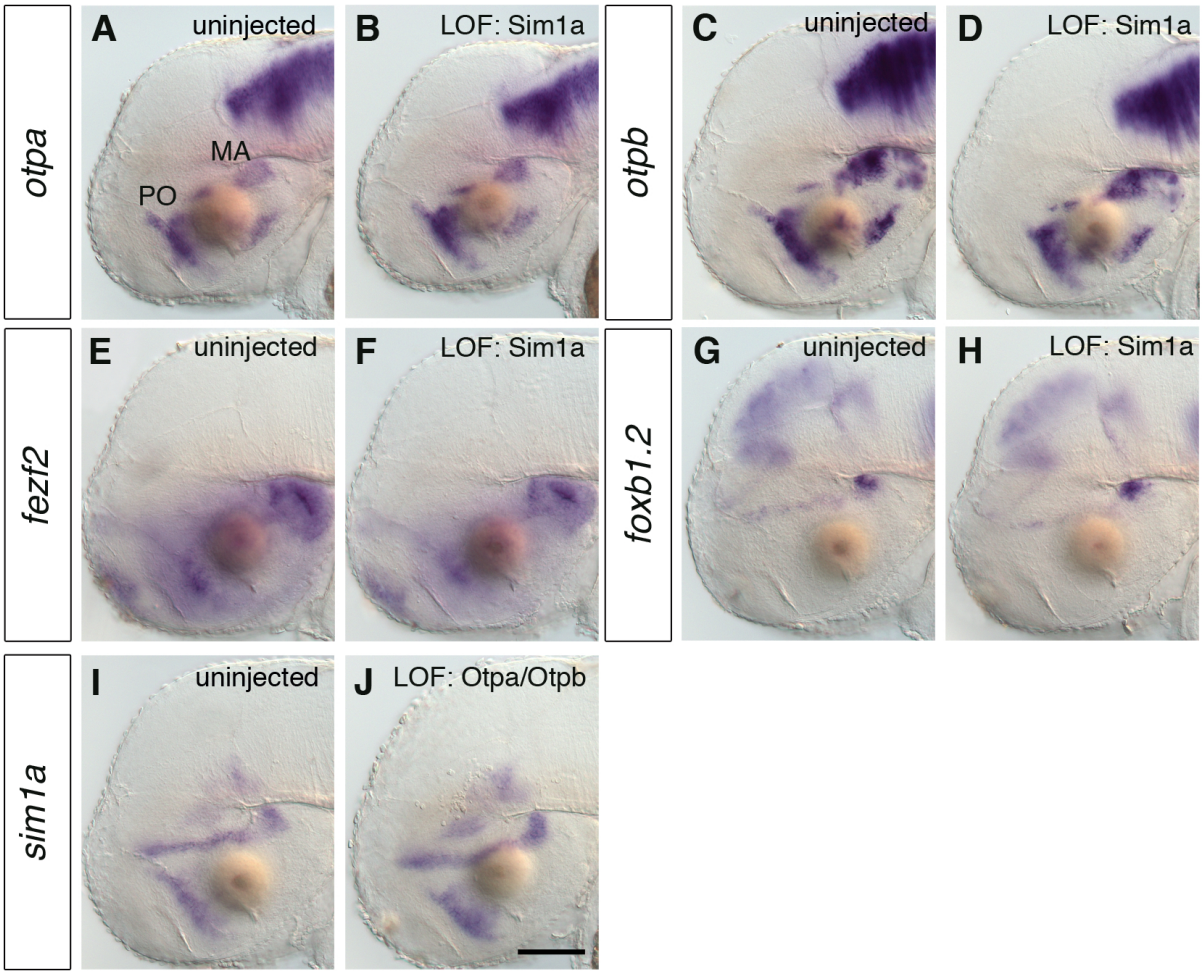

Fig. 3.10: *sim1a* has no cross-regulatory interactions with *otp*, *fezf2* or *foxb1.2*

(A-J) WISH of 2 dpf wild-type embryos (A,C,E,G,I), embryos injected with 1 ng *sim1a* MO (B,D,F,H) and *otpa*^{m866} embryos injected with 4 ng *otpb* MO to detect the expression of *otpa* (A,B), *otpb* (C,D), *fezf2* (E,F), *foxb1.2* (G,H) and *sim1a* (I,J). Lateral views are shown. (A-D)Loss of Sim1a function results in no changes in the expression of *otpa* (n=54) or *otpb* (n=50). (E,F) Additionally, *sim1a* expression is not altered in *fezf2* morphants (n=46) as well as in (G,H) *foxb1.2* morphants (N=43). (I,J) the loss of Otp function led to no changes in *sim1a* expression (n=5). The scale bar represents 200 µm.

In order to test this hypothesis I performed a WISH on embryos injected with a *sim1a* splice-MO (Löhr et al., 2009) and with *otpa*^{m866} embryos injected with 4 ng *otpb* MO. As expected the loss of Sim1a function induced no change in the expression of *otpa* or *otpb* (Fig. 3.10A-D, n=54, n=50). Vice versa, the loss of Otp function embryos displayed no altered *sim1a* expression compared to wild-type (Fig. 3.10I,J, n=5). However, the reduction of Sim1a activity resulted in no apparent changes in the expression of *fezf2* (Fig. 3.10E,F, n=46) or *foxb1.2* (Fig. 3.10G,H, n=43).

These results suggest that, although *otp* and *sim1a* do not regulate each other, their genetic interactions with other transcription factors are not the same. Therefore, they might act in parallel for the specification of certain neuron types but are likely to have also divergent functions.

In summary, the loss-of-function analysis revealed that at 2 dpf a cross-regulatory network of *fezf2*, *otp* and *sim1a* is important for the formation of the preoptic area, while this network together with the additional regulator *foxb1.2* is crucial for the formation of the mammillary area. At 2 dpf, Fezf2 changes its genetic interaction in different areas. In the PO Fezf2 positively regulates *sim1a* expression but in the MA it negatively regulates *sim1a* (Fig. 3.8). Also the genetic interaction of Otp changes in different areas of the hypothalamus. In the PO, Otp positively regulates *fezf2* expression, whereas it negatively regulates *fezf2* in the MA (Fig. 3.1). Further, Foxb1.2 negatively regulates the expression of *fezf2* (Fig. 3.9) and the expression of *otp* and *sim1a* (Fig. 3.8).

Interestingly, the interactions of the transcription factors not only vary in different areas of the brain but also change over time. Foxb1.2 displayed no genetic interactions with *fezf2*, *otp* or *sim1a* at 1 dpf (Fig. 3.4, 3.6), but negatively regulates their expression at 2 dpf (Fig. 3.8, 3.9). In addition, Otp did not regulate the expression of *foxb1.2* and *fezf2* at 1 dpf (Fig. 3.5), whereas at 2 dpf the interaction becomes cross-regulatory (Fig. 3.1). Also the interaction of *fezf2* changes during development. Early on Fezf2 is a key transcription factor for the hypothalamus patterning, but later in development it forms a cross-regulatory network important for the specification of two important nuclei of the hypothalamus, the PO and the MA. Figure 3.11 summarizes and compares the genetic interactions of *fezf2*, *foxb1.2*, *otp* and *sim1a* at 1 dpf and 2 dpf.

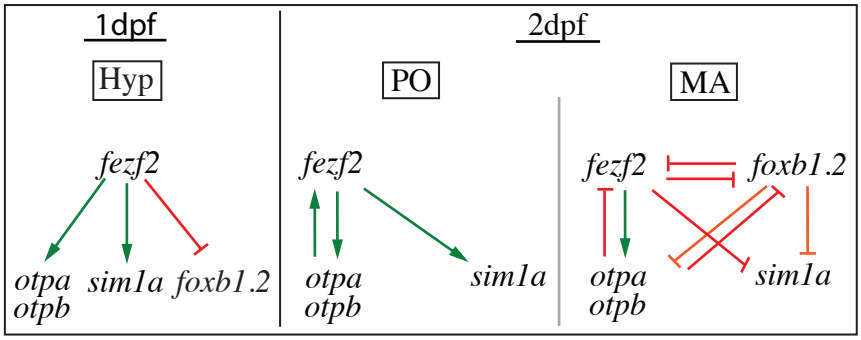

Fig. 3.11: A cross-regulatory network of *fezf2*, *foxb1.2*, *otp* and *sim1a* is crucial for the formation of PO and MA in the zebrafish hypothalamus

A summary of the observed genetic regulatory interactions among *fezf2*, *otp*, *sim1a* and *foxb1.2* at 1 dpf and 2 dpf. In addition, the cross-regulatory network in the MA compared to the interactions observed in the PO at 2 dpf are summarized. Interestingly, the interactions of *otp* and *fezf2* vary over time and are different in the PO and in the MA at 2 dpf. Green arrows indicate positive regulation while red bars represent negative regulation and the orange bars indicate slight negative regulation.

3.1.6 Regulatory interactions of *fezf2*, *foxb1.2*, *otp* and *sim1a* pattern subregions of the MA

Following the identification of the different genetic interactions of *fezf2*, *foxb1.2*, *otp* and *sim1a*, in the MA and the PO, I asked whether these cross-regulatory interactions can pattern different subregions within the MA compared to the PO.

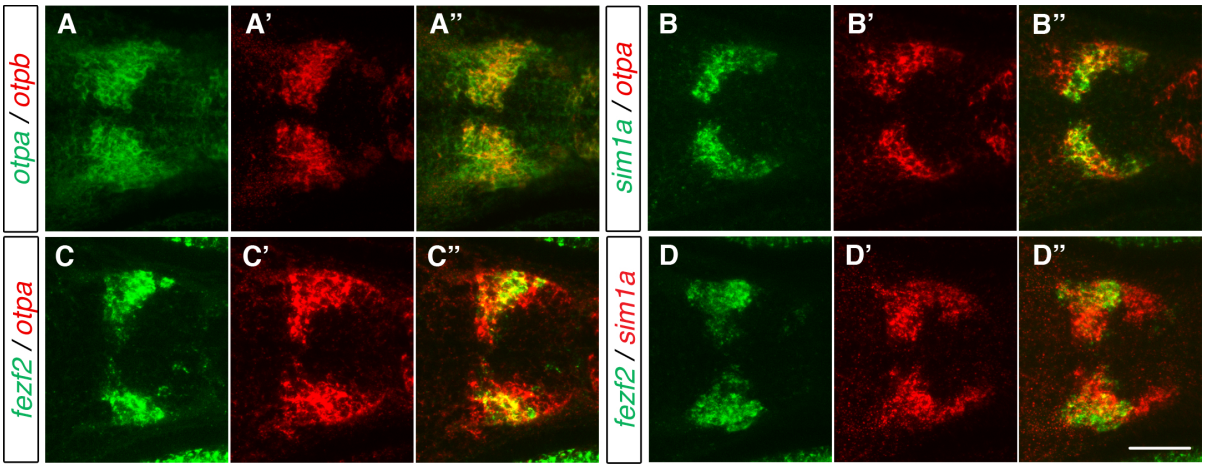

Fig. 3.12: *otpa*, *otpb*, *sim1a* and *fezf2* are mostly co-expressed in the PO

(A-D") FISH of 2 dpf wild-type embryos to reveal the spatial relationship among *otpa* (A,B',C'), *otpb* (A'), *sim1a* (B,D') and *fezf2* (C,D) in the PO. (A-B") The expression of *otpa* mainly overlaps with the expression of *otpb* (A") and *sim1a* (B"). (C-D") The *fezf2*

domain is contained within the *otpa* (C") and *sim1a* (D") expression domain, although slightly smaller. Maximum projections of 10-15 µm in the dorsal view are shown. The scale bar represents 60 µm.

In the PO, where Fezf2 positively regulates *otp* and *sim1a* and where Otp positively regulates *fezf2*, FISH analysis using 2 dpf wild-type embryos showed that these regulators are mainly co-expressed (Fig. 3.12). Since *otpa* and *otpb* were mainly co-expressed in the PO (Fig. 3.12A-A"), I only show the co-expression analysis of *otpa* with *sim1a* and *fezf2*. Consistent with its assumed parallel action in neuron type specification (Löhr et al., 2009), *sim1a* and *otpa* were mainly co-expressed within the PO (Fig. 3.12B-B"). *fezf2* was contained within the *otp* (Fig. 3.12C-C")and *sim1a* (Fig. 3.12D-D") PO expression domain, although the *fezf2* domain was slightly smaller.

In contrast to the PO, the complex regulatory interactions among *fezf2*, *foxb1.2*, *otp* and *sim1a* resulted in the generation of distinct subregions within the MA. In the MA *otpa* and *otpb* expression overlapped in the more anterior part of their expression, but *otpb* had an extra domain in the posterior most MA (Fig. 3.13A-A"). Therefore, I will use *otpb* expression to detect the spatial relationship of *otp* and *fezf2*, *foxb1.2* and *sim1a*. Consistent with their cross-repressive genetic interaction *foxb1.2* and *fezf2* were expressed in a mostly non-overlapping manner. Only the anterior most *fezf2* domain slightly overlapped with the posterior extent of *foxb1.2* (Fig. 3.13B-B"). Since *foxb1.2* was expressed in a compact medially located domain, I will refer to this area as the central MA from now on. The central MA is delineated in figure 3.13 by white dashed lines. The main part of the *fezf2* expression domain formed a separate domain posterior to the central MA. Laterally adjacent to the central MA expression of *fezf2* (Fig. 3.13C-C") and *foxb1.2* (Fig. 3.13D-D"), *otpb* was expressed in the peripheral MA. This finding reflects the cross-repressive genetic interaction of *otp* and *foxb1.2* and *fezf2*. The expression of *sim1a* showed some overlap with *fezf2* (Fig. 3.13E-E") and *foxb1.2* (Fig. 3.13F-F") in the central MA but extended more to the lateral. The *sim1a* expression domain additionally overlapped with that of *otpb* in the peripheral MA (Fig. 3.13G-G").

Taken together, this co-expression analysis showed that strong *foxb1.2* expression demarcates the central MA with contribution by *fezf2* and *sim1a* expression. Domains peripheral to the central MA are delineated by the expression of *fezf2* marking the posterior, *otpa*/*otpb* found in lateral and posterior domains and *sim1a* labeling lateral and anterior domains (Fig. 3.14). Thus, the negative and positive genetic interactions of *fezf2*, *foxb1.2*, *otp* and *sim1a* pattern different subregions within the posterior hypothalamus, while the positive genetic interactions of *otp*, *sim1a* and *fezf2* lead to no distinct subregionalization in the PO (Fig. 3.14).

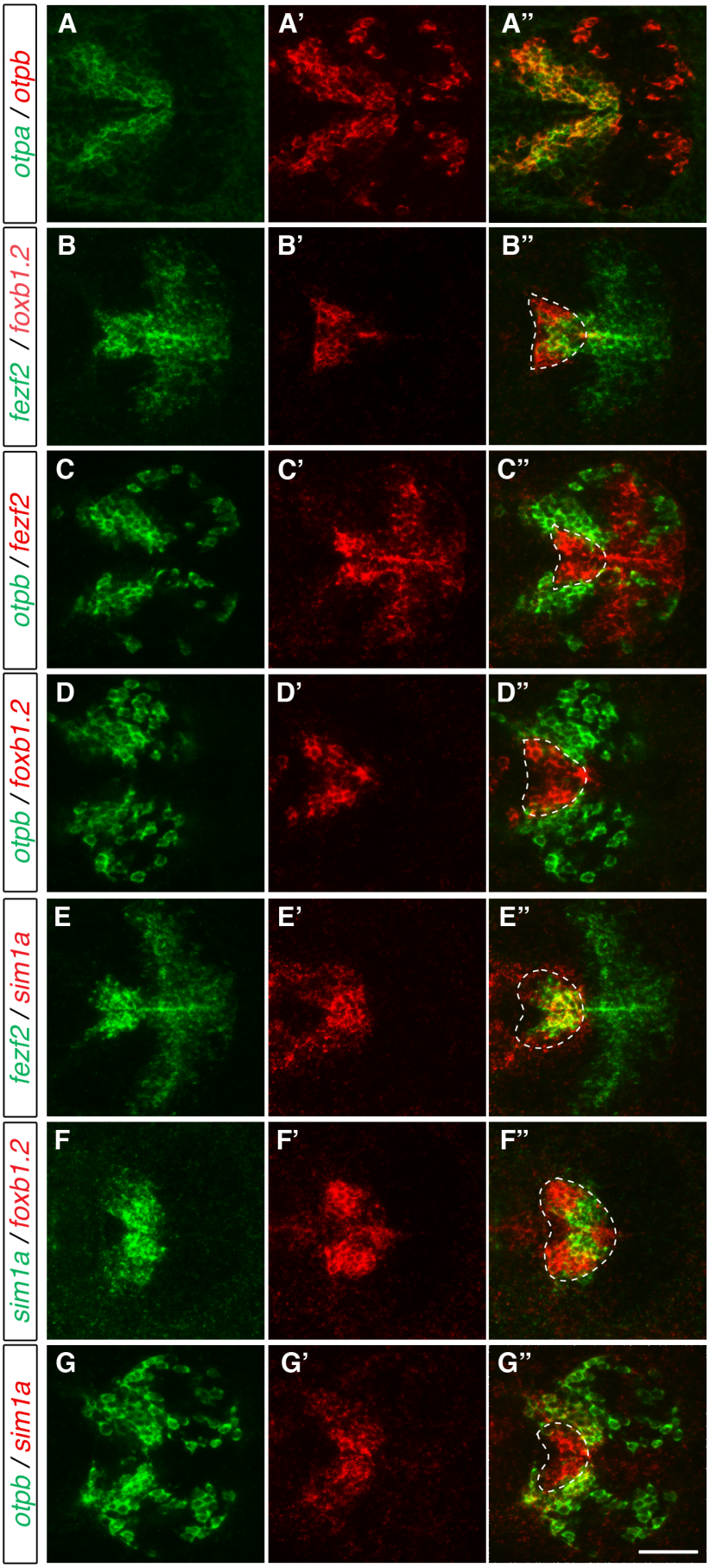
otpa / otpb
A
A'
A''
fezf2 / foxb1.2
B
B'
B''
otpb / fezf2
C
C'
C''
otpb / foxb1.2
D
D'
D''
fezf2 / sim1a
E
E'
E''
sim1a / foxb1.2
F
F'
F''
otpb / sim1a
G
G'
G''

Fig. 3.13: Co-expression analysis of *otp*, *sim1a*, *foxb1.2*, and *fezf2* reveals subregions within the MA

(A-G") Two-color FISH of 2 dpf wild-type embryos with probes for *otpa* (A), *otpb* A',C,D,G), *sim1a* (E',F,G'), *fezf2* (B,C',E,) and *foxb1.2* (B',D',F') reveal their relative expression patterns in the MA. (A-A") *otpa* and *otpb* are mostly co-expressed in the more anterior MA, while *otpb* has an extra domain in the more posterior MA. (B-B") Strong *foxb1.2* expression marks the central MA where *fezf2* expression partially overlaps but extends further towards the posterior. In contrast, (C-C") *otpb* is expressed laterally and adjacent to *fezf2* and (D-D") *foxb1.2*. (E-E") The *fezf2* and *sim1a* domains are partially co-localized in the central MA. (F-F") *sim1a* partially overlaps with *foxb1.2*. (G-G") In the peripheral MA *sim1a* is co-expressed with *otpb*. The white dashed lines demarcate the central MA in B", C", D", E", F" and G". Dorsal views of 10-15 µm maximum projections are shown. Scale bar represents 60 µm.

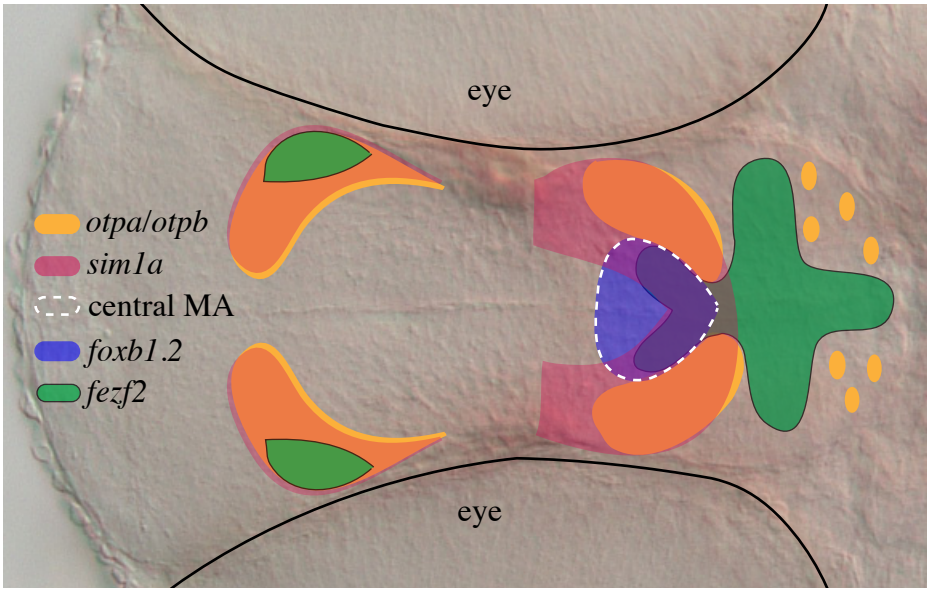

Fig. 3.14: Regulatory interactions among *fezf2*, *foxb1.2*, *otp* and *sim1a* pattern subregions within the MA

A scheme showing the relative expression pattern of the transcription factors in the PO and the MA at 2 dpf. The white dashed line delineates the central MA.

3.1.7 *otp* expression at 1 dpf delineates the prospective preoptic and the mammillary area

The co-expression analysis at 2 dpf showed that in addition to *foxb1.2*, *otp* and *fezf2* could serve as a marker for the MA in the zebrafish hypothalamus. Given the fact that *fezf2* (Levkowitz et al., 2003), *foxb1.2* and *otp* (Ryu et al., 2007) are expressed in the posterior hypothalamus at 1 dpf, I hypothesized that the expression of these regulators may

delineate the prospective MA already at 1 dpf. In addition, a continuous co-expression of *fezf2* and *otp* in the anterior diencephalon could delineate the prospective PO, because in contrast to the MA these two factors are positively regulating each other and they are co-expressed in the PO at 2 dpf.

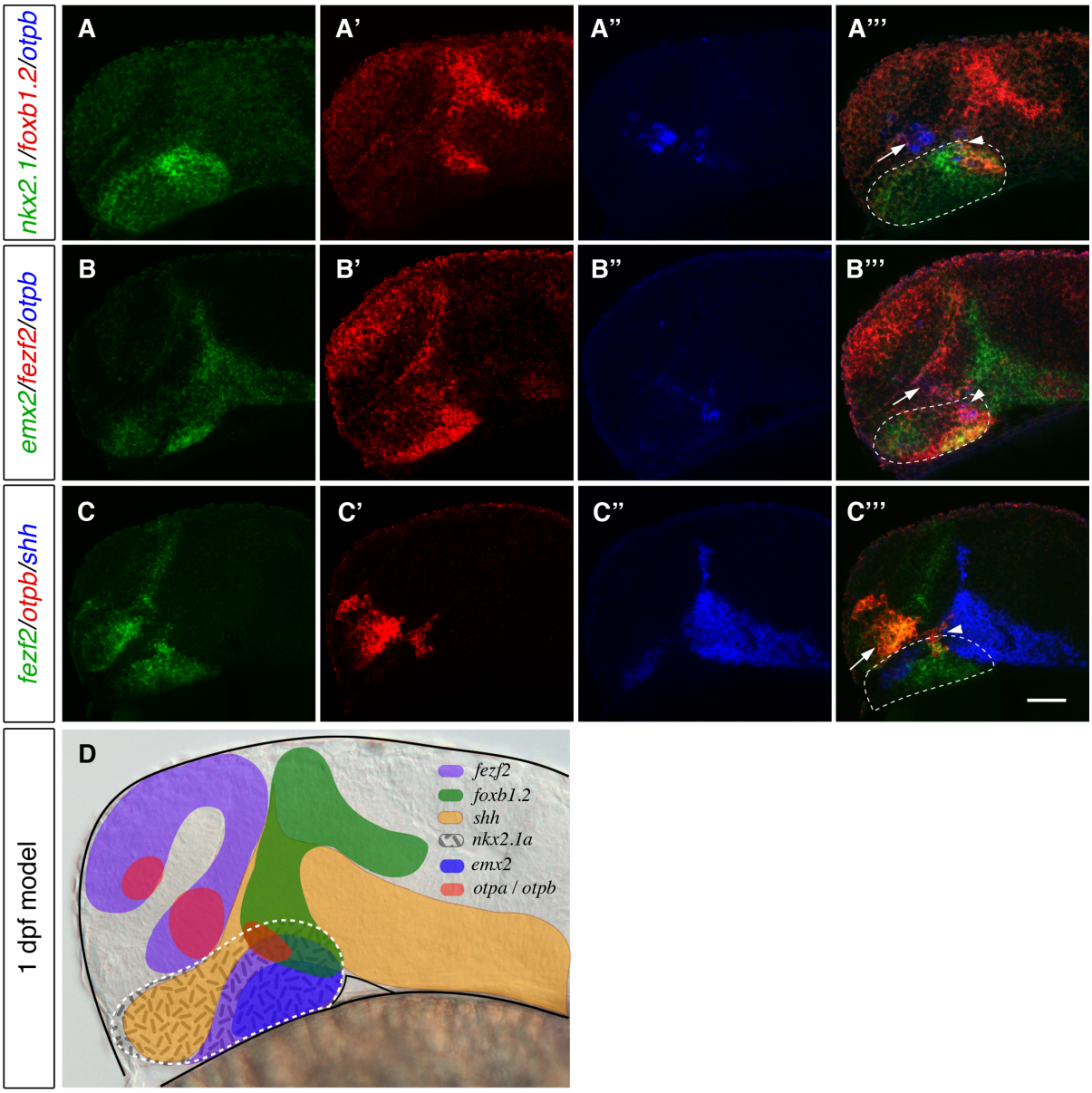

Fig. 3.15: *otp* is expressed in distinct hypothalamic areas at 1 dpf

(A-C"') Three-color FISH to detect the relative expression of *otpb* (A",B",C'), *nkx2.1a* (A), *foxb1.2* (A'), *emx2* (B), *fezf2* (B',C) and *shh* (C") in 1 dpf wild-type embryos. Lateral views of 10 µm projections are shown and the scale bar represents 60 µm. (A-A"') The anterior diencephalic *otpb* expression domain (arrow) is *nkx2.1a*- and *foxb1.2*-negative, while the posterior diencephalic domain (arrowhead) is contained within the *foxb1.2* domain and mainly overlaps with *nkx2.1a*. (B-B"') There is a slight overlap between the posterior *otpb* expression and the posterior-ventral *emx2* domain. (C-C"') The forebrain

otpb expression domains are *fezf2*-positive, while very few posterior *otpb*-positive cells express *shh* as well. (D) A model summarizing the relative expression patterns of *otpb*, *fezf2*, *foxb1.2*, *shh*, *nkx2.1a*, and *emx2*. For simplicity, only the hypothalamic *emx2* expression is shown. White dashed lines in A"', B"', C"' and D delineate the hypothalamus.

To address this, I performed three-color FISH to reveal the spatial relationship between *otp* and *fezf2* and *foxb1.2* in the context of the hypothalamic markers *nkx2.1a* (Rohr et al., 2001), *shh* and *emx2* (Mathieu et al., 2002)(Fig. 3.2). At 1 dpf the expression of *otpa* and *otpb* are comparable. From the anterior to the posterior there are a telencephalic domain, diencephalic domains and a domain in the hindbrain (Ryu et al., 2007). Due to this fact, I only used the expression of the *otpb* probe for the following experiment. Interestingly, at 1 dpf the posterior most diencephalic *otpb* expression domain was contained within the *foxb1.2* domain, while the anterior diencephalic domain was *foxb1.2*- as well as *nkx2.1a*-negative. This posterior *otpb*-, *foxb1.2*-positive domain was only partially *nkx2.1a*-positive (Fig. 3.15A-A"'). Additionally, this posterior *otpb* domain slightly overlapped with that of *emx2* (Fig. 3.15B-B"'). Confirming the positive regulation of Fezf2 on *otpb* in the forebrain at 1 dpf, all forebrain *otpb*-positive domains were also *fezf2* positive, while only a very small proportion of the posterior *otpb* positive cells appeared to express *shh* as well (Fig. 3.15C-C"'). Figure 3.15D shows a model that summarizes the observed co-expression.

These results suggest that indeed *otp* could serve as a marker to delineate prospective hypothalamic regions already at 1 dpf. Although not *nkx2.1*-positive I hypothesized that the anterior diencephalic *otp* domain that is contained within the *fezf2* stripe delineating the ZLI is a marker for the prospective PO (Fig. 3.15 arrows). In contrast, the posterior diencephalic *nkx2.1a*-positive *otp* domain that is additionally *fezf2*- and *foxb1.2*-positive could mark the prospective MA (Fig. 3.15 arrowheads).

To further corroborate this hypothesis I performed two-color FISH at different time points of development to track the changing transcription factor expression domains. At 24 hpf, *fezf2* (Fig. 3.16A-A") and *foxb1.2* (Fig. 3.16B-B") were mainly co-expressed with *otpb* in the posterior hypothalamus or the prospective MA (arrowheads), while *foxb1.2* was not expressed in the prospective PO (arrow). At 30 hpf (Fig. 3.16C-C") the posterior hypothalamic expression domain of *fezf2* started to become more condensed in the prospective MA, where *otpb* was still partially co-expressed with *fezf2*. The *otp* cells in the prospective PO appeared to increase their cell number. At 36 hpf the prospective *otpb*-positive PO still appeared to increase its cell number (Fig. 3.16D", arrow), while the *fezf2* domain was decreasing its size.

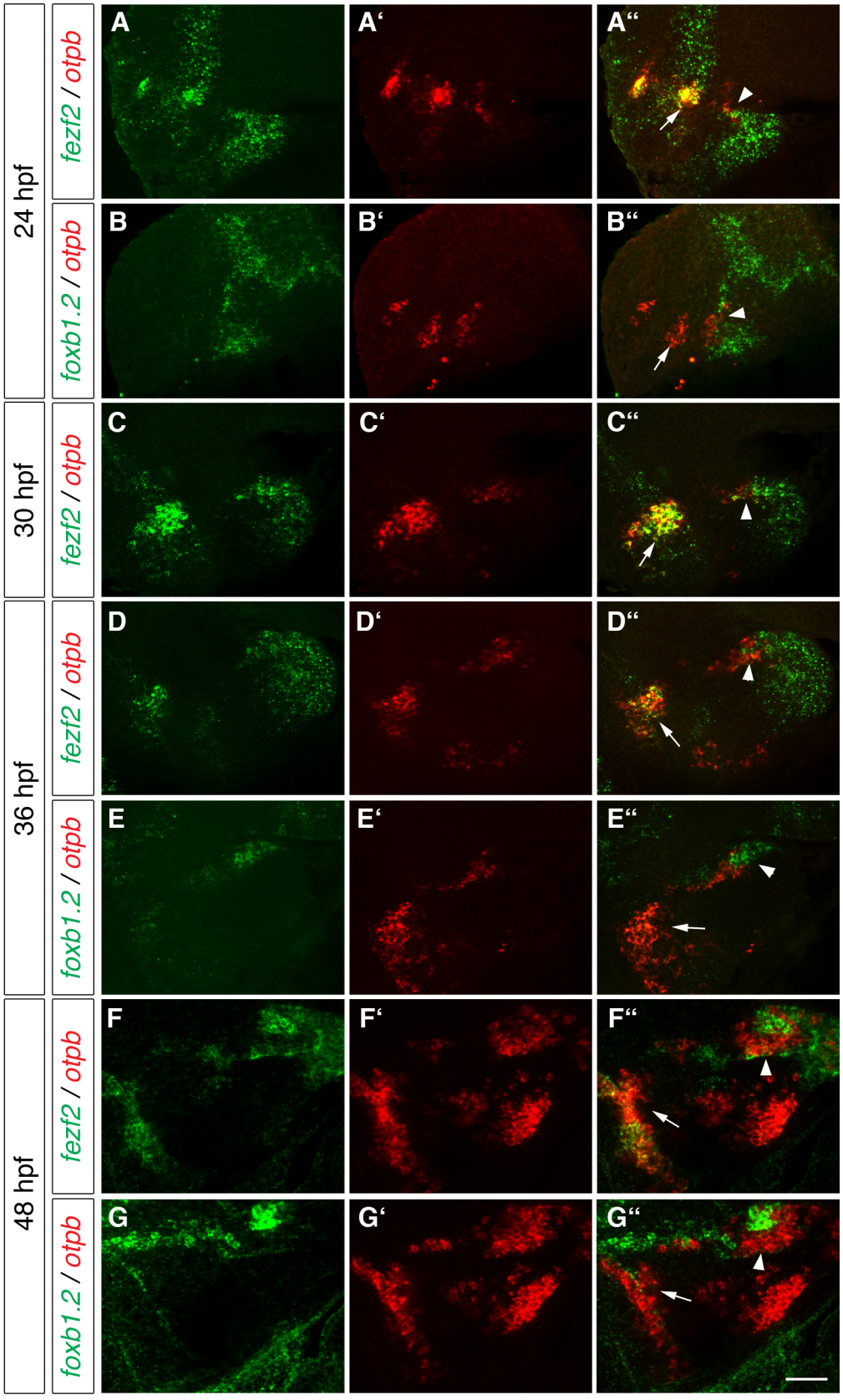

Fig. 3.16: ***otp*** **expression delineates the prospective preoptic and mammillary area from 1 dpf onwards**

(A-G") Two-color FISH with probes for *otpb* (A',B',C',D',E',F',G'), *fezf2* (A,C,D,F) and *foxb1.2* (B,E,G) to reveal their spatial relationship in wild-type embryos at 24 hpf (A-B"), 30 hpf (C-C"), 36 hpf (D-E") and 48 hpf (F-G"). Lateral views of 10 µm projections are shown. (A-A") *otpb* is mainly co-expressed with *fezf2* at 24 hpf, while (B-B") only the posterior hypothalamic *otpb* domain is *foxb1.2* positive. (C-C") At 30 hpf the posterior hypothalamic *fezf2* domain becomes more condensed within the prospective MA. The anterior diencephalic *otpb* domain is *fezf2* positive, while the more posterior domain is partially *fezf2* positive. (D-E") Around 36 hpf the posterior *otpb* domain is separated from the *fezf2* (D-D") and the *foxb1.2* (E-E") domains. Although the size of the prospective PO *fezf2* domain decreases, the *otpb* domain remains *fezf2*-positive. (F-G") In the lateral view at 48 hpf, the PO (arrow) forms a stripe, which is *otpb*- and *fezf2*-positive. The *foxb1.2* expression together with parts of the *fezf2* expression mark the central MA surrounded by the peripheral MA (arrowheads) *otpb* expression domain. Arrows point to the prospective (A-E") and the established (E-G") PO and arrowheads point to the prospective (A-E") and the established (E-G") MA. The scale bar represents 60 µm.

Interestingly, the expression of *otpb* in the prospective MA (arrowheads) started to separate from the *fezf2* (Fig. 3.16D-D") and *foxb1.2* (Fig. 3.16E-E") expression domain, leading to only partially overlapping expression domains. At 48 hpf the PO (arrows) and the MA (arrowheads) were clearly detectable. In the lateral view the PO at 2 dpf formed a dorsal to ventral *otp*-positive stripe. This stripe contained the *fezf2* expression domain that reduced its size during development (Fig. 3.16F-F"). Furthermore, the PO was *foxb1.2*-negative from 24 hpf onwards. In the posterior hypothalamus at 48 hpf, the central MA, which is delineated by the *foxb1.2* expression domain, was clearly visible in the lateral view and surrounded by *otpb* expressing cells (Fig. 3.16G-G") that form the peripheral MA. These *otpb* expressing cells were also adjacent to the central MA *fezf2* domain (Fig. 3.16F-F").

Taken together these results suggest that the preoptic area as well as the mammillary area can already be marked by the expression of *otp* or a combination of *foxb1.2* and *otp* at 1 dpf.

3.2 Transcriptional regulation of the hypothalamic neuronal subtypes

3.2.1 Identification of neuropeptide genes regulated by *fezf2*, *foxb1.2*, *otp* and *sim1a*

To test the roles of *fezf2*, *foxb1.2*, *otp* and *sim1a* on the specification of hypothalamic neuronal subtypes, I first identified markers for neuronal subtypes in the MA and in the PO. Therefore, I searched the zebrafish expression database (www.zfin.org) to find neuropeptides that are expressed in the MA or in the PO. I cloned more than 30 genes coding for different neuropeptides expressed either in the PO or in the MA or both. A complete list of the probes can be found in the Material and Methods chapter 6 in section 6.1.12. To test whether *fezf2*, *foxb1.2*, *otp* and *sim1a* control the specification of the identified neuropeptides, I first examined their expression in loss of Otp function embryos. I choose Otp for this initial screen because Sim1a is assumed to work in parallel to Otp in neuron type specification (Löhr et al., 2009) and should therefore display a similar phenotype. Furthermore, Fezf2 and Foxb1.2 are both important for early patterning of the diencephalon (Jeong et al., 2007; Staudt and Houart, 2007; Toro and Varga, 2007) and the loss of their function would result in a broad effect resulting from the patterning defects. For several neuropeptide-expressing cells it was previously shown that their specification is regulated by Otp. These neuronal subtypes are *th*, *crh*, *oxtl*, *avpl* and *ss* expressing cells (Eaton and Glasgow, 2007; Eaton et al., 2008; Ryu et al., 2007). I confirmed these observations in 2 dpf wild-type embryos and *otpa*^{m866} embryos injected with 4 ng of *otpb* MO; the *th* expression was severely reduced in the posterior tuberculum (PT) in loss of Otp function embryos (Fig. 3.17A,B, arrowheads, n=4/4). Also the *crh* expression was lost in the PO (arrows) while reduced in the posterior tuberculum close to the MA (arrowheads)(Fig. 3.17C,D, n=4/4). Consistent with the published data, the preoptic expression domains of *oxtl* and *avpl* were absent in loss of Otp function embryos (Fig. 3.17E-H, arrows, n=7/7, n=3/3). During this screen I found that embryos injected with 4 ng *otpb* MO that did not carry the *otpa*^{m866} mutation mainly displayed no or only a slight phenotype. Therefore, I concluded that only the complete loss of both Otpa and Otpb function induces severe defects in the specification of neuropeptide-expressing cells. At 3 dpf a complete loss of Otp function resulted in a slight reduction of *ss* (Fig. 3.17K,L, arrows, n=4/4) and *trh* expression in the ventral PO was reduced (Fig. 3.17M,N, arrows, n=4/4). In addition to confirming previous reports, I identified for the first time six neuropeptide-expressing neuronal subtypes that are regulated by Otp.

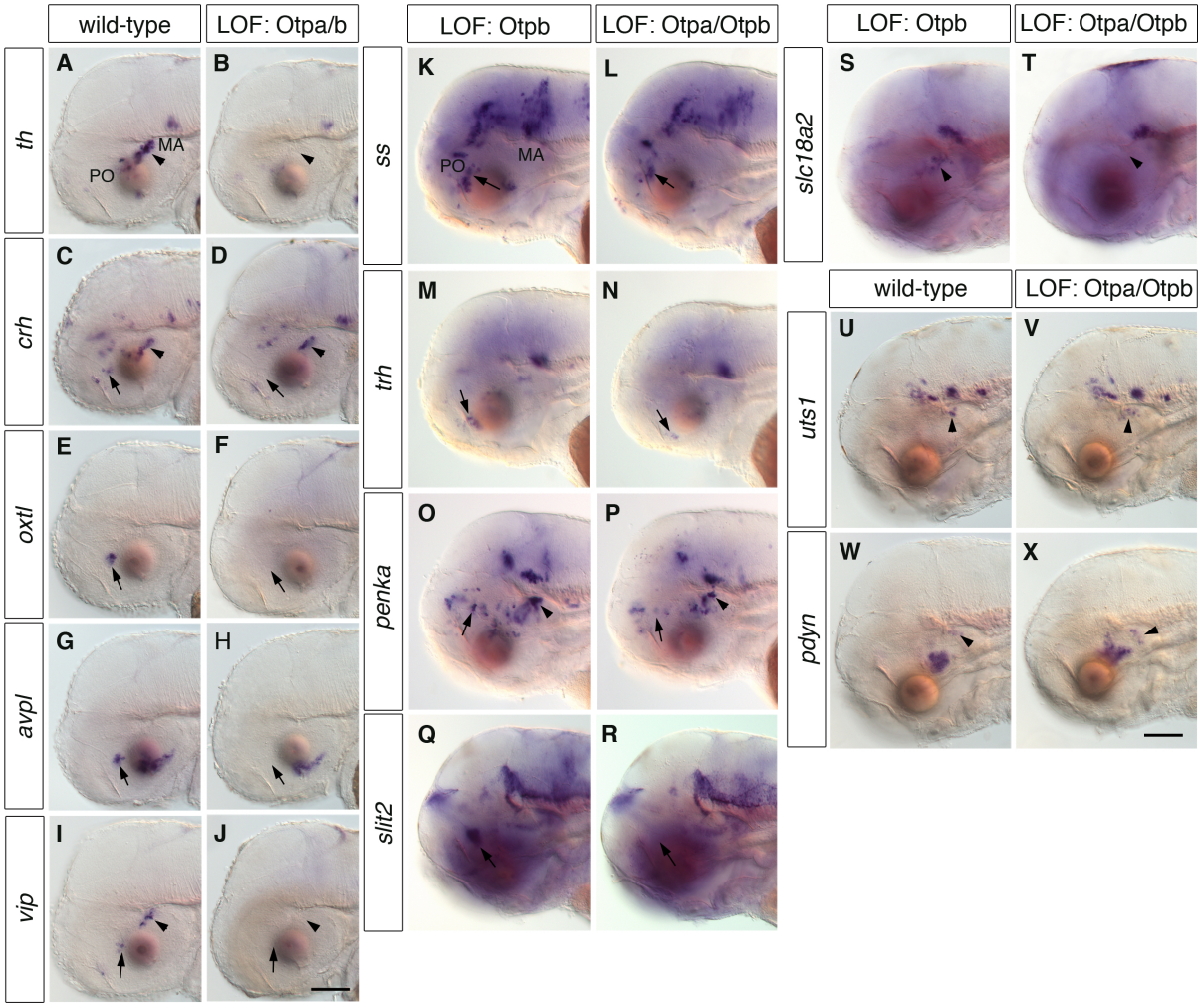

Fig. 3.17: Otp is crucial for the specification of several neuronal subtypes
(A-X) Wild-type embryos (A,C,E,G,I,K,M,O,Q,S,U,W) and *otpa*^{m866} mutant embryos injected with 4 ng *otpb* MO (B,D,F,H,J,L,N,P,R,T,V,X) at 2 dpf (A-J) and 3 dpf (K-X) subjected to WISH to reveal the effect of Otp on the expression of *th* (A,B), *crh* (C,D), *oxtl* (E,F), *avpl* (G,H), *vip* (I,J), *ss* (K,L), *trh* (M,N), *penka* (O,P), *slit2* (Q,R), *slc18a2* (S,T), *uts1* (U,V) and *pdyn* (W,X). Lateral views are shown. (A,B) *th* is reduced in the posterior tuberculum (PT) (arrowheads) in embryos lacking Otp activity (n=4/4), while (C,D) *crh* is reduced in both the PO and the PT upon loss of Otp function (n=4/4). (E-H) *oxtl* (n=7/7) and *avpl* (n=3/3) are both absent in loss of Otp function embryos in the NPO (arrows). (I,J) *vip* expression is lost in embryos lacking Otp activity (n=19/19) both in the PO (arrows) and in the MA (arrowheads). (K-M) The reduction of Otp activity results in a reduced expression of *ss* in the PO (n=4/4) and of *trh* in the ventral PO (n=4/4). (O,P) The expression of *penka* in the PO (arrows) and in the MA (arrowheads) become reduced in loss of Otp function embryos (n=5/5). (Q,R) The expression of *slit2* in the PO (arrows, n=8/8) is absent in embryos lacking Otp activity, which also results in an absent *slc18a2* expression domain in the PT (arrowheads, n=7/7). (U-X) Interestingly, the loss of Otp function leads to an extended expression domain of *uts1* (n=21/21) and *pdyn* (n=5/5) in the MA (arrowheads). The scale bare represents 200 µm.

One of these neuronal subtypes were the *vasoactive intestinal peptide* (*vip*) -expressing cells. *vip* in wild-type embryos appeared to be expressed in the PO (arrows) as well as in the MA (arrowheads) whereas in 2 dpf loss of Otp function embryos its expression was lost in both areas (Fig. 3.17I,J, n=19/19). The expression of proenkephalin a (*penka*) is very broad throughout the hypothalamus. However, *penka* that appeared to be expressed in the PO and in the MA in 3 dpf wild-type embryos was reduced upon complete loss of Otp function both in the PO (arrows) and in the MA (arrowheads) (Fig. 3.17O,P, n=5/5). The expression domains of *slit2* in the PO (arrows) and of *slc18a2* in the PT (arrowheads) were severely downregulated in loss of Otp function embryos (Fig. 3.17Q-T, n=8/8, n=7/7). In contrast, the expression domains of *urotensin1* (*uts1*) and *pdynorphin* (*pdyn*) in the MA (arrowheads) were extended in embryos lacking Otp activity (Fig. 3.17U-X, n=21/21, n=5/5).

Together, this initial screen for neuronal subtypes whose specification is controlled by Otp, shows that Otp is important for the formation of several different neuronal subtypes in mainly the PO, the PT and the MA. In addition, it shows that the expression of the neuropeptides is regulated differentially by Otp in different hypothalamic regions.

3.2.2 Specification of hypothalamic neurons requires Fezf2, Foxb1.2, Otp and Sim1a activity

So far my results show that Otp regulates the specification of numerous neuronal subtypes. Interestingly, I observed that this regulation was not only positive but also negative resulting in more cells expressing a distinct neuropeptide. Therefore, the question arises whether this variable regulatory potential results from the differential regulatory interactions of *otp*, *fezf2*, *foxb1.2* and *sim1a*. To answer this question I performed a comprehensive loss-of-function analysis of the TFs and tested for the expression of the neuropeptide genes that were including *th*-, *crh*-, *vip*-, *oxtl*-, *avpl*-, *uts1*-, *pdyn*- and *penka*-positive neurons. In addition, I tested the expression of *cck* due to its specific expression in the MA. By using probes for *oxtl* and *avpl* I was able to analyze the fate of the magnocellular neurosecretory neurons, while the *crh* expression serves as a marker for the specification of a subset of parvocellular neurosecretory neurons. Since the probes for *th* and *slc18a2* are both labeling dopaminergic neurons and the loss-of-function analysis for *slc18a2* expression displayed the same phenotype (data not shown) as *th* expression I only show the results for *th* in this thesis. Slit2 is implicated in axon midline crossing in zebrafish forebrain (Barresi et al., 2005) a topic, which this thesis did not investigate further.

Most neuropeptide-expressing cells were examined at 2 dpf, the time point at which the regulatory interactions of *fezf2*, *foxb1.2*, *otp* and *sim1a* were investigated. Since not all of the neuropeptides are expressed at this stage I also performed the loss-of-function analysis with these neuropeptides at 3 dpf. Consistent with the published data, th expression was severely downregulated in the hypothalamus in loss of Otp and Sim1a function embryos (Fig. 3.18A-C, arrowheads, n=4/4, n=14/14) (Löhr et al., 2009; Ryu et al., 2007), while the loss of Fezf2 function resulted in a slight reduction of th cells (Fig. 3.18A,D, arrowheads, n=4/11) (Blechman et al., 2007). *foxb1.2* morphants showed no change in *th* expression (Fig. 3.18A,E, n=13). Also for *crh* expression it has been shown that reduced Otp and Sim1a activity results in a lost expression of *crh* in the PO (arrows) and a reduced expression in the PT (arrowheads) (Löhr et al., 2009; Ryu et al., 2007). I confirmed this observation (Fig. 3.18F-H, n=4/4, n=12/12) and showed that *fezf2* morphants display a reduction of the PO (arrows) signal. Further, the reduction of the *crh* PT (arrowheads) expression domain was more severe than in the loss of Otp function embryos (Fig. 3.18F,I, n=13/13). The loss of Foxb1.2 function had no effect on *crh* expression (Fig. 3.18F,J, n=15). Hitherto, the expression of *vip* in zebrafish embryos has not been published. The WISH results (Fig. 3.18K) indicate that *vip* expression domains are found in the PO (arrows) and in the MA (arrowheads). While the loss of Otp, Sim1a and Fezf2 function resulted in an absent PO domain (Fig. 3.18K-N, arrow, n= 14/19, n=47/53, n=39/39), only embryos with reduced Otp activity additionally displayed an absent MA expression domain (Fig. 3.18K,L, n=16/19). *sim1a* morphants showed a slightly reduced MA *vip* domain, while *fezf2* morphants displayed more severe reduction (Fig. 3.18K,M,N, n=30/53, n=37/39). The loss of Foxb1.2 function led to no apparent changes in *vip* expression (Fig. 3.18K,O, n=21). Regulatory action of Otp and Sim1a on *oxtl* and *avpl* neuron specification has been studied previously (Eaton and Glasgow, 2006, 2007; Eaton et al., 2008) as well as the function of Fezf2 in the specification of *oxtl* neurons (Blechman et al., 2007). I confirmed that in loss of Otp, Sim1a and Fezf2 function embryos *oxtl* expression was absent (Fig. 3.18P-S, arrows, n=7/7, n=27/37, n=24/31). Additionally, I showed that the loss of Foxb1.2 function had no impact on *oxtl* expression (Fig. 3.18P,T, n=14). Furthermore, I confirmed the complete absence of PO *avpl* expression in the embryos with reduced Otp and Sim1a activity (Fig. 3.18U-W, arrows, n=6/6, n=40/42). *fezf2* morphants displayed a severe reduction of PO *avpl*-positive cells (Fig. 3.18U,X, arrows, n=28/28) while *foxb1.2* morphant showed no change in *avpl* expression (Fig. 3.18U,Y, n=14).

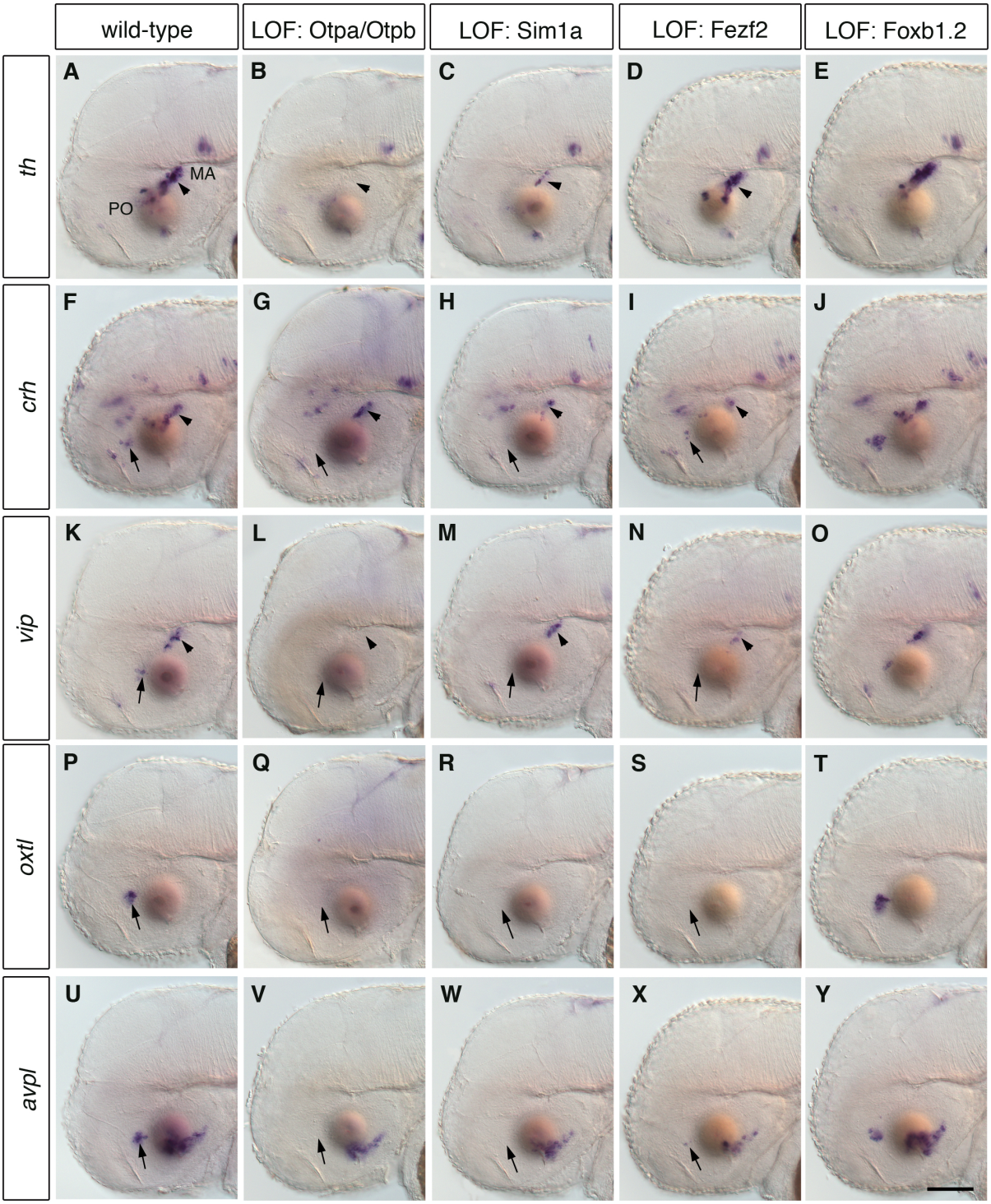

Fig. 3.18: Specification of hypothalamic neurosecretory neurons requires *fezf2*, *foxb1.2*, *otp* and *sim1a* at 2 dpf

(A-Y) WISH of 2 dpf wild-type embryos (A,F,K,P,U), *otpa*^{m866} mutant embryos injected with 4 ng *otpb* morpholino (B,G,L,Q,V), embryos injected with 1 ng *sim1a* MO (C,H,M,R,W), 4ng *fezf2* MO (D,I,N,S,X) and 8 ng *foxb1.2* MO (E,J,O,T,Y) to detect changes in the expression of *th* (A-E), *crh* (F-J), *vip* (K-O), *oxtl* (P-T) and *avpl* (U-Y). Lateral views are shown. (A-E) The expression of *th* is highly reduced (arrowheads) in loss of Otp (B, n=4/4) and Sim1a (C, n=14/14) function embryos, while slightly

reduced in *fezf2* morphants (D, n=4/11) and not changed in *foxb1.2* morphants (E, n=13). (F-J) The expression of *crh* in the PO (arrows) is absent in embryos with reduced Otp (G, n=4/4) and Sim1a (H, n=12/12) activity, while reduced in *fezf2* morphants (I, n=13/13). The PT *crh* expression domain (arrowheads) is reduced in loss of Otp, Sim1a and Fezf2 function embryos, whereas the loss of Foxb1.2 function results in no apparent change in *crh* expression (J, n=15). (K-O) Embryos with reduced Otp (L, n=15/19), Sim1a (M, n=47/53) and Fezf2 (N, n=39/39) activity display an absent *vip* expression domain in the PO (arrows), while loss of Otp function only resulted in a loss of the *vip* MA expression domain (L, n=16/19) whereas *sim1a* and *fezf2* morphants display a reduced MA *vip* domain (M,N, n=30/53, n=37/39). *foxb1.2* morphants shown no change in *vip* expression (O, n=21). (P-T) *oxtl* expression is absent in loss of Otp (Q, n=7/7), Sim1a (R, n=27/37) and Fezf2 (S, n=24/31) function and not changed in loss of Foxb1.2 function (T, n=14). (U-Y) Embryos with reduced Otp and Sim1 activity show no PO *avpl* expression (V,W, arrows, n=6/6, n=40/42), while *fezf2* morphants display a highly reduced PO *avpl* domain (X, arrow, n=28/28) and *foxb1.2* morphants show no apparent change in *avpl* expression (Y, n=14). The scale bar represents 200 µm.

Nothing is known about the transcription factors necessary for the specification of Uts1-, Pdyn-, Penka- or Cck-positive neurons. At 3 dpf, *uts1* is expressed in several distinct hindbrain domains and a single hypothalamic domain. This domain appeared to be within the MA (Fig. 3.19A, arrowhead). The loss-of-function analysis showed that a reduced Sim1a and Fezf2 activity led to a reduced *uts1* domain in the MA (Fig. 3.19A,C,D, arrowheads, n=29/42, n=17/42), while, in contrast, the loss of Otp and Foxb1.2 function resulted in an expansion of the hypothalamic *uts1* domain (Fig. 3.19A,B,E, arrowheads, n=21/21, n=58/76). *pdyn* has a broad expression domain in the ventral hypothalamus and a faint expression domain in the MA (Fig. 3.19F, arrowheads). Comparable to the *uts1* phenotype, this faint MA expression domain of *pdyn* was highly reduced in embryos lacking Sim1a and Fezf2 function (Fig. 3.19F,H,I, arrowheads, n=23/23, n=26/26). Interestingly, the loss of Otp and the loss of Foxb1.2 function resulted in a ventrally expanded *pdyn* domain in the MA (Fig. 3.19F,G,J, arrowheads, n=5/5, n=16/21). In contrast, the expression of *penka* is broadly distributed throughout the zebrafish hypothalamus. Although very scattered one can detect a preoptic or slightly dorsal to PO expression domain (Fig. 3.19K, arrow) and a domain, which appears to be expressed in the MA (Fig. 3.19K, arrowheads). Upon loss of Otp, Sim1a and Fezf2 function the preoptic expression domain of *penka* was reduced (Fig. 3.19K-N, arrows, n=5/5, n=10/22, n=11/17) while the mammillary expression domain (arrowheads) of *penka* appeared to be reduced in embryos with reduced Otp activity but not in *sim1a* and *fezf2* morphants. The loss of Foxb1.2 function showed no apparent effect on *penka* expression (Fig. 3.19K,O, n=22). *cck* appears to be specifically expressed within the hypothalamus of zebrafish at 3 dpf. In the hypothalamus it is expressed in the ventral part and it appeared to be expressed in the MA or slightly anterior to the MA

(arrowheads). The loss Fezf2 function resulted in a reduced *cck* expression domain (Fig. 3.19P,S, arrowheads, n=20/25), while the reduction of Otp, Sim1a and Foxb1.2 activity resulted in no apparent change in *cck* expression (Fig. 3.19P-R,T, n=6, n =25, n=23). The expression of *cck* appears to be slightly reduced in loss of Otp and Sim1a function embryos but this is due to the reduced age of the embryo and therefore no reliable result.

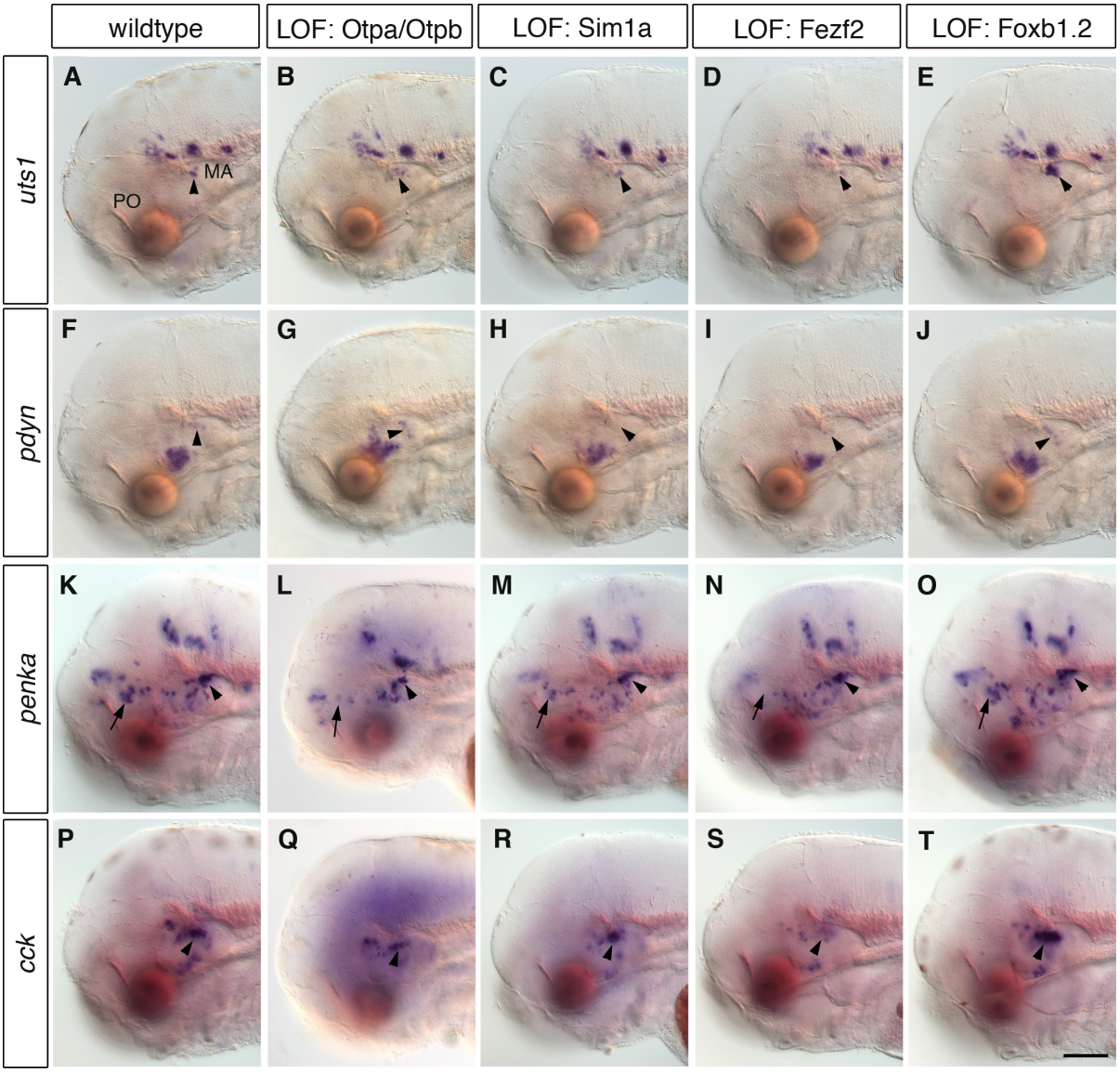

Fig. 3.19: The regulatory action of Fezf2, Foxb1.2, Otp and Sim1a is necessary for the specification of *uts1*, *pdyn*, *penka* and *cck* expressing neurons

(A-T) 3 dpf old wild-type embryos (A,F,K,P), *otpa*^{m866} mutant embryos injected with 4 ng *otpb* morpholino (B,G,L,Q), embryos injected with 1 ng *sim1a* MO (C,H,M,R), 4ng *fezf2* MO (D,I,N,S) and 8 ng *foxb1.2* MO (E,J,O,T) subjected to WISH to detect changes in the expression of *uts1* (A-E), *pdyn* (F-J), *penka* (K-O) and *cck* (P-T). Lateral views are shown. (A-E) *sim1a* and *fezf2* morphants display a reduced *uts1* expression in the MA

(C,D, arrowheads, n=29/42, n=17/42), while embryos with reduced Otp and Foxb1.2 activity show an expanded MA *uts1* domain (B,E, arrowheads, n=21/21, n=58/76). (F-J) While the loss of Sim1a and Fezf2 function results in a severe reduction of *pdyn* expression (H,I, arrowheads, n=23/23, n=26/26), loss of Otp and Foxb1.2 function leads to an expanded *pdyn* expression domain in the MA (G,J, arrowheads, n=5/5, n=16/21). (K-O) The expression of *penka* in the PO (arrows) is reduced upon loss of Otp (L, n=5/5), Sim1a (M, n=10/21) and Fezf2 (N, n=11/17) function, while in the MA (arrowheads) the *penka* expression appears to be diminished in loss of Otp function embryos but not changed in *sim1a* and *fezf2* morphants. The loss of Foxb1.2 had no apparent effect on the *penka* expression (O, n=22). (P-T) While the loss of Otp (Q, n=6), Sim1a (R, n=25) and Foxb1.2 (T, n=23) function results in no change of *cck* expression in the MA (arrowheads), the loss of Fezf2 function reduced *cck* expression in the MA (S, arrowheads, n=20/25). The scale bar shows 200 µm.

The previous results demonstrate, that the activity of Otp, Sim1a and Fezf2 was necessary for the formation of *oxtl*-, *avpl*-, *vip*- and *uts1*-expressing neurons (Fig. 3.18K-Y; Fig. 3.19A-E). To further investigate how the loss of Otp, Sim1a, Fezf2 and Foxb1.2 function affects the specification of these neuron types I analyzed their cell number in the PO (Fig. 3.20A-C) and in the MA (Fig. 3.21A,B) at 2 dpf and 3 dpf using Wilcoxon Signed rank test.

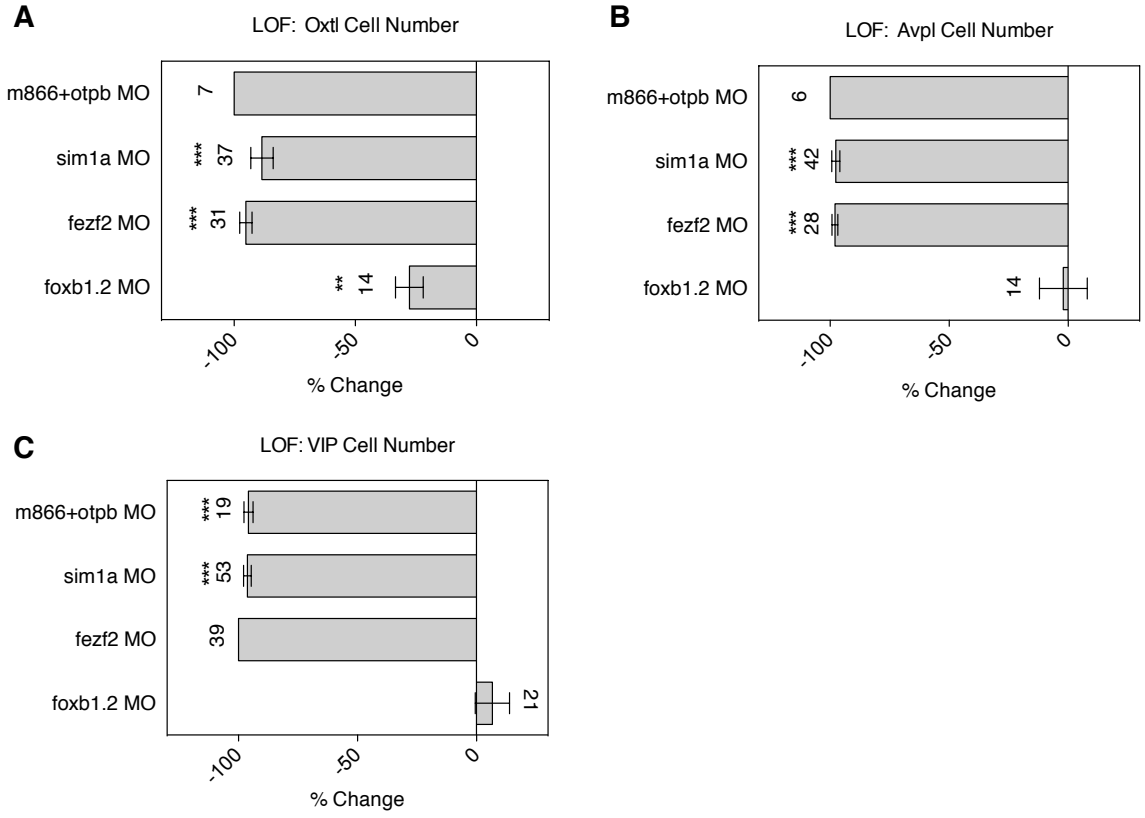

Fig. 3.20: Otp, Sim1a and Fezf2 activity is necessary for the formation of *oxtl*, *avpl* and *vip* expressing neurons in the PO

(A-C) Chart bars indicate the percent change in the number of (A) Oxtl-, (B) Avpl- and (C) VIP-positive neurons in *otpa*^{m866} mutant embryos injected with 4 ng *otpb* morpholino, wild-type embryos injected with 1 ng *sim1a* MO, 4ng *fezf2* MO and 8 ng *foxb1.2* MO compared to uninjected wild-type embryos. The number of *oxtl*-, *avpl*- and *vip*-expressing cells was compared using the Wilcoxon Signed rank test, which uses the medians of wild-type embryos as a reference. At 2 dpf the number of *oxtl*-expressing cells in wild-type embryos has median and mean (± s.e.m.) values of 16 and 15.5 (± 0.37), respectively. (A) The loss of Otp, Sim1a, and Fezf2 function leads to a reduction in *oxtl*-expressing cells (no P value, complete loss; P<0.001; P<0.001), whereas *foxb1.2* morphants show a slight decrease compared to the wild-type embryos (P=0.0032). The number of *avpl*-expressing cells in wild-type embryos has median and mean (± s.e.m.) values of 7 and 6.5 (± 0.44), respectively. (B) Embryos lacking Otp, Sim1a and Fezf2 activity have a decreased number of *avpl*-expressing cells (no P value, complete loss; P<0.001; P<0.001). The loss of Foxb1.2 leads to no changes in Avpl cell number (P=0.8582). The number of *vip*-expressing cells in the PO of wild-type embryos has median and mean (± s.e.m.) values of 5 and 4.7 (± 0.25), respectively. (C) The loss of Otp, Sim1a and Fezf2 function results in the reduction of *vip*-expressing cells (P<0.001; P<0.001; no P value, 100 % loss), whereas the reduction of Foxb1.2 activity results in no changes of VIP cell number (P=0.3973). Error bars indicate standard error of the means. The number of embryos analyzed is shown in the graph. Abbreviations used: loss-of-function (LOF).

In the PO, Oxtl cell number is highly reduced in embryos lacking Otp, Sim1a and Fezf2 function (no P value because loss of OTP function results 100 % loss of *oxtl* cells; P<0.001; P<0.001)(Fig. 3.20A). In contrast the loss of Foxb1.2 function resulted in slight changes in Oxtl cell number (P=0.0032) that could result from the delayed development that the embryos injected with 8 ng of MO display. The embryos with reduced Otp function showed a lack of *avpl*-expressing neurons (Fig. 3.20B), whereas the loss of Sim1a and Fezf2 function resulted in a severe reduction in Avpl cell number (P<0.001; P<0.001) and no changes were observed in embryos with reduced Foxb1.2 activity (P=0.8582). Similar to Avpl cell number, *vip*-expressing neurons in the PO are highly reduced in loss of Otp and Sim1a function (P<0.001; P<0.001)(Fig. 3.20C). The reduction of Fezf2 activity resulted in the the loss of all *vip*-expressing neurons, whereas *foxb1.2* morphants show no changes in VIP cell number (P=0.3973).

In the MA, Embryos with reduced Otp, Sim1a and Fezf2 activity showed a variable reduction of *vip* expressing cells in the MA (Fig. 3.18K-N). To further assess this finding, I analyzed the number of *vip*-expressing cells in wild-type embryos and embryos with reduced Otp, Sim1a, Fezf2 and Foxb1.2 activity in the MA (Fig. 3.21A). The loss of Otp, Sim1a and Fezf2 function resulted in a decreased number of *vip*-expressing cells in the MA compared to wild-type embryos (Wilcoxon Signed rank test, P<0.001; P<0.001; P<0.001), whereas the reduction of Foxb1.2 activity had no effect in *vip*-expressing cells (P=0.7423). In contrast, the number of *uts1*-expressing neurons is increased in loss of Otp and Foxb1.2 function embryos (P<0.001; P<0.001) (Fig. 3.21B) and decreased in

sim1a and *fezf2* morphants (P<0.001; P<0.001).

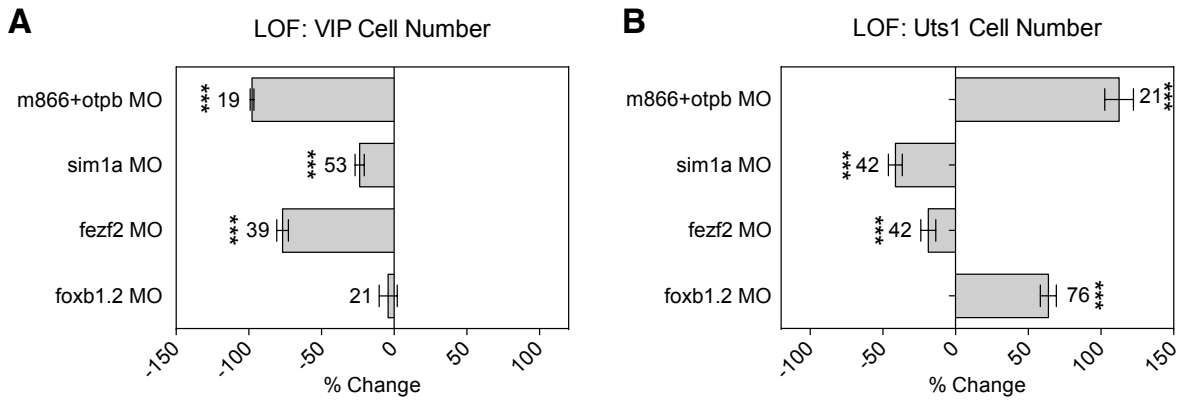

Fig. 3.21: Otp, Sim1a, Fezf2 and Foxb1.2 activity is necessary for the formation of *vip* and *uts1* expressing neurons in the MA

(A,B) Chart bars indicate the percent change in the number of (A) VIP- and (B) Uts1-positive neurons in loss-of-function embryos compared to uninjected wild-type embryos. The number of *vip*- and *uts1*-expressing cells was compared using the medians of wild-type embryos as a reference (Wilcoxon Signed rank test). At 2 dpf the number of *vip*-expressing cells in wild-type embryos has median and mean (± s.e.m.) values of 14 and 14.6 (± 0.36), respectively. (A) The loss of Otp, Sim1a, and Fezf2 function leads to a reduction in VIP cell number in the MA compared to the wild-type embryos (P<0.001; P<0.001; P<0.001), whereas the loss of Foxb1.2 shows no difference compared to the wild-type (P=0.7423). The number of *uts1*-expressing cells in wild-type embryos has median and mean (± s.e.m.) values of 8 and 8.5 (± 0.22), respectively. (B) Embryos lacking Otp and Foxb1.2 activity have an increased number of Uts1-positive cells (P<0.001; P<0.001), whereas the loss of Sim1a and Fezf2 leads to a reduction (P<0.001; P<0.001). Error bars indicate standard error of the means. The number of embryos analyzed is shown in the graph. Abbreviations used: loss-of-function (LOF).

In summary, these analyses of the cell numbers of distinct neuron types in loss-of-function embryos shows the necessity of Otp, Sim1a and Fezf2 in the PO and of Otp, Sim1a, Fezf2 and Foxb1.2 in the MA for the specification of neuronal subtypes. Interestingly, the regulation of the specification of *vip*-expressing neurons in the PO compared to the MA is different. To investigate the regulatory action of the transcription factors on neuron type specification further and to detect which of the regulators are sufficient to induce a distinct neuron type I performed a co-expression and a gain-of-function analysis.

3.2.3 Different MA and PO domains give rise to distinct neuronal subtypes

The observed changes in the expression of the neuropeptides upon loss of Fezf2, Foxb1.2, Otp and Sim1a function reveal the necessity of these transcription factors for the specification of the neuronal subtypes. However, to what extent the variable interactions among these transcription factors and the resulting subdomains within the PO and the MA play a role in the specification of distinct neuron types remains unknown. To answer this question, I first performed co-expression analyses using two color FISH with probes of selected neuropeptides in combination with *fezf2*, *foxb1.2*, *otp* or *sim1a*.

In the MA *penka* and *cck* were expressed lateral to the *otpa* expression domain, which marks the peripheral MA (Fig. 3.22A-B"). Hence, these neuropeptide are expressed outside of the so far described MA and its regulatory network that they are therefore excluded from following experiments. The regulatory action of Fezf2 on the expression of both neuropeptides can be explained by the early regulatory interactions of Fezf2 in the posterior hypothalamus at 1 dpf.

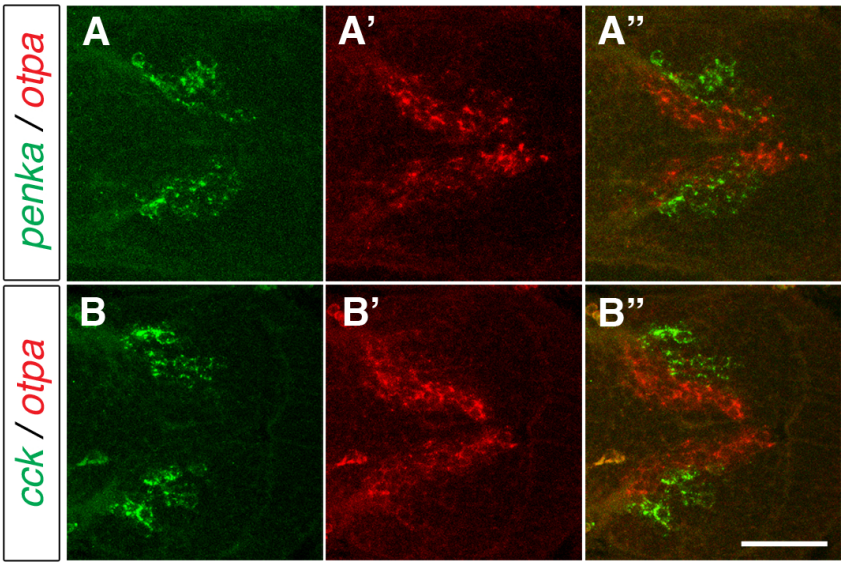

Fig. 3.22: *penka* and *cck* are expressed lateral to the peripheral MA
(A-B") 2 dpf wild-type embryos subjected to two color FISH to reveal the spatial relationship of *otpa* (A',B') and both *penka* (A) and *cck* (B) in the MA. The maximum projections of 10-15 µm are shown in the dorsal view. (A-B") *penka* (A-A") and *cck* (B-B") are expressed lateral to the *otpa* expression domain. The scale bar represents 60 µm.

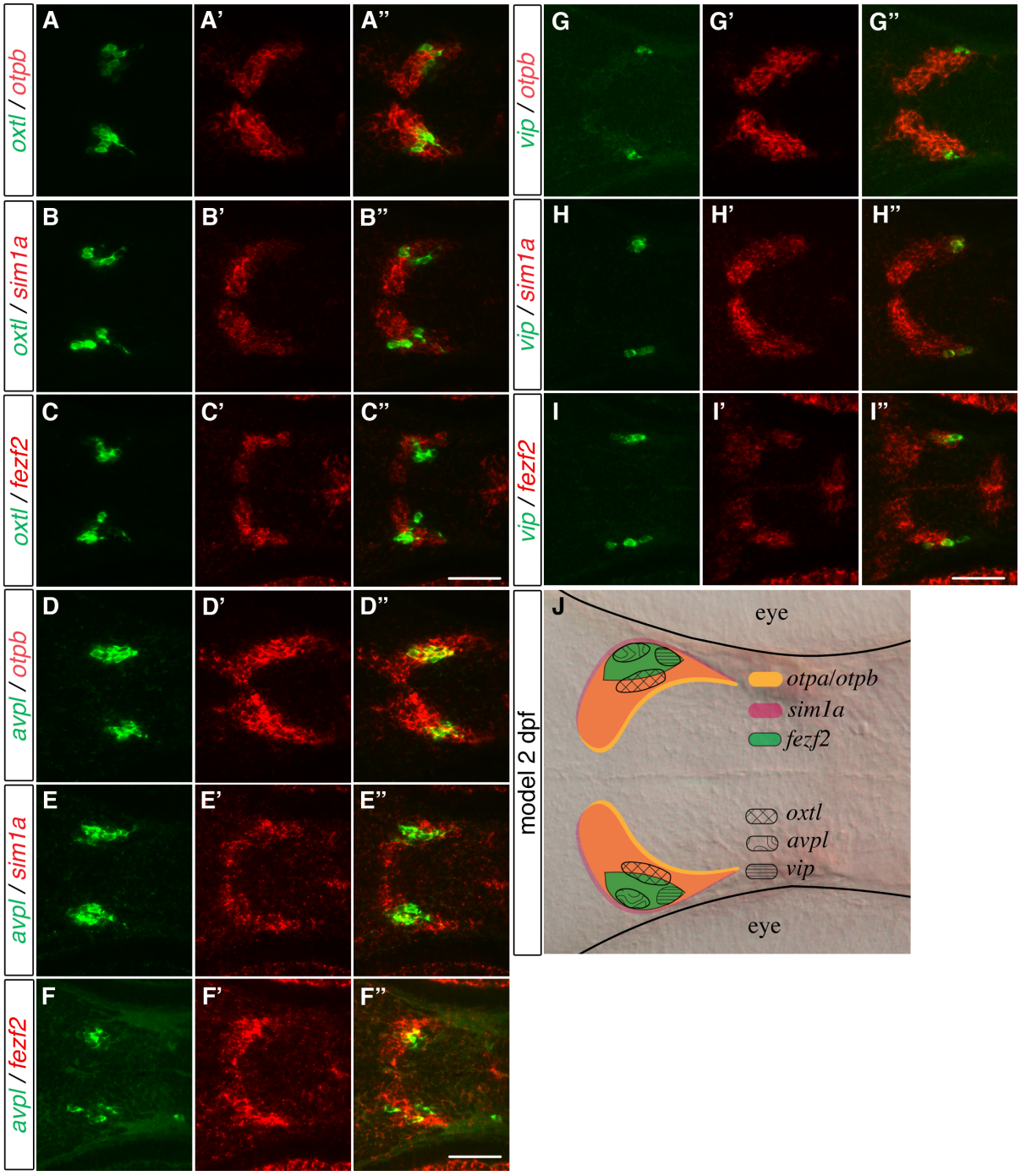

Fig. 3.23: The co-expression of transcription factors with *oxtl*, *avpl* and *vip* displays regulatory actions

(A-I) Two-color FISH with probes for *otpb* (A',D',G'), *sim1a* (B',E',H'), *fezf2* (C',F',I'), *oxtl* (A-C"), *avpl* (D-F") and *vip* (G-I") in 2 dpf wild-type embryos to reveal their spatial relationship. Maximum projections of 10 µm in the dorsal view are shown. (A-B") The expression of *oxtl* is contained within the *otpb* (A-A") and *sim1a* (B-B") expression domains. (C-C") *fezf2* and *oxtl* are mostly not co-expressed. (D-E") Also the preoptic *avpl* expression is contained within the *otpb* (D-D") and *sim1a* (E-E") expression domain and (F-F") *avpl* is mostly co-expressed with *fezf2*. (G-I") *vip* is co-expressed with *otpb* (G-G"), *sim1a* (H-H") and mostly with *fezf2* (I-I"). (J) A model summarizing the spatial relationship among the transcription factors and *oxtl*, *avpl* and *vip*. The scale bar shows 60 µm.

The co-expression analysis in the PO was performed using probes of *oxtl*, *avpl* and *vip* in combination with probes of the transcription factors *otpb* (as representative of the two *otp* paralogs), *sim1a* and *fezf2*. As expected from the loss-of-function analysis, *oxtl* was co-expressed with *otpb* (Fig. 3.23A-A") and *sim1a* (Fig. 3.23B-B"), consistent with the fact that the loss of both Otpa and Sim1a activity led to a loss of *oxtl*-expressing cells (Fig. 3.18P-R). Only a few *oxtl*-expressing cells were contained within the *fezf2* expression domain (Fig. 3.23C-C"), although the loss of Fezf2 function resulted in an absent *oxtl* domain (Fig. 3.18P,S). This suggests that Fezf2 regulates *oxtl* expression by its effect on *otp* expression. Alternatively, Fezf2 might be present in *oxtl* progenitor cells.

avpl expression was contained within the *otpb* and *sim1a* expression domain (Fig. 3.23D-E"), consistent with the findings that the loss of Otp and Sim1a function resulted in an absent preoptic *avpl* domain (Fig. 3.18U-W). The loss of Fezf2 function resulted in a highly reduced preoptic *avpl* expression (Fig. 3.18U,X), which suggested co-expression. Indeed, most of the *avpl*-positive neurons were *fezf2*-positive (Fig. 3.23F-F"). Since *vip* expression in the PO is absent in embryos with reduced Otp, Sim1a and Fezf2 activity (Fig. 3.18K-N), I expected that *vip* is contained within all three expression domains. In fact, *vip* was co-expressed with *otpb* (Fig. 3.23G-G"), *sim1a* (Fig. 3.23H-H") and mainly co-expressed with *fezf2* (Fig. 3.23I-I"). The model in figure 3.23J summarizes the results of the co-expression analysis of the PO. Consistent with the fact that *foxb1.2* is not expressed in the PO throughout embryonic development (Fig. 3.1E and Fig. 3.2) a loss of Foxb1.2 function resulted in no apparent change of *oxtl*, *avpl* and *vip* expression at 2 dpf (Fig. 3.18K,O,P,T,U,Y).

In the MA the regulatory interactions of *fezf2*, *foxb1.2*, *otp* and *sim1a* formed distinct subregions within the MA. To test whether these subregions and the defined combination of transcription factors expressed there give rise to distinct neuronal subtypes, I performed a co-expression analysis. The spatial relationship of *vip* and *uts1* with *otpb*, *sim1a*, *fezf2* and *foxb1.2* was investigated. Since the loss of Otp function resulted in a complete loss of *vip* expression in the MA (Fig. 3.18K,L) I hypothesized that *vip* is expressed in the *otp* expression domain and is therefore situated in the peripheral MA. Indeed, *vip* was contained within the *otpb* expression domain at 2 dpf (Fig. 3.24A-A") as well as within the *sim1a* expression domain (Fig. 3.24B-B"). This finding is also consistent with the fact that embryos with reduced Sim1a activity show a slightly reduced *vip* expression in the MA (Fig. 3.18K,M). Although *vip* is not co-expressed with *fezf2* (Fig. 3.24C-C") the loss of Fezf2 function resulted in a severe reduction of *vip*-expressing cells in the MA (Fig. 3.18K,N). This finding might be explained by the earlier positive regulation of Fezf2 on the *otp* expression in the MA, where *fezf2* morphants display a severe reduction of *otp* expression at 1 dpf and at 2 dpf (Fig. 3.6A,B,D,E and Fig. 3.8A,B,D,E). The loss of Foxb1.2 activity resulted

in no changes of *vip* expression in the MA (Fig. 3.18K,T) which is consistent with the finding that *vip* expression was found outside of the *foxb1.2* expression domain (Fig. 3.24D-D"). In the case of *uts1*, loss of Sim1a and Fezf2 function embryos displayed a reduced number of *uts1*-expressing cells in the MA (Fig. 3.19A,C,D), which suggests that *uts1* is expressed in a *sim1a*- and *fezf2*-positive domain. Indeed, the co-expression analysis at 3 dpf revealed that *uts1* is contained within the medially located *sim1a* (Fig. 3.24F-F") and *fezf2* expression domain (Fig. 3.24G-G").

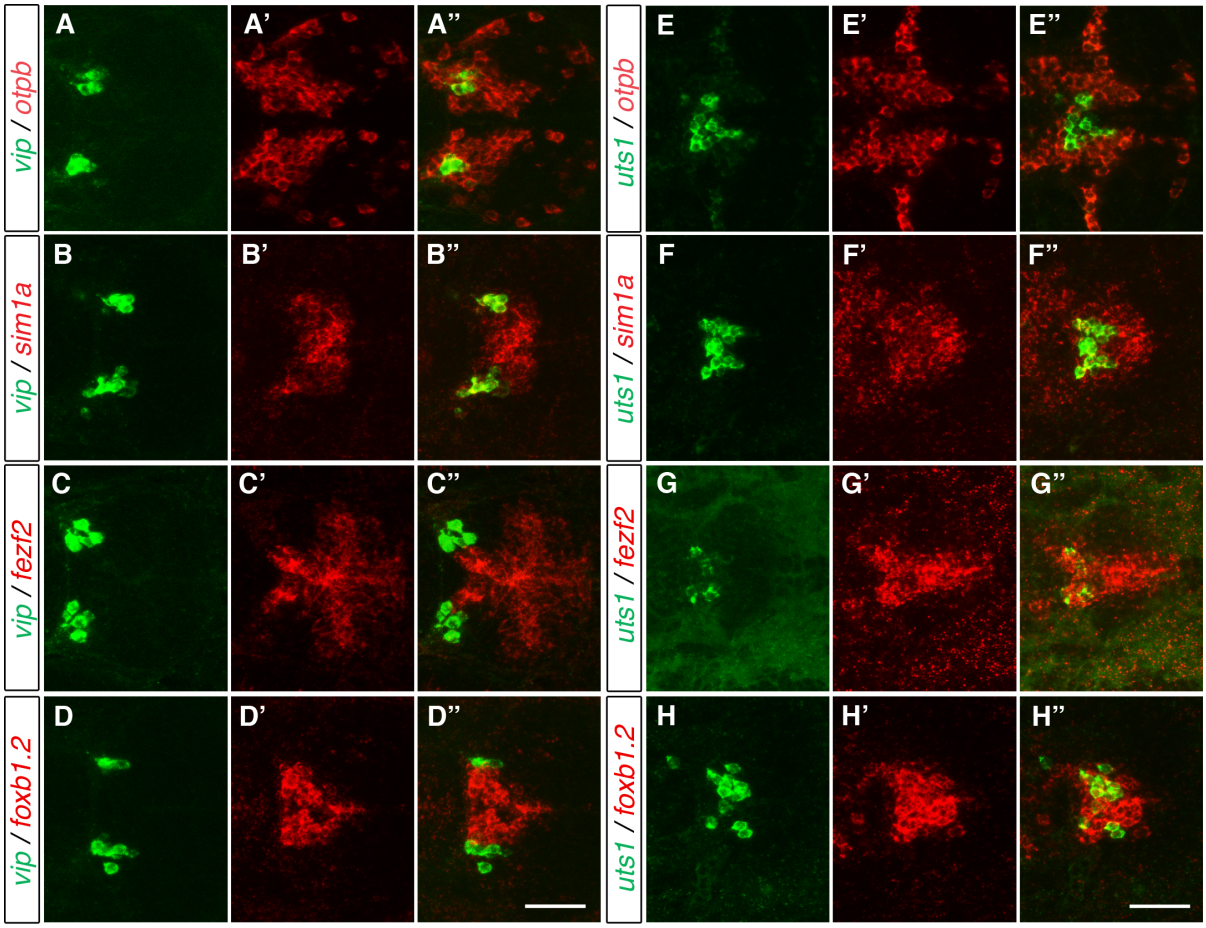

Fig. 3.24: VIP- and Uts1 positive neurons develop within different MA domains

(A-H") Two-color FISH of 2 dpf (A-D") and 3 dpf wild-type embryos (E-H") to detect the relative expression of *vip* (A-D") and *uts1* (E-H") in comparison to the expression of *otpb* (A',E'), *sim1a* (B',F'), *fezf2* (C',G') and *foxb1.2* (D',H'). (A-D") *vip* is contained within the *otpb* (A-A") and the *sim1a* (B-B") expression, while it is not co-expressed with *fezf2* (C-C") and *foxb1.2* (D-D"). (E-H") In contrast, *uts1* is not co-expressed with *otpb* (E-E"), but is co-expressed with *sim1a* (F-F"), *fezf2* (G-G") and *foxb1.2* (H-H"). 10-15 µm maximum projections in the dorsal view are shown. Scale bars represent 60 µm.

In addition, *otpb* is expressed adjacent to the *uts1* expressing cells with nearly no overlap, showing that *uts1* is expressed in the central MA that is surrounded by the lateral *otpb* expression (Fig. 3.24E-E"). This finding together with the loss-of-function analysis illustrates clearly the regulatory interactions between *otp* and *fezf2* at 2 dpf, where the loss of Otp function resulted in an expanded *fezf2* expression domain (Fig. 3.1C,D), that contains *uts1*-expressing cells, and results in more *uts1* cells (Fig. 3.19A,B). Although the co-expression analysis revealed *uts1* expressing cells to be expressed in the central MA and to be *foxb1.2*-positive (Fig. 3.24H-H"), the loss of Foxb1.2 function resulted in an expanded *uts1* expression domain in the MA. This result can be explained by the genetic regulatory interaction of *foxb1.2* and *fezf2*. The loss of Foxb1.2 function resulted in an expanded *fezf2* expression domain in the MA (Fig. 3.9C,D). Since Fezf2 activity is important for the specification of *uts1*-expressing cells in the MA (Fig. 3.19A,D), an expanded *fezf2* expression domain could lead to more *uts1* cells.

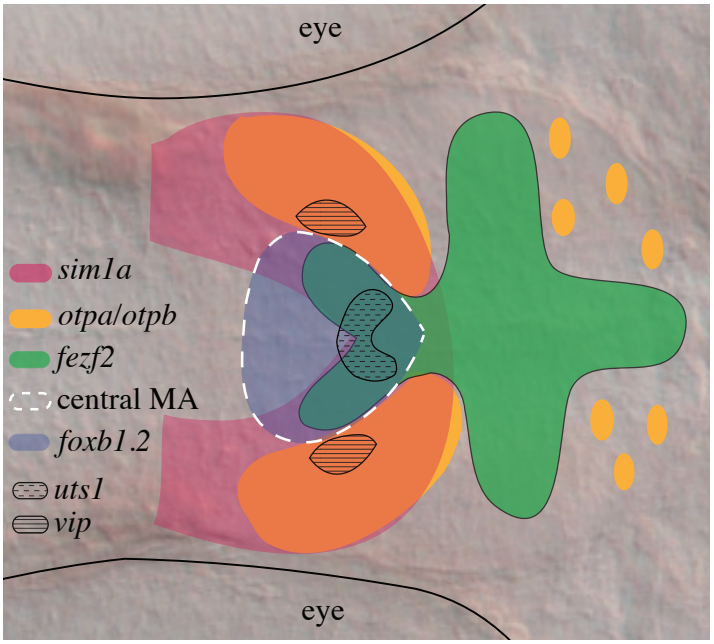

Fig. 3.25: *vip* and *uts1* are expressed in subregions of the MA
A model illustrating the co-expression of *fezf2*, *foxb1.2*, *otp*, and *sim1a* with *vip* and *uts1* in different subregions of the MA. The white dashed line demarcates the central MA.

In summary, VIP and Uts1-positive cells develop in different subregions of the MA, which reflect the requirement of the distinct transcription factors for the specification of these neuropeptide producing cells. VIP cells are situated in the peripheral MA and are *otp*+ and *sim1*+. Uts1 cells can be found in the central MA and are *sim1a*+, *fezf2*+ and *foxb1.2*+, although Foxb1.2 is not necessary for the specification of *uts1* expressing cells (Fig. 3.25).

3.2.4 Transcription factor requirement for the specification of Oxtl, Avpl, VIP and Uts1 neurons

3.2.4.1 Establishment of inducible transgenic lines over-expressing *fezf2*, *foxb1.2*, *otpa*, *otpb* or *sim1a* cDNA

To test which of the transcription factors might be sufficient to induce the fate of a neuron type I generated transgenic lines over-expressing the cDNA of *fezf2*, *foxb1.2*, *otpa*, *otpb* and *sim1a* fused with either GFP or tdTomato fluorophores via the viral 2A peptide (Tang et al., 2009) to allow easy identification of embryos carrying the transgenes. These transgene cassettes are under the control of an inducible basal heat shock promoter (Halloran et al., 2000). To test whether the transgenic lines do over-express the distinct cDNAs I performed WISH of the lines with strongest fluorophore over-expression after over night (ON) heat shock treatment. All of the analyzed transgenic lines showed a strong over-expression of the cDNAs contained within the transgenic construct (Fig. 3.26A-J).

3.2.4.2 Otp, Sim1a, Fezf2 or Foxb1.2 are sufficient to induce distinct neuronal subtypes

To test whether the transcription factors expressed in Oxtl, Avpl, VIP and Uts1 neurons are sufficient to promote their fates I performed temporally controlled gain-of-function (GOF) analyses. The ON heat shock was carried out either at 34 hpf for Oxtl, Avpl and VIP or at 2,5 dpf for Uts1 in order not to interfere with early patterning of the hypothalamus. Neurons expressing *oxtl* express *otpa*, *otpb* and *sim1a* while they do mostly not express *fezf2* (Fig. 3.23A-C"). Therefore, I hypothesized that the over-expression of Otp and Sim1a might be sufficient to induce supernumerary *oxtl*-expressing cells. Indeed, I found more *oxtl*-expressing cells in embryos over-expressing *otpa* ($P<0.001$, Wilcoxon Signed rank test) and *sim1a* ($P<0.001$) while over-expression of *fezf2* ($P=0.6926$) and *otpb* ($P=0.2105$) had no effect on *oxtl* cell number when compared to wild-type embryos that were heat-shocked (Fig. 3.27A). Similar to *oxtl*-expressing cells, *avpl*-expressing cells are present within the *otp* and *sim1a* expression domains. In addition, *avpl* cells also mostly co-express *fezf2* (Fig. 3.23D-F"). The analysis of the *avpl* cells revealed that only the over-expression of *sim1a* ($P<0.001$, Wilcoxon Signed rank test) and a combination of *otpa* and *fezf2* ($P<0.001$) was sufficient to induce supernumerary *avpl* cells compared to heat-shocked wild-type embryos (Fig. 3.27B). In contrast, neither the over-expression of *otpa* ($P=0.3428$), *otpb* ($P=0.1293$), *fezf2* ($P=0.6253$) nor other transcription factor combinations were sufficient. Similar to *avpl*-expressing cells, *vip* in the PO is co-expressed with *otp*, *sim1a* and *fezf2* (Fig. 3.23G-I"), which suggests that

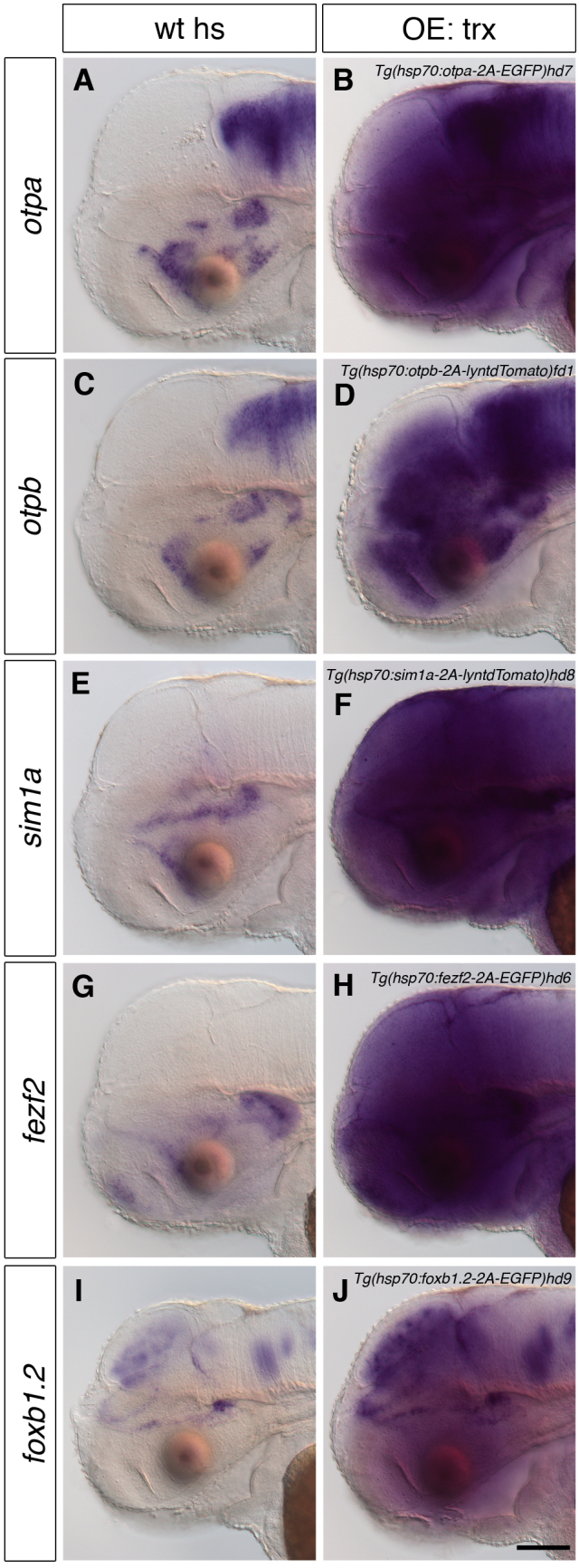

Fig. 3.26: Heat shock induces over-expression of the transgenes

(A-J) After an overnight heat shock wild-type embryos (A,C,E,G,I) and embryos containing (B) *Tg(hsp70:otpa-2A-EGFP)hd7*, (C) *Tg(hsp70:otpb-2A-lyntdTomato)fd1*, (F) *Tg(hsp70:sim1a-2A-lyntdTomato)hd8*, (H) *Tg(hsp70:fezf2-2A-EGFP)hd6* and (J) *Tg(hsp70:foxb1.2-2A-EGFP)hd9* have been subjected to WISH to detect the over-expression of *otpa* (A,B), *otpb* (C,D), *sim1a* (E,F), *fezf2* (G, H) or *foxb1.2* (I,J). Transgenic lines expressing *otpa* (A, B, n=24/24), *otpb* (C,D, n=21/29), *sim1a* (E,F, n=29/29), *fezf2* (G,H, n=37/37) or *foxb1.2* (I,J, n=34/34) showed an over-expression of the specific transgenes. Lateral views are shown. The scale bar is 200 µm.

the over-expression of these three transcription factors individually or in combination might be sufficient to induce supernumerary *vip* cells. Interestingly, while the over-expression of *otpa* (P=0.0331, Wilcoxon Signed rank test) and *fezf2* (P=0.0016) resulted in fewer preoptic *vip* cells the over-expression of *otpb* led to ectopic *vip* expression throughout the brain (Fig. 3.29A,B), only the over-expression of *sim1a* resulted in a higher *vip* cell number compared to heat-shocked wild-type embryos (P=0.0116). Different combinations of the transcription factors had no effect on *vip* cell number (Fig. 3.27C).

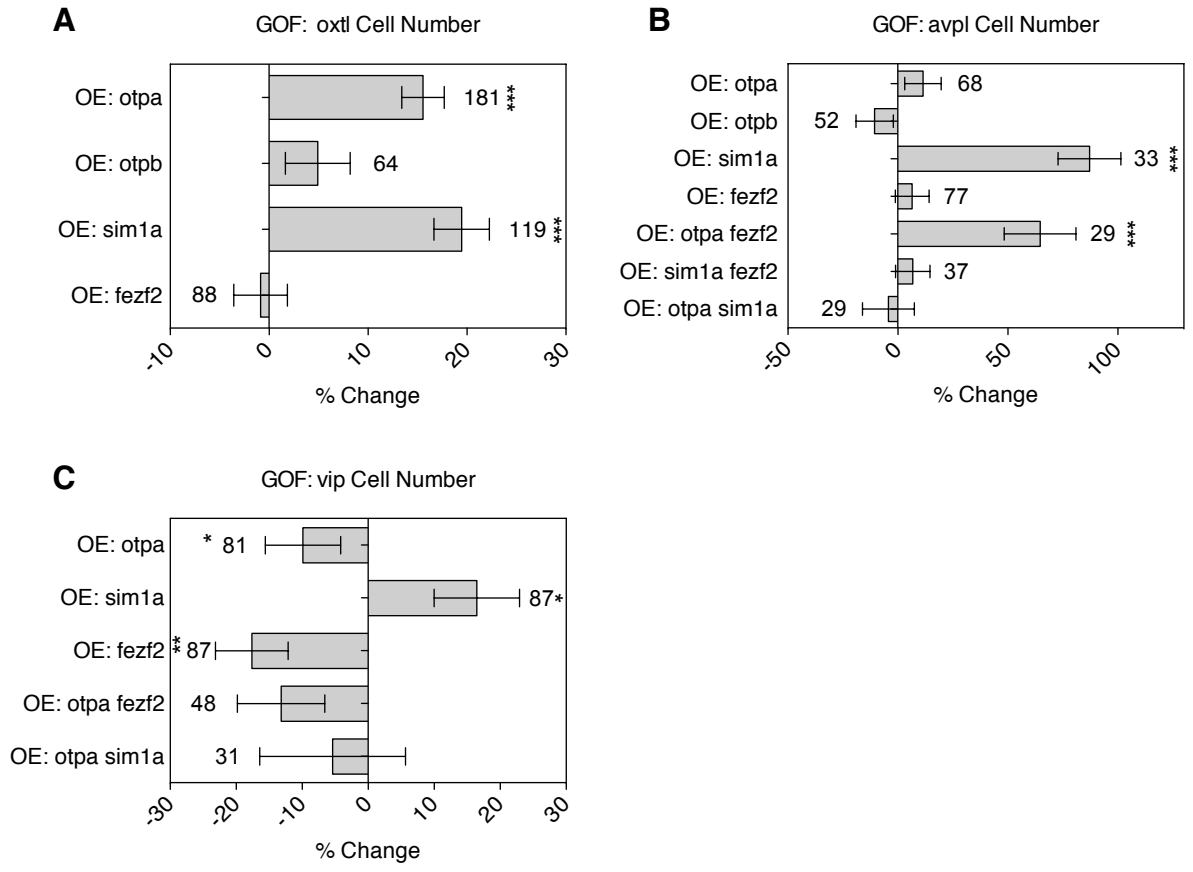

Fig. 3.27: Otp and Sim1a are sufficient to induce supernumerary *oxtl*, *avpl* and *vip* cells in the PO.

(A-C) Chart bars indicate the percent change in the number of Oxtl- (A) and Avpl- (B) and VIP-positive (C) neurons in transgenic embryos over-expressing cDNAs for *otpa*, *sim1a* or *fezf2* in comparison to wild-type embryos. Cell number changes were compared to the theoretical median cell number of wild-type embryos and analyzed using Wilcoxon Signed rank test. Heat-shocked wild-type embryos have median and mean (± s.e.m.) values of 13 and 13.2 (± 0.18) Oxtl-positive cells, respectively. (A) Over-expression of *otpa* (P<0.001) and *sim1a* (P<0.001) results in a higher *oxtl* cell number whereas over-expression of *otpb* (P=0.2105) and *fezf2* (P=0.6926) leads to no changes in *oxtl* cell number. Wild-type embryos subjected to ON heat shock displayed median and mean (± s.e.m.) values of 4 and 4.1 (± 0.12) Avpl-positive cells, respectively. (B) Over-expression of *sim1a* (P<0.001) and combined over-expression of *otpa* and *fezf2* (P<0.001) results in a higher number of *avpl*-expressing cells while the over-expression of *otpa* (P=0.3428), *otpb* (P=0.1293), *fezf2* (P=0.6352), *sim1a* plus *fezf2* (P=0.4083) and *otpa* plus *sim1a* (P=0.5287) has no effect. Heat-shocked wild-type embryos showed median and mean (± s.e.m.) values of 3 and 3.1 (± 0.11) VIP-positive cells, respectively. (C) While only the over-expression of *sim1a* (P=0.0116) leads to supernumerary *vip* cells the over-expression of *otpa* (P=0.0331) and *fezf2* (P=0.0016) results in a reduction of *vip* cells. The combination of *otpa* plus *fezf2* (P=0.0607) and *otpa* plus *sim1a* (P=0.5024) has no effect on *vip* cell number. Error bars indicate standard error of the means. The number of embryos analyzed is shown in the graph.

In summary, the co-expression and loss- and gain-of-function experiments demonstrate that Oxtl-positive neuron specification requires Otp and Sim1a, Avpl-positive neurons require Otp, Sim1a and Fezf2 and the preoptic VIP cells require Sim1a activity. In addition, Otp and Fezf2 function seems to be crucial demonstrated by the strong reduction of VIP-positive cells in loss of Otp and Fezf2 function embryos.

In contrast to the PO, in the MA *vip* is co-expressed with *otp* and *sim1a* but not with *fezf2* and *foxb1.2* (Fig. 3.24A-D"). Hence, I hypothesized that the over-expression of *otpa*, *sim1a* and/or a combination of both may lead to supernumerary VIP-positive cells. Indeed, I found more VIP-positive cells in embryos over-expressing *otpa* (P<0.001, Wilcoxon Signed rank test), *sim1a* (P<0.001) or a combination of both (P<0.001) compared to wild-type embryos that were heat-shocked (Fig. 3.28A). Notably, the over-expression of *fezf2* (P=0.2170) or *foxb1.2* did not lead to an increase in the number of VIP-positive cells consistent with the fact that *vip* cells are not co-expressed with *fezf2* and *foxb1.2* at this stage. Moreover, the over-expression of *foxb1.2* led to a reduction in *vip* cell number (P<0.001). Since *uts1* was co-expressed with *sim1a*, *fezf2*, and *foxb1.2* in the central MA (Fig. 3.24F-H"), I tested the effect of over-expression of these factors on the number of *uts1*-expressing cells. Although the over-expression of *sim1a* (P=0.5645, Wilcoxon signed rank test) alone had no effect on the number of Uts1-positive cells, over-expression of *fezf2* (P=0.0137), *sim1a* plus *fezf2* (P=0.0099), and of *foxb1.2* (P<0.001) led to an increase in the number of *uts1*-expressing cells. Consistent with the fact that *uts1* is not co-expressed with *otp* the over-expression of

otpa did not change *uts1* cell number (P=0.4485) (Fig. 3.28B). The increase in the number of Uts1-positive cells was relatively small, which was likely due to the fact that I performed the heat shock induction at 2.5 dpf, when most of the neurons are already specified. However, the increase was nonetheless significant and consistent across multiple experiments. Thus taken together, the loss-of-function and over-expression analyses demonstrate that VIP-positive neuron specification requires Otp and Sim1a whereas Uts1-positive neurons require Fezf2, Sim1a and Foxb1.2 activity. In addition, although not expressed in VIP-positive neurons in later developmental stages, Fezf2 is crucial for their development, shown by the strong reduction of *vip* expressing cells in *fezf2* morphants.

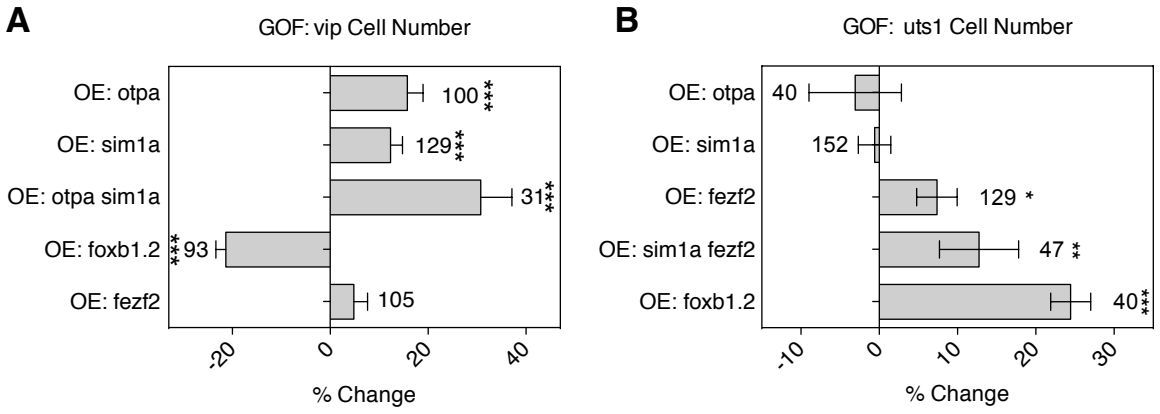

Fig. 3.28: Otpa and Sim1a are sufficient to induce VIP neurons and Fezf2 and Foxb1.2 are sufficient to induce Uts1 neurons

(A,B) Chart bars indicate the percent change in the number of VIP- (A) and Uts1 (B) -positive neurons in transgenic embryos over-expressing cDNAs for *otpa*, *sim1a*, *fezf2* or *foxb1.2* in comparison to wild-type embryos. Cell number changes were compared to the theoretical median cell number of wild-type embryos and analyzed using the Wilcoxon Signed rank test. Heat-shocked wild-type embryos have median and mean (± s.e.m.) values of 13 and 12.8 (± 0.17) VIP-positive cells, respectively. (A) Over-expression of *otpa* (P<0.001) and *sim1a* (P<0.001) leads to a higher number of VIP-positive cells. Combined over-expression of *otpa* and *sim1a* also leads to a higher number of VIP-positive cells (P<0.001) whereas over-expression of *foxb1.2* results in a lower number of VIP-positive cells (P<0.001). Over-expression of *fezf2* leads to no change in the number of VIP-positive cells (P=0.2170). The number of Uts1-positive cells in heat-shocked wild-type embryos has median and mean (± s.e.m.) values of 9 and 8.8 (± 0.19), respectively. (B) The over-expression of *fezf2*, a combination of *fezf2* and *sim1a*, and of *foxb1.2* leads to a higher number of Uts1-positive cells at 3 dpf in the MA compared to the wild-type (P<0.05; P<0.01; P<0.001) whereas over-expression of *sim1a* showed no changes in the number of Uts1-positive cells (P=0.5645). Error bars indicate standard error of the means. The number of embryos analyzed is shown in the graph.

3.2.4.3 Otpb is sufficient to induce ectopic *vip* expression

The over-expression of *otpb* resulted in ectopic expression of *vip* broadly in the brain at 34 hpf before the onset of endogenous *vip* expression and also at 48 hpf (Fig. 3.29A-D, n=27/27, n=112/112). Interestingly, in addition embryos over-expressing *sim1a* displayed an ectopic *vip* expression domain at 34 hpf before endogenous *vip* starts to be expressed (Fig. 3.29E,F, n=25/25) and also at 48 hpf (data not shown) in the hindbrain.

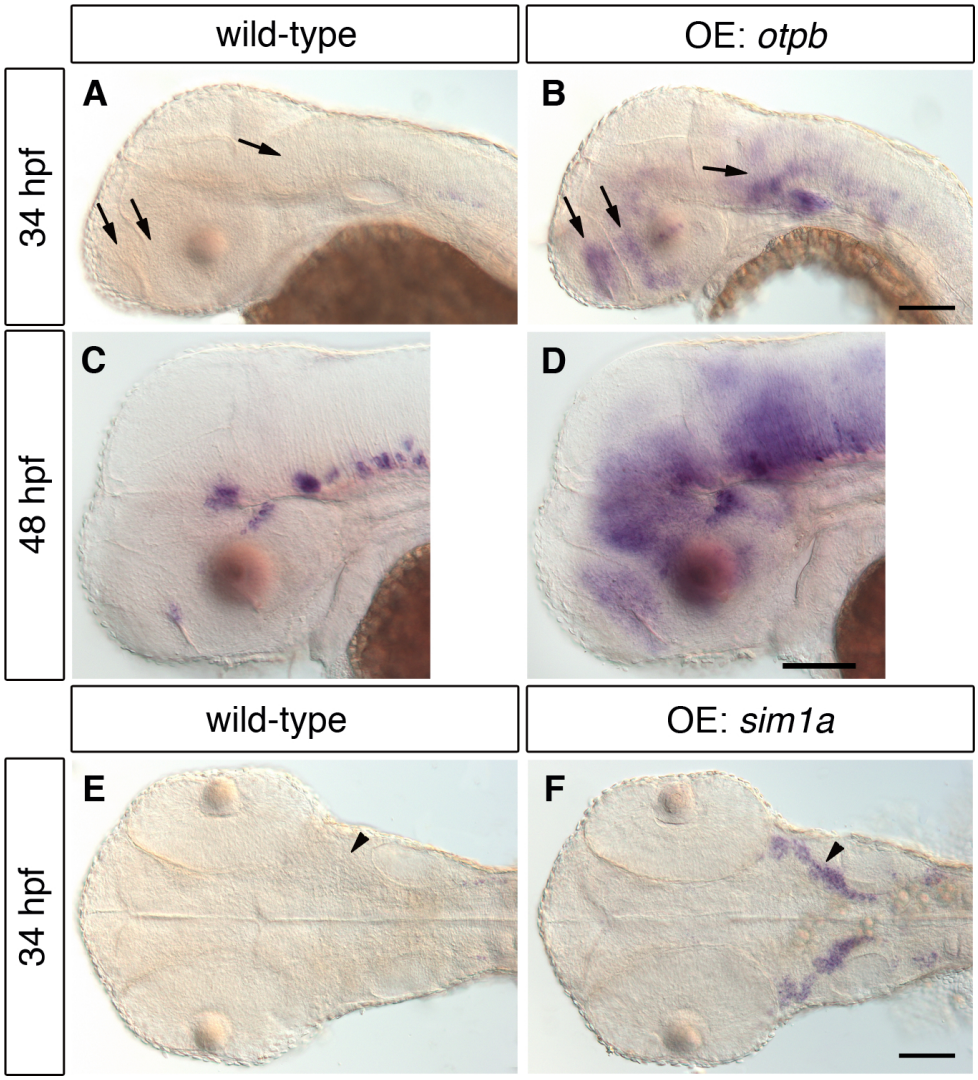

Fig. 3.29: Over-expression of *otpb* is sufficient to induce ectopic *vip* expression
(A-F) WISH to detect the expression of *vip* in heat-shocked wild-type embryos (A,C,E), in heat-shocked *Tg(hs:otpb-2A-tdTomato)fd1* embryos (B,D) and in heat-shocked *Tg(hs:sim1a-2A-tdTomato)hd8* embryos at 34 hpf (A,B,E,F) and at 2 dpf (C,D). (A-D) Ectopic *vip* expression can be induced by *otpb* over-expression at 34 hpf (n=27/27) and at 2 dpf (n=112/112). (E,F) Over-expression of *sim1a* induces an extra *vip* expression domain in the hindbrain (n=25/25). The Scale bar represents 200 µm.

Embryos over-expressing *sim1a* displayed a higher degree of over-expression of the transgene in a domain in the hindbrain (Fig. 3.30A,B, arrow, n=29/29), this domain also showed ectopic expression of *otpb* (Fig. 3.30C,D, arrow, n=20/20). This suggests that the ectopic *otpb* expression in turn led to ectopic expression of *vip* in this hindbrain domain at 2 dpf (data not shown, n=129/129) and at 3 dpf (Fig. 3.30E,F, arrow, n=70/70). Thus, a high over-expression of *sim1a* induces ectopic expression of *otpb*, which in turn induces ectopic expression of *vip*.

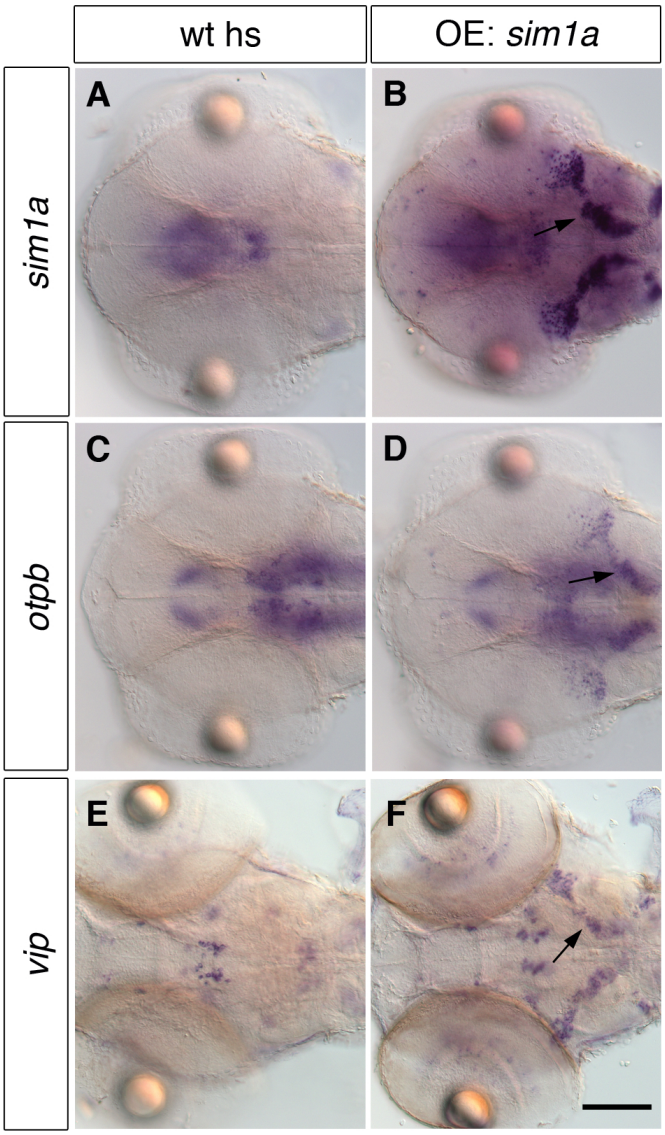

Fig. 3.30: High-level over-expression of *sim1a* induces ectopic *otpb* expression that subsequently results in ectopic *vip* expression

(A-F) The expression of *sim1a* (A,B), *otpb* (C,D) and *vip* (E,F) was detected after ON heats shock in wild-type embryos (A,C,E) and embryos containing *Tg(hsp70:sim1a-2A-lyntdTomato)hd8* (B,D,F) at 2 dpf (A-D) and 3 dpf (E,F) by using WISH. (A,B) Short staining of embryos over-expressing *sim1a* revealed a

high-level over-expression domain of *sim1a* in the hindbrain (arrow, n=29/29). (C,D) Over-expression of *sim1a* results in ectopic expression of *otpb* in the hindbrain (arrow, n=20/20). (E,F) A comparable hindbrain domain shows ectopic *vip* expression upon *sim1a* over-expression (n=70/70). Dorsal views are shown. The scale bar represents 200 µm.

To determine if the over-expression of the transcription factors results in the upregulation or downregulation of other transcription factors as well, I performed a gain-of-function analysis. I found that none of the over-expressed transcription factor was sufficient to change the expression of another one (Fig. 3.31A-B2) except of *sim1a*, which showed ectopic *otpb* expression in the hindbrain (Fig. 3.30C,D).

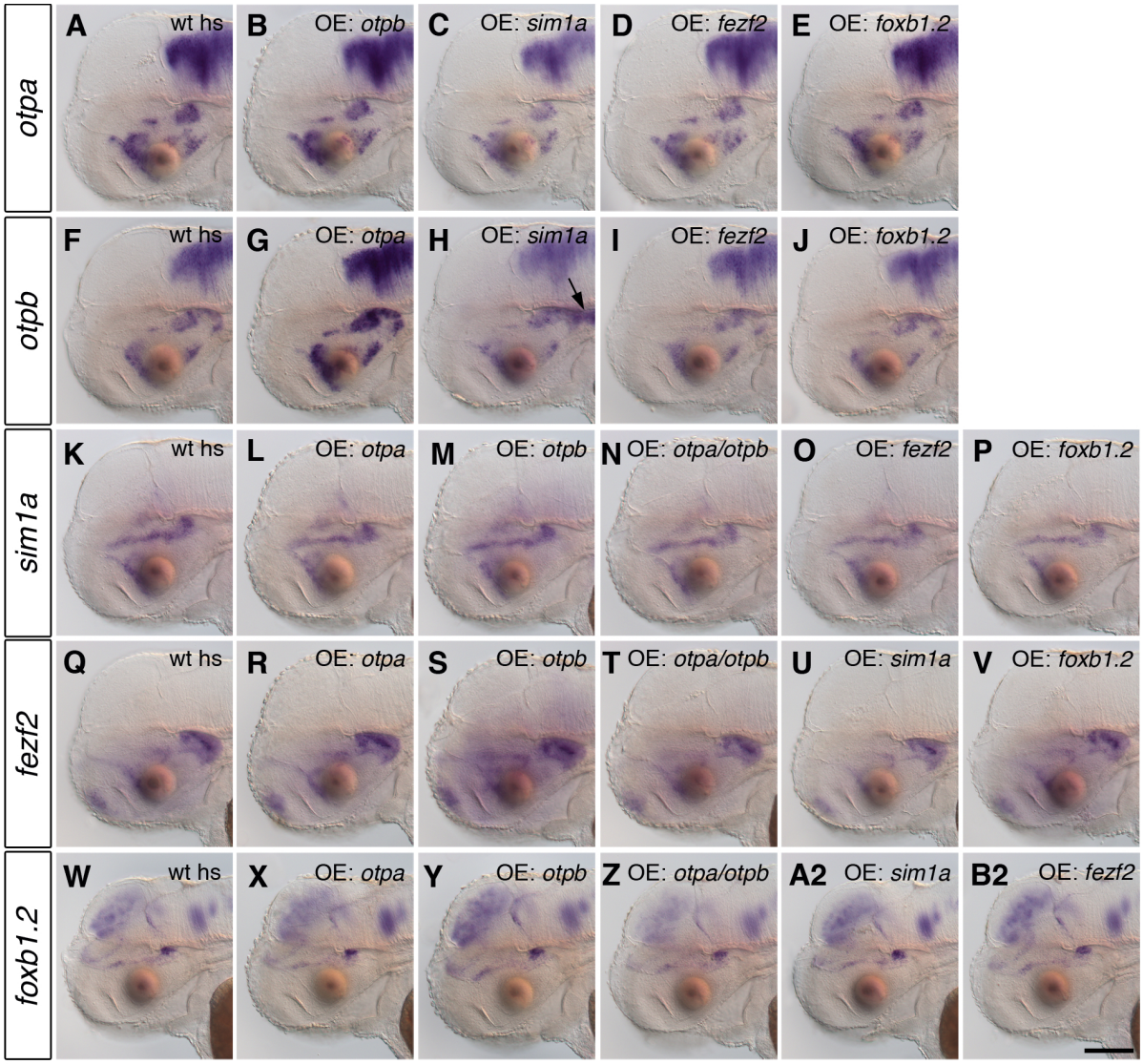

Fig. 3.31: Over-expression of *otpa*, *otpb*, *sim1a*, *fezf2* and *foxb1.2* revealed nearly no genetic regulatory interactions among those transcription factors

(A-B2) WISH to detect the expression of *otpa* (A-E), *otpb* (F-J), *sim1a* (K-P), *fezf2* (Q-V) and *foxb1.2* (W-B2) in 2 dpf wild-type embryos and embryos containing *Tg(hsp70:otpa-2A-EGFP)hd7* (G,L,R,X), *Tg(hsp70:otpb-2A-lyntdTomato)fd1* (B,M,S,Y), *Tg(hsp70:otpa-2A-EGFP)hd7* plus *Tg(hsp70:otpb-2A-lyntdTomato)fd1* (N,T,Z), *Tg(hsp70:sim1a-2A-lyntdTomato)hd8* (C,H,U,A2), *Tg(hsp70:fezf2-2A-EGFP) hd6* (D,I,O,B2) and *Tg(hsp70:foxb1.2-2A-EGFP)hd9* (E,J,P,V) after ON heat shock to detect alterations in transcription factor expression patterns. Lateral views are shown. (A-E) Neither the over-expression (OE) of *otpb* (B, n=16), *sim1a* (C, n=27), *fezf2* (D, n=16) nor of *foxb1.2* (E, n=30) results in a change in *otpa* expression. (F-J) The expression of *otpb* is not affected by over-expression of *otpa* (G, n=17), *fezf2* (I, n=20) or *foxb1.2* (J, n=36), while the over-expression of *sim1a* results in an ectopic *otpb* domain in the hindbrain (H, arrow, n=20/20). (K-P) *sim1a* expression is not changed upon over-expression of *otpa* (L, n=18), *otpb* (M, n=19), a combination of *otpa* and *otpb* (N, n=25), *fezf2* (O, n=22) and *foxb1.2* (P, n=33). (Q-V) Over-expression of *otpa* (R, n=18), *otpb* (S, n=17), a combinatorial over-expression of *otpa* and *otpb* (T, n=24), *sim1a* (U, n=35) and *foxb1.2* (V, n=27) results in no alteration of *fezf2* expression. (W-B2) No effect on *foxb1.2* expression is achieved by over-expressing *otpa* (X, n=13), *otpb* (Y, n=12), a combination of *otpa* and *otpb* (Z, n=22), *sim1a* (A2, n=32) and *fezf2* (B2, n=24). The scale bar shows 200 µm.

The fact that there was no detectable upregulation or downregulation of other transcription factors in the gain-of-function analysis might be due to the fact that the level of over-expression is very low. A real-time PCR analysis to detect the fold change of *otpa* expression in the F1 and F2 generation of *Tg(hsp70:otpa-2A-EGFP)hd7* embryos showed no significant increase in *otpa* expression (Chen-Min Yeh, unpublished data). In addition, the low level of over-expression is further decreasing in the following generation (Fig. 3.32).

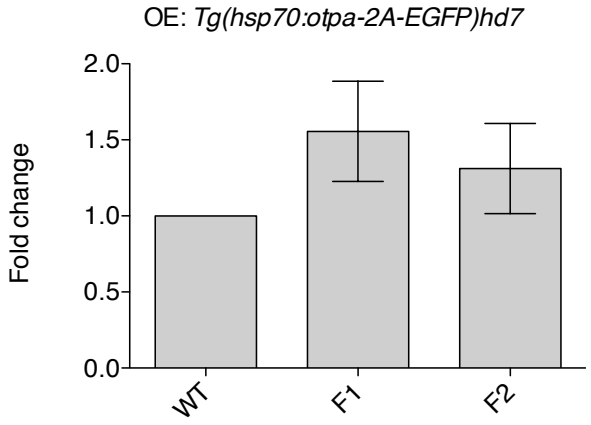

Fig. 3.32: ON activation of *Tg(hsp70:otpa-2A-EGFP)hd7* results in a slight increase in *otpa* mRNA

Chart bars indicate the fold change of *otpa* mRNA after ON heat shock of wild-type and *Tg(hsp70:otpa-2A-EGFP)hd7* embryos of the F1 and F2 generation. Four individual

samples of each genotype have been analyzed and compared using paired t-test. In the F1 generation a 1.6 fold non-significant increase in *otpa* mRNA compared to heat-shocked wild-type embryos could be detected (P=0.1902) while the F2 generation only shows an increase of 1.3 fold (P=0.3704). Error bars indicate standard error of the means.

3.3 In vivo analysis of regulatory interactions in preoptic and posterior hypothalamus neurons

3.3.1 Generation of stable transgenic lines labeling transcription factor expression domain

One of the advantages of zebrafish is the transparency of its embryos. Using this advantage one can utilize *in vivo* time-lapse imaging to observe even slight differences in the birthdates of distinct neurons and to track a labeled lineage at single cell resolution. To further investigate the hypothalamic cell lineages, it is necessary to induce the expression of a fluorophore in distinct sub-lineages of the developing hypothalamus. Otp in zebrafish is one of the key transcription factors for the development of the neuroendocrine hypothalamus (Blechman et al., 2007; Eaton and Glasgow, 2007; Eaton et al., 2008; Ryu et al., 2007). Reflecting its complex function for the specification of several neuronal sub-types, Otp is broadly expressed from 2 days post fertilization (dpf) onwards in the zebrafish diencephalon, including parts of the PO, the posterior tuberculum (PT), the ventral hypothalamus (vHyp) and the posterior hypothalamus (pHyp) (Fig. 3.33A-D). Therefore, the regulatory region of *otp* should contain enhancers specific for distinct Otp-positive regions and for several neuron types. Contrary to Otp, *fezf2* has a broad expression in the diencephalon at 1 dpf but a more specific expression from 2 dpf onwards. The diencephalic expression of *fezf2* at 2 dpf is restricted to the PO and to the posterior hypothalamus (Fig. 3.33E,F). Therefore, also the *fezf2* regulatory region should contain enhancers specific for distinct Fezf2 positive regions and neuron types.

It is already known that some cells are Fezf2- and Otp-positive until they are fully differentiated. *fezf2* for example is expressed in Oxtl neurons (Blechman et al., 2007) in the preoptic area, which suggests that Fezf2 may defines distinct lineages of differentiated neurons. Furthermore, *otp* is expressed in progenitors as well as in differentiated neurons (Ryu et al., 2007). This suggests that early *otp* is expressed broadly in progenitor cells and may define a specific hypothalamic lineage. To investigate the relationship between Otp-positive and Fezf2-positive neurons as well as their final fates I generated constructs containing the regulatory region of these transcription factors controlling the expression of a fluorescent protein.

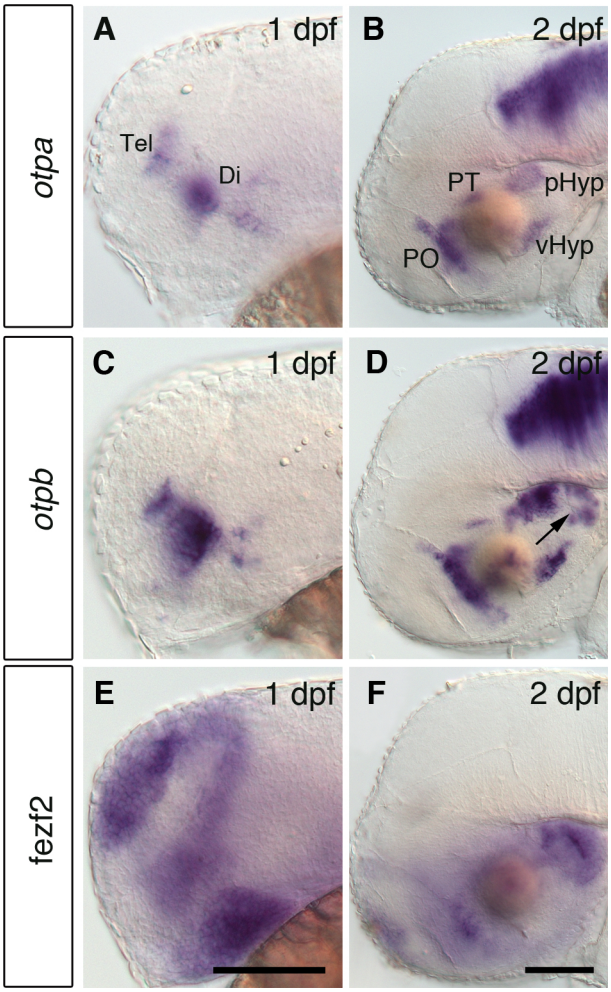

Fig. 3.33: The expression of *otpa*, *otpb* and *fezf2* at 1 and at 2 dpf
(A-F) WISH of the expression of *otpa* (A,B), *otpb* (C,D) and *fezf2* (E,F) at 1 dpf (A,C,E) and at 2 dpf (B,D,F) to reveal their expression pattern in wild-type embryos. (A-D) At 1 dpf the expression of *otpa* and *otpb* is spatially restricted to a small telencephalic (Tel) domain, diencephalic (Di) domains and a hindbrain domain (not shown). Both factors show a broad expression at 2 dpf including the preoptic area (PO), the posterior tuberculum (PT), the ventral hypothalamus (vHyp) and the posterior hypothalamus (pHyp). (E,F) In contrast, *fezf2* has a broad expression at 1 dpf and spatially restricted expression at 2 dpf including a small domain in the PO and a bigger domain in the vHyp. Dorsal views are shown and the scale bar represents 200 µm.

Due to a genome duplication event there are 2 paralogs of the *otp* gene in zebrafish, *otpa* and *otpb*. Since both regulators have a similar expression pattern except for a domain in the posterior hypothalamus (Ryu et al., 2007) (Fig. 3.33D, arrow), the regulatory region of one of the paralogs should be sufficient to identify enhancers specific for distinct Otp-positive domains. The upstream regulatory region of *otpa* contains a region highly conserved among human, mouse, chicken, frog and zebrafish (hs 262) with the size of approximately 400 bp (Visel et al., 2007). In a mouse embryo, this non-coding

DNA fragment induces a lacZ expression specific to the hypothalamus and no additional lacZ expression in other brain regions known to express *Otp* in mouse. Based on this finding I used this highly conserved regulatory region flanked by neighboring 100 bp or 1300 bp to generate approximately 600 bp or 3 kb DNA fragments. These two fragments were cloned into a vector, containing a basal heat shock promoter from the *hsp70* gene (Halloran et al., 2000) (obtained from Laurence Ettwiller, Ettwiller Lab) and GFP, membrane-bound Dendra or membrane-bound Cerulean (MbmCerulean). Dendra is a green-to-red photoconvertible fluorescent protein (Gurskaya et al., 2006), whose photoconversion is not reversible making it possible to trace photoconverted single cells or a group of cells. The constructs were injected into single-cell stage zebrafish embryos and subsequently screened for germ-line integration of the transgene. I analyzed the obtained transgenes *Tg(600bp-otpa:hsp-egfp)Fd13* and the *Tg(3kb-otpa:hsp-egfp)Fd2* using αOtpa, αOxtl and αAvpl antibodies generated in the laboratory in combination with a commercial GFP antibody. Both constructs label specific neurons within the PO but not neurons in the posterior hypothalamus. Additionally, I observed that the 3 kb-*otpa* constructs reliably labels the majority of Otpa-positive cells in the PO (Fig. 3.34A-A").

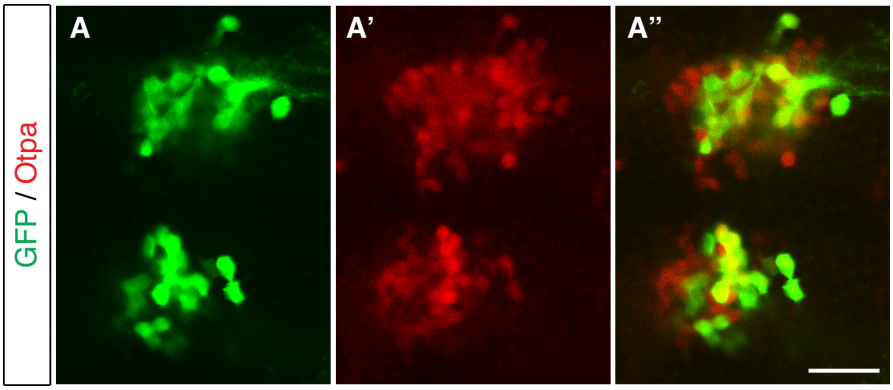

Fig. 3.34: The 3 kb fragment of the *otpa* regulatory region induces GFP expression in the majority of Otp-positive neurons
(A-A") Immunohistochemistry of *Tg(3kb-otpa:hsp-egfp)Fd2* embryos at 3 dpf with antibodies against GFP (A) and Otpa (A') to investigate the specificity of the GFP labeled cells. A major proportion of the Otpa-positive cells are GFP positive as well. A single plane in the dorsal view is shown and the scale bar represents 60 µm.

In Contrast, the 600 bp-*otpa* construct labels only a subset of the Otpa-positive cells in the PO. Interestingly, these few GFP-positive cells include nearly all Oxtl neurons and nearly all Avpl neurons (Fig. 3.35A-B"), suggesting that a part of the *otpa* promoter region can label different neuronal subtypes within this area. In order to label other subsets of Otp-positive neurons, I generated 4 additional constructs containing

fragments of the 3kb promoter region combined with MbmCerulean. Since the 600 bp promoter fragment labels mainly Oxtl and Avpl neurons, I reasoned that remaining parts of the 3kb fragment might label other neuronal subtypes within the PO. Several stable transgenic lines generated from these four constructs were analyzed, but none of them showed specific expression in the hypothalamus.

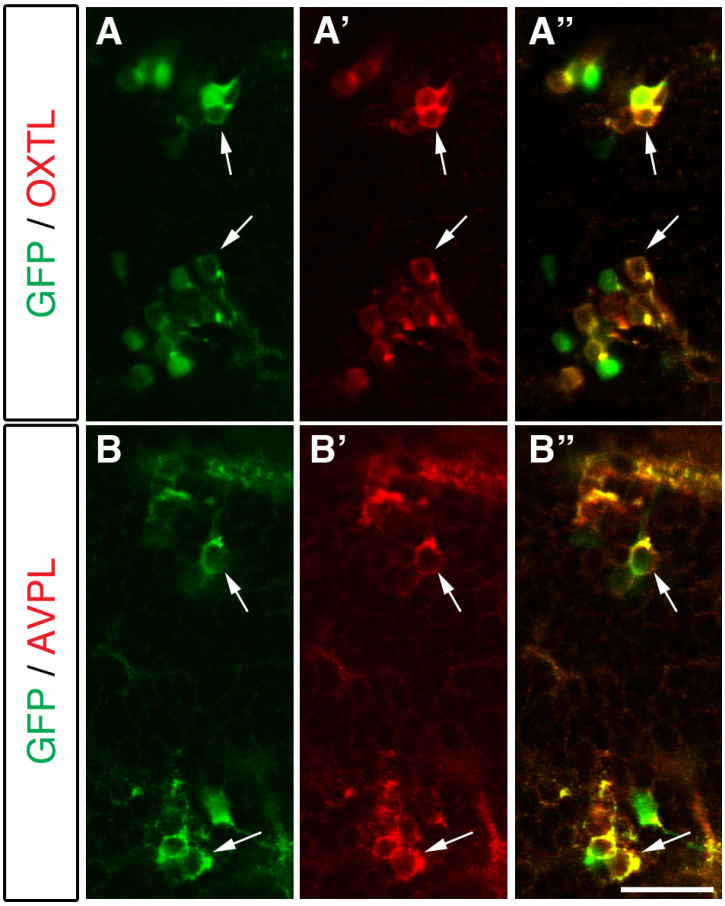

Fig. 3.35: The 600 bp-*otpa* fragment is sufficient to induce GFP expression in Oxtl- and Avpl-positive cells
(A-B") Immunohistochemistry on *Tg(600bp-otpa:hsp-egfp)Fd13* 3 dpf old embryos using antibodies against GFP (A,B), Oxtl (A') and Avpl (B') to determine the identity of the GFP-positive cells. The 600 bp *otpa* promoter region induces GFP expression mainly in Oxtl- (A-A") and Avpl-positive cells (B-B"). White arrows point to examples of co-expressing cells. Single planes in the dorsal view are shown and the scale bar represents 60 µm.

To generate transgenic lines labeling Fezf2-positive neurons I used a 3 kb promoter region obtained from Dr. Josh Bonkowski (University of Utah) driving membrane-bound Dendra. Analysis of transgenic lines with three different insertions showed a strong expression at 1 dpf similar to the observed *fezf2* expression at 1 dpf. However, there

was no detectable Dendra signal at later stages of development. Therefore, none of the obtained lines are useful for lineage analysis of Fezf2-positive neurons.

These results show that the regulatory regions of *otpa* and *fezf2* are sufficient to mimic their endogenous specific expression. Since the *fezf2* promoter element is only active until 1 dpf and the *otpa* fragments are only labeling parts of the PO a comprehensive lineage and birthday analysis of different hypothalamic cell lineages especially within the posterior hypothalamus was not possible.

3.4 Summary

In this thesis, I showed that the genetic regulatory interactions of Fezf2 are crucial for the regionalization of the hypothalamus at 1 dpf and that at 2 dpf a variable regulatory network of *fezf2* together with *otp*, *sim1a* and *foxb1.2* is important for the regionalization of the preoptic and the mammillary area in zebrafish embryos leading to defined subregions. I also to showed that this variable regulatory network and the resulting subregions are crucial for the formation of distinct neuronal subtypes. Lastly, I showed that a highly conserved regulatory region upstream of the *otpa* gene is sufficient to induce reliably specific expression in the preoptic area.

I demonstrated that Fezf2 at 1 dpf is crucial for the regionalization of the hypothalamus. *fezf2* is expressed in the posterior hypothalamus where is positively regulates the expression of *emx2*, a marker of the posterior-ventral hypothalamus. Further, the repressive interactions to *shh* in the ZLI appears to subsequently influence the regionalization of the anterior-dorsal hypothalamus. Fezf2 negatively regulates the expression of *foxb1.2*, which is present in the posterior-dorsal hypothalamus. Together these results show the crucial role of Fezf2 at 1 dpf for the regionalization of the hypothalamus and demonstrate the existence of distinct subregions within the early hypothalamus that have not been described so far. Furthermore, at 1 dpf Fezf2 positively regulates the expression of the transcription factors *otp* and *sim1a* that are important for the specification of neuronal subtypes. A summary of the observed genetic regulatory interactions and the anatomy of the hypothalamus at 1 dpf is shown in figure 3.36.

Moreover, 3 kb including a highly conserved regulatory region upstream of the *otpa* gene together with a basal heat shock promoter are sufficient to induce the expression of a fluorophore reliably and specific in the preoptic area of zebrafish. Since a smaller construct that contains the 400 bp highly conserved regulatory region labels mainly magnocellular neurosecretory neurons, this regulatory region might be helpful for further functional characterization of these neurons.

At 2 dpf Fezf2 is important for the formation of the preoptic area, the homologous structure to the mammalian PVN and SON, and the posterior most region of the

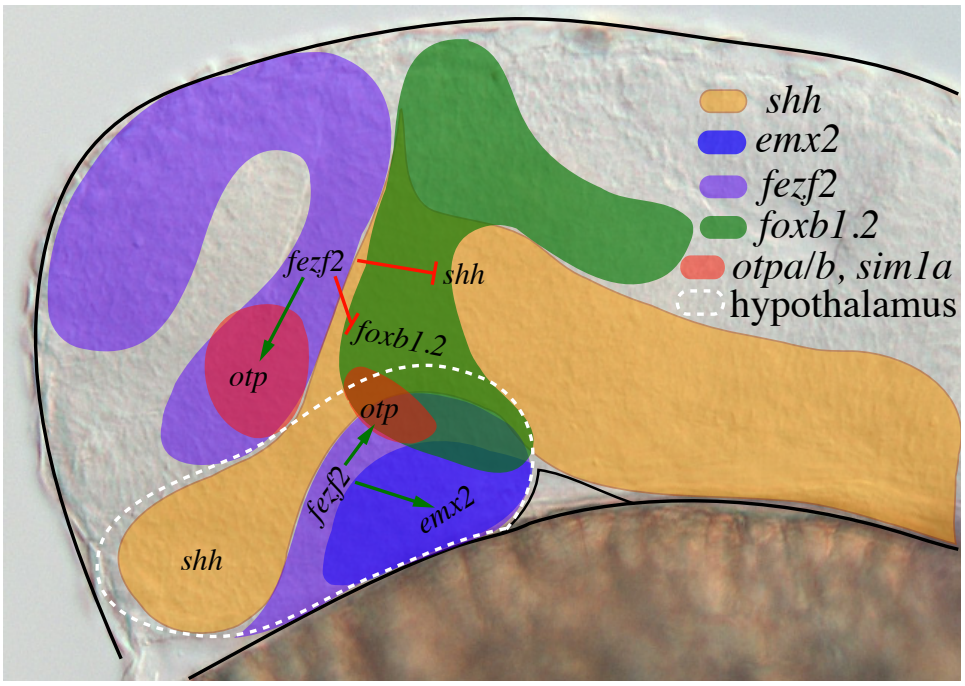

Fig. 3.36: A map summarizing the interaction and expression of *fezf2*, *foxb1.2*, *emx2*, *shh* and *otp* at 1 dpf

A schematized map displaying the relative expression patterns of the transcription factors at 1 dpf and a summary of their genetic interactions. Red bars indicate negative regulation while green arrows indicate positive regulation. The white dashed line delineates the prospective hypothalamus demarcated by *nkx2.1a* expression.

hypothalamus, the mammillary area. To my knowledge, this is the first study describing the mammillary area in zebrafish embryos and providing a molecular map of mammillary subregions.Similar to mouse, where FOXB1 is the marker for the mammillary body (MB), *foxb1.2* was found to be expressed in the central MA of zebrafish. Together with the expression domains of *fezf2*, *otp* and *sim1a*, *foxb1.2* delineates distinct subregions of the MA in zebrafish. Loss-of-function analyses showed that these subregions of the MA may results from the complex regulatory interactions of *fezf2*, *foxb1.2*, *otp* and *sim1a*. In a temporally- and spatially-controlled manner these factors negatively regulate each other contributing to the formation of the separated subregions. Similarly, in the preoptic area the positive regulatory interactions of *otp*, *sim1a* and *fezf2* are reflected in the anatomy, which shows mostly co-expression. Interestingly, while at 1 dpf Fezf2 acts upstream of *otp* at 2 dpf Otp also regulates *fezf2* positively in the PO and negatively in the MA.

Further, in this study I investigated the specification of Oxtl-, Avpl-, VIP- and Uts1-positive neurons. By combining the information about genetic regulatory interactions of the transcription factors and their co-expression, I identified necessary transcription factors for the formation of these neuronal subtypes. Taking advantage of the transgenic heat shock lines, which over-express temporally controlled cDNAs of

fezf2, *foxb1.2*, *otp* and *sim1a*, I demonstrated which transcription factors are sufficient for the specification of Oxtl-, Avpl-, VIP- and Uts1-positive neurons. I found that key transcription factors sufficient for the formation of Oxtl-positive neurons, are Otpa and Sim1a, whereas for Avpl-positive neurons in the PO a combination of Otpa, Sim1a and Fezf2 appears to be crucial. Further, my studies revealed that, although Otp, Sim1a and Fezf2 are necessary for the specification of VIP-positive neurons in the PO, only Sim1a activity induces a higher amount of *vip*-expressing cells. Remarkably, Otpb activity is sufficient to induce ectopic *vip* expression broadly in the brain. In addition, in the MA Otpa and Sim1a activity is sufficient to induce supernumerary VIP-positive cells. Notably, since the loss of Fezf2 function results in a severe reduction of *vip* expressing cells also this transcription factors appears to play a crucial role for VIP-neurons specification. Lastly, for the Uts1-positive neurons my results show that consistent with the co-expression data, Fezf2 alone and in combination with Sim1a or Foxb1.2 are sufficient to induce supernumerary Uts1-positive neurons in the central MA. A summary of the observed genetic regulatory interactions and the resulting complex anatomy important for the formation of the discussed neuronal subtypes is shown in figure 3.37.

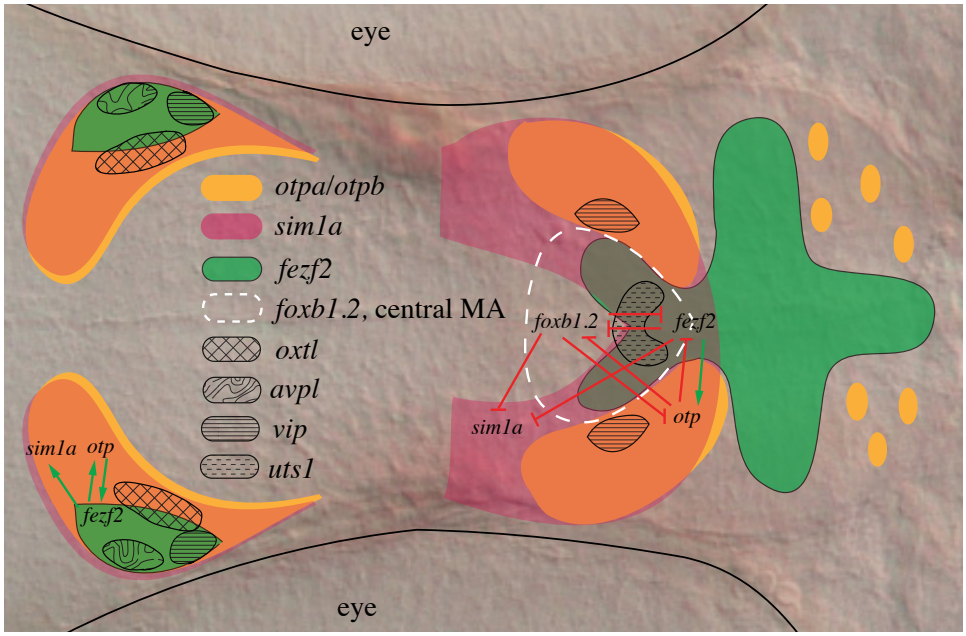

Fig. 3.37: A summary of the relative expression and interaction of the transcription factors and neuropeptides at 2 dpf

A schematized map showing the relative expression of the transcription factors and the neuropeptides in the PO and the MA at 2 dpf and a summary of the genetic interactions of the transcription factors. Red bars indicate negative regulation and green arrows indicate positive regulation. The white dashed line delineates the central MA.

4 Discussion

4.1 A molecular map of the posterior hypothalamus

To my knowledge, this is the first study to define a comprehensive molecular map of the MA based on the underlying genetic regulatory interactions. This genetic regulatory network involving *fezf2*, *foxb1.2*, *otp* and *sim1a* leads to the delineation of distinct subdomains within the MA where subsequently distinct neuronal subtypes are specified. The posterior most region of the hypothalamus, the mammillary area is an evolutionary highly important and conserved hypothalamic area, whose existence is even discussed in lamprey (Nieuwenhuys et al., 1998). It is a crucial hypothalamic region in the control of spatial memory and cognitive behavior in mammals (Pan and McNaughton, 2004; Vann, 2010). Although anatomists distinguished 5 different nuclei within the MA since decades (Swanson, 1987), the role of these nuclei or neuron types within them have been difficult to analyze. This difficulty persists, because lesions studies (Beracochea, 2005; Canteras et al., 2001; Pan and McNaughton, 2004; Vann, 2010; Vann and Aggleton, 2004) and mutants affecting MA formation (Alvarez-Bolado et al., 2000a; Marion et al., 2005; Wehr et al., 1997) mostly affect more than one defined nucleus. So far the MA has been regarded as a homogenous non-laminar region with few cell types with different morphology and electrophysiological properties (Allen and Hopkins, 1988; Alvarez-Bolado et al., 2000a; Vann, 2010; Veazey et al., 1982). This study revealed an unexpected complexity of the MA and provides molecular marker to distinguish its subregions. A recent study provided a list of markers for different hypothalamic nuclei in the developing mouse brain (Shimogori et al., 2010). In future studies these marker can be incorporated to the molecular map defined in this study in order to generate an even higher resolution molecular map and more defined regulatory codes for the specification of distinct neuron types. Based on Shimogori et al. (2010) and my preliminary results, these additional markers include *dbx1a*, *dlx1a*, *dlx2a*, *dlx5a*, *isl1*, *tbx2b*, *arx* and *lhx6*. Since the tuberomammillary area is the sole seat of histaminergic neurons (Haas and Panula, 2003) the expression of histidine decarboxylase (*hdc*) would serve as a perfect marker to delineate the position of the tuberomammillary area in zebrafish embryos.

These subdomains, which have been identified in this thesis and that express a defined combination of transcription factors might reflect the functionally different nuclei of the MA known from mammals. In addition, the knowledge gained in this thesis may

allow a more precise manipulation for a functional analysis of these different posterior hypothalamic nuclei or even distinct neuron types.

4.2 The mammillothalamic tract in zebrafish?

The principal mammillary tract is the major axon bundle from the MB, sprouting at a specific point to become the mammillotegmental and the mammillothalamic tract (Szabo et al., 2011). As the name indicates, the mammillothalamic tract connects the MB with the thalamus, which makes it important in spatial and working memory and in object and scene discrimination (Radyushkin et al., 2005; Vann, 2010). Studies showed that the mammillothalamic tract fails to develop in mutants for Foxb1 and Sim1/Sim2 (Alvarez-Bolado et al., 2000b; Marion et al., 2005). This thesis showed that the activity of Foxb1.2 and Sim1a is crucial for the formations of the MA or neuron types of the MA in zebrafish. Further in the lateral view an extension of the mammillary *foxb1.2* and also *sim1a* expression domain towards the thalamus is clearly detectable. Therefore, I hypothesize that this stripe of transcription factor expression might delineate the mammillothalamic tract in zebrafish, but further experiments are necessary to confirm this. This could represent the first description of such an important connection in a non-mammalian species.

4.3 Conserved regulatory interactions in the PO and in the MA

It has been known that the function of distinct transcription factors important for the formation of the preoptic area is evolutionary conserved (Caqueret et al., 2005; Machluf et al., 2011; Ryu et al., 2007). In this study I showed that transcription factors important for the formation of the mammillary area, the putative homologue to the mammalian MA, are also highly conserved. The transcription factor FOXB1 in rodents is crucial for the proper formation of the MB (Wehr et al., 1997). This thesis shows that in zebrafish the orthologous transcription factor Foxb1.2 is important for the formation of the central MA. Furthermore, it was found that SIM1/SIM2 is expressed in the MB of rodents and that these regulators are necessary for the formation of the mammillothalamic tract, although the formation of the MB itself in Sim1/Sim2 mutants is not altered (Marion et al., 2005). Consistent with the data in rodents, Sim1a is expressed in the MA in zebrafish. A loss of Sim1a function did not alter the expression of other transcription factors, which suggests that there is no alteration of the development of the MA. Sim1a in zebrafish might be important for the formation of an equivalent to the mammillothalamic tract of rodents.

A recently published study, which provides a list of markers expressed in distinct hypothalamic nuclei in the developing mouse brain (Shimogori et al., 2010) shows that

Otp is expressed in several mammillary nuclei like the premammillary nucleus, the mammillary body and the tuberomammillary area at different stages of the development of the mouse brain. This thesis showed that the posterior hypothalamic *otp* expression in zebrafish (Ryu et al., 2007) is found in the peripheral MA, where it plays an important role for the proper formation of the MA of zebrafish. In rodents the activity of SIM1/SIM2 in the MA is independent of ARNT2 function (Marion et al., 2005). Consistent with this finding, in zebrafish *arnt2* is not expressed in the posterior hypothalamus (Löhr, 2009). All these observations argue for the existence of conservation of the regulatory actions of transcription factors in the hypothalamus development. One exception might be represented by SIM1/SIM2. In mouse SIM1/SIM2 appear to be acting upstream of Foxb1 because a mutation of SIM1/SIM2 is leading to a reduced Foxb1 expression (Marion et al., 2005). However in zebrafish, *sim1a* morphants display no change in *foxb1.2* expression.

In summary, most of the transcription factor activities appear to be highly conserved between rodents and zebrafish. Therefore, findings obtained in zebrafish like the molecular map of the MA or the transcription factors contribution to the specification of distinct neuron types will be worthwhile to analyze in rodents in order to gain insight in the hypothalamus development in mammals.

4.4 Evolutionary conservation of VIP- and Uts1 positive neurons in zebrafish

To the best of my knowledge this is the first study analyzing the expression pattern of *vip* and *uts1* cells in the embryonic zebrafish hypothalamus. In mammals VIP is broadly expressed and has pleiotropic regulatory functions throughout the whole body. In the central nervous system regions of highest VIP expression are the suprachiasmatic nucleus where it is implicated in the generation and synchronization of the circadian oscillation clocks (Vosko et al., 2007) and another site of strong VIP expression is the PVN where it is co-expressed with CRH (Ceccatelli et al., 1989; Hokfelt et al., 1987) and can enhance ACTH secretion in the pituitary (Nussdorfer and Malendowicz, 1998). Other VIP-immunoreactive as well as VIP-binding sites in the mammalian hypothalamus are situated in the mammillary nuclei (Saper, 2000; Sarrieau et al., 1994). However, the function of those neurons in the MA is poorly understood. In zebrafish, I observed mainly two sites of VIP expression in the embryonic zebrafish hypothalamus: in the PO and in the MA, suggesting an important and evolutionary conserved function of VIP within these hypothalamic areas.

Uts1 is the teleost ortholog of the mammalian urocortin 1 (UCN1) (Vaughan et al., 1995) that belongs to the urocortins, which are structurally related to CRH and consist of UCN1, UCN2 and UCN3 (Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2000; Vaughan et al., 1995). UCN1 is widely distributed in the brain and also in the periphery where it has important physiological functions in various tissues. In the central nervous system it is implicated in the modulation of energy and water homeostasis as well as in stress adaptation while in the periphery it associated with immunomodulation and cardioprotection (Oki and Sasano, 2004). A low-level expression of Ucn1 in colchicine-treated rats was found in the lateral mammillary and in the supramammillary nuclei (Bittencourt et al., 1999), whereas in adult zebrafish a homogeneously distributed expression of *uts1* was detected within the corpus mamillare (Alderman and Bernier, 2007). Although the function of UCN1 in the MA of rats and of Uts1 in the MA of zebrafish is not known, this conserved expression indicates an important evolutionary conserved function, which still needs to be investigated. In contrast to the high conservation of Uts1 expression domains among mammals and zebrafish, a substantial number of descending Uts1-ir fibers but no or very few CRH-ir fibers form in the spinal cord of teleosts. In contrast, in mammals the opposite it true (Pepels et al., 2002).

4.5 Molecular mechanism of VIP- and Uts1-positive neuron specification

In this thesis I showed for the first time transcription factor requirements for the specification of VIP and Uts1-positive neurons. Fezf2 was already known to be required for the formation of several neuron types both in mouse and in zebrafish. In mouse, early in development Fezf2 is broadly expressed throughout the dorsal telencephalon but becomes restricted to the cortical deep layers later in development where it is important for the specification of subcerebral neurons (Chen et al., 2005a,b; Molyneaux et al., 2005; Shimizu and Hibi, 2009). In zebrafish, Fezf2 is important for the specification of 5-Hydroxytryptamine (5-HT), DA and Oxtl neurons (Blechman et al., 2007; Guo et al., 1999; Jeong et al., 2006; Levkowitz et al., 2003). In this thesis I showed that Fezf2 is also crucial for the development of VIP- and Uts1 positive neurons. Since *fezf2* is only co-expressed with *uts1* but not with *vip* in the zebrafish MA the mechanism by which Fezf2 affects their specification is likely to be different. Fezf2 controls neuron type specification non-cell-autonomously for the development of 5-HT and DA neurons since *fezf2* is not expressed in these cell types (Levkowitz et al., 2003). It might be possible that for VIP neuron specification Fezf2 also acts non-cell-autonomously. Alternatively, Fezf2

might affect development of the early VIP progenitor cells either directly or by regulating *otp* expression. I consider the latter possibility to be more likely because *fezf2* is broadly expressed in the posterior hypothalamus at earlier stages and it down-regulates the expression of *otp*. Also in the PO where *vip* is co-expressed with *fezf2*, the over-expression of *fezf2* resulted in no supernumerary VIP cells while *fezf2* morphants display a severe reduction of VIP cells in the PO and the MA. In contrast, for the Uts1-positive neurons the loss of Fezf2 function caused fewer *uts1*-expressing cells while the gain of Fezf2 function resulted in more *uts1*-expressing cells. Therefore, it is likely that Fezf2 directly regulates the late aspects of specification of *uts1*-expressing cells.

The regulatory action of Otp and Sim1a on the specification of VIP-positive neurons appears to be directly linked. The loss of Sim1a and Otp function caused a reduction of *vip*-expressing cells in the PO and an increase in *vip*-expressing cells in the MA. In contrast, Foxb1.2 appears to regulate *vip* expression indirectly because they are not co-expressed and the over-expression of *foxb1.2* results in a decrease in the number of *vip*-expressing cells whereas *foxb1.2* morphants display no change in the number of *vip*-expressing cells. Since *foxb1.2* and *otp* appear to negatively regulate each other, a gain of Foxb1.2 function could result in a decreased activity of Otp, which is a key regulator for VIP neuron specification, and therefore in a fewer VIP cell number.

This negative cross-regulatory interaction of *otp* and *foxb1.2* might also play a role in the specification of *uts1*-expressing neurons. In loss of Otp function embryos the number of *uts1*-expressing neurons increases whereas the over-expression of *otp* causes no change in *uts1* cell number. Since the gain of Foxb1.2 functions results in more *uts1*-expressing neurons and the loss of Otp function results in an expanded *foxb1.2* domain, the increase of *uts1* neurons in loss of Otp function embryos might be indirect through the negative genetic interaction of *otp* and *foxb1.2*. Interestingly, also *foxb1.2* morphants display an increase in *uts1*-expressing neurons. Foxb1.2 appears to have an early and a late role in forebrain development similar to other transcription factors (Baumgardt et al., 2007). Early in development it is important for the regionalization of the forebrain (Staudt and Houart, 2007; Toro and Varga, 2007) but this thesis showed that later in development it is important for the formation of the central MA and for the specification of neuron types. Since the loss-of-function analysis with morpholinos results in the loss of the protein activity from one-cell stage onwards both early and late functions are disrupted and it might be impossible to detect the late function of this transcription factor. Increase in Uts1 cell number both in loss and in gain of Foxb1.2 function might be due to the fact that the loss-of-function experiment reveals the effects on the regionalization of the posterior hypothalamus while the gain-of-function experiment shows the effects on cell type specification.

4.6 Molecular mechanism of the specification of magnocellular neurosecretory neurons

Several studies (Blechman et al., 2007; Eaton and Glasgow, 2006, 2007; Eaton et al., 2008) together with this thesis revealed the necessity of Otp, Sim1a and Fezf2 for the formation of Oxtl- and Avpl-positive neurons. The co-expression analysis suggested that Fezf2 might directly regulate Avpl neuron specification and indirectly the specification of Oxtl neurons, because *avpl* is mainly co-expressed with *fezf2* while *oxtl* is not. With the gain-of-function analysis I was able to confirm this hypothesis that *oxtl* is indirectly regulated by *fezf2*, because the over-expression of *fezf2* resulted in no changes in *oxtl* cell number. Another possible mechanism for the regulatory action of *fezf2* on *oxtl*-expressing cells is that *fezf2* acts on early progenitors of *oxtl*-expressing neurons. In addition, the gain-of function analysis shows that Fezf2 does not act non-cell-autonomously in the specification of the preoptic and the mammillary neuron types. Interestingly, the ectopic over-expression of *fezf2* in this study does not induce phenotypes in neurons, which do not express *fezf2*. However, this thesis did not identify key transcription factors similar to a terminal selector gene known from *C. elegans* (Hobert, 2008), which are exclusively sufficient for the formation of Oxtl- or Avpl-positive neurons in the zebrafish hypothalamus. Otp, Sim1a and Fezf2 are important for the formation of several neuronal subtypes, making it likely that a underlying regulatory network is important for the formation of *avpl*- or *oxtl*-expressing neurons.

4.7 Fezf2 is a key transcription factor for early hypothalamus patterning

This study provides the first insight into the importance of Fezf2 on the patterning of the hypothalamus is zebrafish. Although it was known already that *fezf2* is expressed in the hypothalamus early on, the function of Fezf2 in this area has not been clear so far. A previous study assumed that the patterning of the hypothalamus in *fezf2* morphants is largely normal, because *fezf2* morphants display no change in *nkx2.1a* expression (Jeong et al., 2007). In this study, I found that although *nkx2.1a* expression was not changed in *fezf2* morphants, Fezf2 has important functions for the patterning of the hypothalamus at 1 dpf. *fezf2* is expressed in the posterior half of *nkx2.1a* expression domain, perfectly abutting but not overlapping with the expression of *shh* in the anterior hypothalamus. The *fezf2* expression domain contains the smaller expression domain of *emx2*, the posterior-ventral hypothalamic marker (Mathieu et al., 2002) indicating that *fezf2* is labeling a larger proportion of the posterior hypothalamus, whereas *emx2* delineates the posterior-ventral hypothalamus. In *fezf2* morphants the expression of *emx2*

in the posterior-ventral hypothalamus is severely reduced. In addition, the anterior-dorsal hypothalamic *shh* expression domain is more dorsal and more compact (Jeong et al., 2007), which might be an indirect effect of the observed shift of the ZLI *shh* expression because *fezf2* is not co-expressed with *shh*.

Another factor highly important for the formation of the posterior hypothalamus is Nodal. Mutants of the Nodal pathway display a severe reduction of *emx2* expression while *shh* expression is retained (Mathieu et al., 2002). Therefore, it is possible that *fezf2* is affected by the Nodal pathway and represses *shh*, which is known to suppress the posterior-ventral hypothalamic fate by promoting the anterior-dorsal hypothalamus (Wilson and Houart, 2004). Interestingly, mutant of the nodal pathway display a highly reduced *otp* expression in the PO and no *otp* expression in the posterior hypothalamus comparable but weaker than what is observed in *fezf2* morphants (Del Giacco et al., 2006).

Fezf2 was originally identified as a gene upregulated upon Dkk1 over-expression, indicating that *fezf2* expression is repressed by canonical *wnt/β-catenin* pathway (Hashimoto et al., 2000). More recent studies indeed found Fezf2 to be part of the canonical Wnt signaling of Wnt8b mediated by Lef1 (Jeong et al., 2007; Lee et al., 2006; Russek-Blum et al., 2008). In the case of DA neuron specification, it was found that *fezf2* is downstream of Wnt8b, a key regulator for DA neuron development. These findings suggests that a balanced activity of Wnt8b and Fezf2 is needed to form the correct number of DA neurons (Russek-Blum et al., 2008). Since *wnt8b* has been reported to be expressed in the mammillary and the retromammillary hypothalamus in mice and humans (Lako et al., 1998) this upstream regulatory function of Wnt8b on *fezf2* expression might also be important for the formation of the MA in zebrafish.

As it was already proposed in recent studies (Eaton and Glasgow, 2006, 2007) this thesis showed no evidence that Otp or Sim1a are involved in the patterning of the hypothalamus. Therefore, I concluded that Otp and Sim1a regulate differentiation and cell fate rather than patterning. Supporting this statement, is the fact that Otp is only expressed in non-proliferating progenitor cells (Ryu et al., 2007) and that my initial screen to find transcription factors regulated by Otp only revealed three factors out of more than 80 different transcription factors.

4.8 Variable regulatory interactions of a common transcription factor set generates hypothalamic neuronal diversity

Neuronal subtype specification is thought to involve combinatorial usage of a limited repertoire of transcription factors (Shirasaki and Pfaff, 2002). To explain how such an

amazing neuronal diversity can be achieved by using a small set of common transcription factors several mechanisms have been suggested. For example, feed-forward regulatory loops segregate the fate of motor neurons and interneurons in the developing spinal cord although they are specified by related LIM-complexes (Lee et al., 2008). In the Drosophila nervous system in contrast, the progenitor competence is limited over time by a temporal cascade of transcription factors (Baumgardt et al., 2009). Although several transcription factors have been identified, which are implicated in the development of the hypothalamus and its contained neuronal subtypes it remains unknown how these regulators interact to generate distinct neuron types. In this study I found that one mechanism for the generation of neuronal diversity with a limited set of transcription factors is their dynamic interaction. Transcription factors can act as an activator or a repressor (Babu et al., 2004). I demonstrate that the genetic cross-regulatory network composed of *fezf2*, *foxb1.2*, *otp* and *sim1a* changes in spatially and temporally dependent manner. At 1 dpf, Fezf2 acts upstream of *otpb* (Blechman et al., 2007), *otpa*, *sim1a* and *emx2*, controlling thereby the regionalization, neurogenesis and/ or the initial steps of neuronal differentiation in the hypothalamus. Strikingly at 2 dpf, the genetic regulatory interactions of the transcription factors changes at multiple levels. First, the *fezf2*-*otp* interactions become cross-regulatory, now Otp positively regulates the expression of *fezf2* in the PO while it negatively regulates the expression of *fezf2* in the MA. Sim1a that is proposed to work in parallel to Otp (Löhr et al., 2009) remains downstream of Fezf2. Second, Fezf2 positively regulates the expression of *sim1a* in the PO while in contrast, it negatively regulates it in the MA. Third, *fezf2* and *foxb1.2* interactions as well as *otp* and *foxb1.2* interactions become cross-repressive in the MA. The functional consequences of these dynamic genetic regulatory interactions are the definition of the subdomains in the PO and in the MA that are important for the generation of distinct neuronal subtypes. Although Otp and Sim1a are proposed to work in a parallel pathway, they are regulated differentially and their sufficiency to induce neuropeptide expression is different as well. Further, Otp and Sim1a are not completely co-expressed which allows Sim1a and Otp to exert different effects on Uts1 cell number. Taken together, these results do not support the existence of completely parallel functions of Otp and Sim1a. In addition, SIM1 heterozygous mutant mice display a dosage dependent obesity phenotype (Duplan et al., 2009) whereas OTP mutant mouse do not show such a phenotype.

This study provides important mechanistic insights into the generation of neuronal diversity in the hypothalamus. In hypothalamus development early regulators such as Fezf2 and Foxb1.2 are important for the regionalization and then later in development become re-deployed in the generation of different neuronal subtypes. This economical way of using transcription factors has the disadvantage that the loss-of-function analysis with Morpholinos interferes with both functions and the effects of the earlier function

might still have impact on the detection of the later function. Hence it might be possible that Fezf2 at 2 dpf does not positively regulate the expression of *otp* in the MA. It is more likely that the *otp-fezf2* interactions becomes cross-repressive since their two expression domains separate beginning at 1 dpf. This statement is supported by the fact that cross-repressive regulatory interactions are used to sharpen boundaries of domains (Shirasaki and Pfaff, 2002).

4.9 The limitations of gain-of-function analyses

The usage of gain-of-function analysis has clear advantages. Using an inducible promoter like a heat shock shock promoter the time when the gene of interest should be over-expressed is controlled. Especially with transcription factors that have early and late functions in development, the investigation of the later roles with loss-of-function analysis remains difficult. So far, only the usage of techniques like the usage of PhotoMorphs (Tomasini et al., 2009) makes it possible to achieve an temporal controlled loss-of-function situation. Another advantage of gain-of-function experiments is the fact that the sufficiency of the gene of interest can be investigated. Loss-of-function studies only provide information on the necessity of the gene of interest for the investigated processes. Despite its advantages, the temporal control of the gain-of-function constructs using an inducible heat shock promoter has disadvantages. Several studies showed that the time point of giving the heat shock is critical to induce an over-expression phenotype (Blechman et al., 2007; Jeong et al., 2007). To find this time point extensive tests using different induction times are necessary. In addition, knowledge about the earlier roles of the investigated transcription factors are necessary to avoid interference with this function.For example, the relatively mild effect of *fezf2* over-expression on Uts1 cell number is likely due to the heat shock that was performed at 2.5 dpf. At this time point much of the hypothalamic differentiation processes have been initiated already, making it more difficult to induce significant changes than at earlier stages. Furthermore the expression level of the induced transgene is of high importance. The expression level of Fezf2 transgenic line established in this study is likely to be significantly lower than in *fezf2* over-expression systems published earlier, where the Gal4-UAS system has been used for a strong transgene induction (Jeong et al., 2007). Additionally, the real-time PCR analysis performed by Chen-Min Yeh (Ryu Laboratory) testing the expression level of the established *otpa* over-expression line revealed a very low fold change of the mRNA level of the induced gene. Another issue to take into consideration is the question whether the investigated transcription factors act together with other proteins in protein complexes or whether they need other factors to bind to the distinct DNA binding site to induce the investigated phenotype. It might be possible that only so called terminal selector

genes (Hobert, 2008) are able to induce a broad or ectopic expression of a marker for a distinct neuronal subtype when over-expressed alone. So far, to my knowledge, terminal selector genes in vertebrates as such have not been described. If the fact that cell identity is achieved by the active repression of other possible neuronal fates (Shirasaki and Pfaff, 2002) is true in the brain as well then the induction of other cell types needs to overcome the repressors of the distinct cell types that should be achieved by over-expression of a distinct transcription factor. Therefore, over-expression of a distinct transcription factor might only induce a neuronal fate in a very small subset of neurons where this active repression does not take place and the neurons are still competent for the induction of another fate. This hypothesis is supported by the fact that I achieved only very slight changes of cell number in the gain-of-function experiments.

4.10 Ectopic *vip* expression reveals different roles of Otpa vs. Otpb

In this study I showed for the first time that the over-expression of *otpb* is sufficient to induce the ectopic expression of *vip* broadly in the brain. So far studies utilizing over-expression of transcription factors in the zebrafish hypothalamus usually showed a limited number of extra cells observed very close or within the original cell cluster (Blechman et al., 2007; Löhr et al., 2009; Ryu et al., 2007). Only very high levels of over-expression of cmv-*otpa* transiently induced a limited amount of ectopic expression of *th* in areas where no *th* expression is usually found (Ryu et al., 2007). In this study I showed that a long induction of low-level over-expression of *otpb* is sufficient to induce ectopic *vip* expression broadly in several areas in the brain that do not normally express *vip*. This effect might be due to of the fact that neurons are competent to express *vip* in addition to their normal fate. Alternatively *otpb* might be a terminal selector gene that is able to induce the specification of VIP neurons in the whole brain. I think the first option is more likely because VIP has very broad functions including its neurotrophic properties. This neurotrophic or neuroprotection function includes the stimulation of astrocytic mitosis, the increase in neuronal survival and the neuronal differentiation of embryonic stem cells (Passemard et al., 2011). Therefore, it is very likely that neurons are capable of producing VIP in addition to their original expression profile. Interestingly, only the over-expression of *otpb* is sufficient to induce this phenotype although its expression is limited to distinct regions in the brain. This implies that *otpa* and *otpb* are not only working in parallel but that their functions differ from each other where one is more important for the neuron type specification and the other one might be more important for the neuronal survival.

5 Conclusion

Although several transcription factors important for hypothalamus development are known, one big question remains. How do these transcription factors act in concert to form the specific nuclei of the hypothalamus and the various neuron types within them? In order to contribute to our understanding of the development of the hypothalamus, the aim of this thesis was to identify mechanisms important for the formation of distinct neuron types in the preoptic area and the posterior hypothalamus. In this thesis, I identified that a limited set of transcription factors and their dynamic interactions are crucial for the formation of these two functionally different nuclei. I found that the regulatory interactions involving Fezf2, Otp, Sim1a and Foxb1.2 vary between anterior and posterior hypothalamus resulting in subdomains with distinct transcription factor expression. Within each of these subdomains different neuron types with defined transcription factor co-expression form. Therefore, this study provides an important mechanistic insight into the formation of neuronal diversity in the developing hypothalamus. Further, the subdomains defined by the TF expression allow for the first time the generation of a regulatory and a molecular map of the mammillary area. These maps will contribute significantly to the assignment of distinct functions to different mammillary nuclei and cell types by aiding future functional investigations or interference.

The assigned genetic regulatory interactions in this thesis are based on the observed changes in the expression of the transcription factors. Therefore, it not known whether these interactions are direct or indirect. Further studies are needed to show if other transcription factors or signaling pathways are involved in these regulatory networks. Similarly, the precise mechanism by which the transcription factors regulate the specification of the neuron types need to be further investigated. Nevertheless, with this thesis, important first steps are made to define the transcription network governing the spatio-temporal development of the mammillary area. In addition, it provides an important entry point for further investigation of the hypothalamic transcription factor interactions, the role of different signaling pathways and the functional relevance of the investigated neuropeptides.

In conclusion, this thesis achieved the following major findings: First, it defined for the first time the transcriptional network that regulates the mammillary area development. Second, it identified hitherto uncharacterized regulatory interactions of the hypothalamic

regulators. Third, it identified that one important mechanism underlying the generation of hypothalamic neuronal diversity is the temporally and spatially dynamic regulatory interactions of a common transcription factor set.

6 Material and Methods

6.1 Materials

6.1.1 Buffers

Buffer	Composition
AP staining buffer	100 mM Tris-HCL pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1 % Tween20
Danieau's Solution (30x)	1.74 M NaCl, 21 mM KCl, 12 mM $MgSO_4$, 18 mM $Ca(NO_3)2x4(H_2O)$, 150 mM Hepes, adjust to pH 7.6
Egg water	3 g Red Sea Salt in 10 l dd H_2O
Gel loading buffer	50 % (v/v) Glycerin, 50 mM EDTA pH8.0, 10 mM Tris/HCl pH8.0, bromophenol blue
Hybridization mix	50 % Formamide, 5x SSC, 5 mg/ml torula RNA, 50 µg/ml heparin, 0.1 % Tween 20
Hybridization mix +D	Hybridization mix, 5 % Dextran sulfate
Lysis buffer (10x)	100mM Tris pH 8, 10 mM EDTA pH 8, 50 0mM KCl, 3 % Tween 20, 3 % NP-40
Maleic acid buffer	100 mM Maleic acid, 150 mM NaCl, adjust to pH 7.5 (with NaOH)
PBS (20x)	160 g/l NaCl, 4 g/l KCl, 28,8 g/l Na_2HPO_4, 4.8 g/l KH_2PO_4
PBT	1x PBS, 0.1 % Tween20
PBTX	1x PBT, 0.1 % Tween20, 0.1 % TritonX100
4 % Paraformaldehyd (PFA)	40 g PFA in 1 l PBS
PTU	0.03 % 1-phenyl-2-thiourea in egg water
Roche blocking solution	10 % (w/v) blocking reagent in maleic acid buffer
SSC (20x)	175.3 g/l NaCl, 88.2 g/l NaCitrate, adjust to pH 7.0

SSCT	1x SSC, 0.1 % Tween20
TAE-buffer (50x)	242 g/l Tris Base, 57.1 ml/l glacial acetic acid, 0.05 M EDTA (pH 8)
Tricaine Solution	4 g/l Tricaine powder, 25 ml 1 M Tris-HCL (pH 9), adjust to pH 7
TSA Reaction buffer	100 mM borate, 2 % dextran sulfate, 0.1 % Tween, 0.003 % H_2O_2, 150-450 µg/ml 4-Iodophenol, adjust to pH 8.5

Table 6.1: Buffers

6.1.2 Computer software

Software	Company
EndNote X5	Thomson Scientific, USA
Fiji	Max Planck Society, Germany
Generunner	Hastings Software, Inc.
Lasergene	DNASTAR, USA
Leica LAS AF	Leica Microsystems, Germany
Leica Application Suite V3.7	Leica Microsystems, Germany
Prism 5	GraphPad Software, Inc., USA

Table 6.2: Software

6.1.3 Bacterial strains

Bacterial strains used in this thesis are Top10 cells from Invitrogen.

6.1.4 Bacterial media

Medium	Composition
Luria Bertani (LB) Medium	Recipe according to Sambrook and Russel (2001)
SOC Medium	10 g/l bacto-tryptone, 5 g/l yeast extract, 0.5 g/l NaCl, 0.186 g/l KCl, 12.5 ml/l 1M $MgCl_2$, 12.5 ml/l 1M $MgSO_4$, after autoclaving add 20 ml/l 1 M Glucose (sterile filtered)

Table 6.3: Bacterial media

All media were autoclaved prior to use. Media for the plates contained in addition 20 g agar per liter. Antibiotics were added if necessary (50 mg/l Ampicillin, 25 mg/l Kanamycin).

6.1.5 Enzymes

Enzyme	Company
Big Dye Terminator V3.01	ABI Prism
DNase I	New England Biolabs
Phusion DNA Polymerase	NEB
Proteinase K	Sigma-Aldrich
Restriction Enzymes	New England Biolabs
RNA Polymerases (T7, Sp6)	Roche
RNA Polymerase (T3)	Fermentas
RiboLock RNase Inhibitor	Fermentas
T4 DNA Ligase	New England Biolabs
Taq DNA Polymerase	New England Biolabs

Table 6.4: Enzymes

6.1.6 Reaction kits

Kit	Company
ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
DIG RNA labeling mix	Roche
GenElute PCR Clean-Up Kit	Sigma-Aldrich
GenElute Plasmid Miniprep Kit	Sigma-Aldrich
mMessage mMachine Sp6, T7	Ambion
Qiagen Plasmid Midi Kit	Qiagen
Qiaquick Gel extraction Kit	Qiagen
Rneasy Mini Kit	Qiagen
PureLink RNA Mini Kit	Invitrogen/Ambion
Superscript III Reverse Transcriptase	Invitrogen
Dual Promoter TA Cloning Kit, pCRII vector	Invitrogen
Power SYBR Green RNA-to-CT	Applied Biosystems

Table 6.5: Kits

6.1.7 Instruments and special equipment

Equipment	Supplier
Bacterial incubator	Heraeus Brutschrank B 6060
Cameras	Leica DFC500
Centrifuges	Eppendorf Centrifuge 5417R, Heraeus Labofuge 400R, Beckman Avanti J-25
DNA sequencer	ABI Prism 3100-Avant Genetic Analyzer
Fish incubator	Rubarth Apparate GmbH RuMed 3101
Gel documentation system	Intas Gel iX Imager
Microinjector	Eppendorf Femto Jet, Transjector 5246
Microscopes	Leica SP5, Leica DM5500B, Leica MZ16
Needle puller	Flaming/Brown Micropipette Puller
PCR machines	Eppendorf Mastercycler epgradient, Applied Biosystems 7500 Real-Time PCR System
PH-meter	Knick pH-Meter 766 Calimatic
Spectrophotometer	Eppendorf BioPhotometer
Stereomicroscope	Leica MZ6, M3Z, Zeiss Stemi DV4

Table 6.6: Equipment

6.1.8 Fish strains

Fish strains that have been used in this study despite of the wild-type strain ABTL:

Fish strain	Reference
Tg(3kb-otpa:hsp70-EGFP)fd2	this thesis
Tg(600bp-otpa:hsp70-EGFP)fd13	this thesis
$otpa^{m866}$	Ryu et al. (2007)
Tg(hsp70:fezf2-2A-EGFP)hd6	this thesis
Tg(hsp70:otpa-2A-EGFP)hd7	this thesis
Tg(hsp70:otpb-2A-lyntdTomato)fd1	this thesis
Tg(hsp70:sim1a-2A-lyntdTomato)hd8	this thesis
Tg(hsp70:foxb1.2-2A-EGFP)hd9	this thesis

Table 6.7: Fish strains

6.1.9 Morpholinos

Morpholino based antisense oligonucleotides were synthesized by Gene Tools LLC (Philomath, OR, USA). Names, Sequences and binding sites are shown in the table below.

Morpholino	**Sequence**	**Reference**
otpb (e2i2) splice MO	5'-GAGCAAGTTCATTAAG TCTCACCTG-3'	Ryu et al. (2007)
sim1a (e2i2) splice MO	5'-TGTGATTGTGTACCTG AAGCAGATG-3'	Löhr et al. (2009)
fezf2 / sp3 (e2i2) splice MO	5'-TATTTTAACCTACCTG TGTGTGAAT-3'	Jeong et al. (2006)
foxb1.2 ATG MO	5'-TCTGGGCATCCTGTC CTAATCGCGT-3'	this thesis
Standard control MO	5'-CCTCTTACCTCAGTTA CAATTTATA-3'	Gene Tools LLC

Table 6.8: Morpholinos

6.1.10 Vectors

Standard cloning vectors:

Vector	**Company**
pCRII	Invitrogen
pCS2+	Rupp et al. (1994)

Table 6.9: Vectors

6.1.11 Constructs generated and/or used in this thesis

Construct	Reference
Hsp339-3kb-*otpa*:*hsp70*-EGFP	Ettwiller laboratory and this thesis
Hsp339-600bp-*otpa*:*hsp70*-EGFP	Ettwiller laboratory and this thesis
Hsp339-*fezf2*:*hsp70*-Dendra-ras	Dr. Josh Bonkowski and this thesis
pCS2+-ATG-EGFP	Wittbrodt Laboratory
pCS2+-*foxb1.2*MO-EGFP	this thesis
pCS2FA-transposase	Kwan et al. (2007)
pSceIn-*hsp70*:*otpb*-2A-lyntdTomato	Arturo Gutierrez-Triana, Ryu laboratory and this thesis
pSceIn-*hsp70*:*sim1a*-2A-lyntdTomato	Arturo Gutierrez-Triana, Ryu laboratory and this thesis
pT2G12A-*hsp70*:*fezf2*-2A-EGFP	Arturo Gutierrez-Triana, Ryu laboratory and this thesis
pT2G12A-*hsp70*:*foxb1.2*-2A-EGFP	Arturo Gutierrez-Triana, Ryu laboratory and this thesis
pT2G12A-*hsp70*:*otpa*-2A-EGFP	Arturo Gutierrez-Triana, Ryu laboratory and this thesis

Table 6.10: Constructs

6.1.12 Constructs for RNA Probe synthesis

Constructs for transcription factor probes:

Gene	Enzyme	RNA Pol.	Reference
pCRII-*a2bp1*	EcoRV	Sp6	this thesis
pZL-*arx*	SalI	Sp6	Miura et al. (1997)
pCRII-*ascl1a*	EcoRV	Sp6	this thesis
pCRII-*barhl1.1*	EcoRV	Sp6	this thesis
barhl2	SalI	Sp6	Colombo et al. (2006)
pCRII-*creb1*	EcoRV	Sp6	this thesis
pCRII-*dact1*	EcoRV	Sp6	this thesis
pCRII-*dbx1a*	NotI	Sp6	this thesis
pCRII-*dxb1b*	BamHI	T7	this thesis
pCRII-*dlx1a*	BamHI	T7	this thesis

pBSKS-*dlx2a*	BamHI	T7	Akimenko et al. (1994)
pBSKS-*dlx5a*	BamHI	T7	Akimenko et al. (1994)
pCRII-*dmrt3a*	NotI	Sp6	this thesis
pCRII-*dnmt4*	EcoRV	Sp6	this thesis
pBSKS-*emx2*	XhoI	T7	Morita et al. (1995)
pBSKS-*emx3*	AccI	T7	Morita et al. (1995)
pCRII-*erm*	BamHI	T7	this thesis
pCRII-*esr2b*	EcoRV	Sp6	this thesis
pCRII-*fezf2*	EcoRV	Sp6	this thesis
pCRII-*foxa1*	BamHI	T7	this thesis
pCRII-*foxb1.1*	EcoRV	Sp6	this thesis
pBSSK-*foxb1.2*	EcoRI	T7	Thisse et al. (2001)
pCRII-*foxp2*	EcoRV	Sp6	this thesis
pBSSK-*gli3*	NotI	T7	Tyurina et al. (2005)
pCRII-*grhl1*	EcoRV	Sp6	this thesis
pCRII-*gsh1*	BamHI	T7	this thesis
pCRII-*her8a*	BamHI	T7	this thesis
pCRII-*hmx3*	NotI	Sp6	this thesis
pCRII-*id3beg*	EcoRV	Sp6	this thesis
pCRII-*id3end*	BamHI	T7	this thesis
pBSKS-*isl1*	XbaI	T3	Inoue et al. (1994)
pCRII-*lcorl*	BamHI	T7	this thesis
pCRII-*lef1*	EcoRV	Sp6	this thesis
pCRII-*lhx1*	XhoI	Sp6	this thesis
pCRII-*lhx5*	BamHI	T7	this thesis
pCRII-*lhx6*	BamHI	T7	this thesis
pCRII-*lhx7*	NotI	Sp6	this thesis
pCRII-*lhx9*	KpnI	T7	this thesis
pCRII-*lmo1*	EcoRV	Sp6	this thesis
pCRII-*lmx1b*	NotI	Sp6	this thesis
pCRII-*mbd3b*	NotI	Sp6	this thesis

mcm5			Ryu et al. (2005)
pCRII-*neurod4*	BamHI	T7	this thesis
ngn1	XhoI	T7	Blader et al. (1997)
pCRII-*ngn3*	XbaI	Sp6	Wang et al. (2001)
pBSSK-*nkx2.1a*	SacII	T3	Rohr and Concha (2000)
pCRII-*nkx2.1b*	BamHI	T7	this thesis
pCRII-*nkx2.2a*	EcoRV	Sp6	this thesis
pCRII-*nr2e1*	EcoRV	Sp6	this thesis
pCRII-*nr2f1a*	BamHI	T7	this thesis
pCRII-*nr2f1b*	BamHI	T7	this thesis
pCRII-*nr4a2a*	XhoI	SP6	Filippi et al. (2007)
pCRII-*nr4a2b*	SacI	T7	this thesis
pCRII-*nr5a1a*	EcoRV	Sp6	this thesis
pCRII-*nr5a2*	NotI	Sp6	this thesis
pCRII-*nr6a1b*	EcoRV	Sp6	this thesis
olig2			Park et al. (2002)
pCRII-*otpa*	XhoI	Sp6	Ryu et al. (2007)
pCRII-*otpb*	KpnI	T7	Ryu et al. (2007)
otx1	EcoRI	T7	Li et al. (1994); Mori et al. (1994)
pCRII-*otx1l*	NotI	Sp6	this thesis
otx2	EcoRI	T7	Li et al. (1994); Mori et al. (1994)
pCRII-*pax3b*	BamHI	T7	this thesis
pCRII-*pax6a*	BamHI	T7	this thesis
pCRII-*pax6b*	NotI	Sp6	this thesis
pCMVSport-*pcna*	SalI	Sp6	Koudijs et al. (2005)
pBSKS-*pitx3*	NotI	T7	Dutta et al. (2005)
pou12			Spaniol et al. (1996)
pBSKS-*prox1*	XbaI	T3	Glasgow and Tomarev (1998)
pCRII-*rfx2*	EcoRV	Sp6	this thesis
pCRII-*roraa*	EcoRV	Sp6	this thesis
pBSSK-*rx3*	XbaI	T3	Chuang et al. (1999)

pCRII-*sall1a*	EcoRV	Sp6	this thesis
pCRII-*satb2*	EcoRV	Sp6	this thesis
pCRII-*sb:cb306*	EcoRV	Sp6	this thesis
pCRII-*sb:cb382*	BamHI	T7	this thesis
pCRII-*sb:cb458*	EcoRV	Sp6	this thesis
pCRII-*sb:eu863*	BamHI	T7	this thesis
pUC18-*shha*	HindIII	T7	Krauss et al. (1993)
pCRII-*sim1a*	XhoI	Sp6	recloned from Löhr et al. (2009)
pCMVSport-*sim2*	EcoRI	T7	RZPD clone (IRBOp991C0138D)
six3b	BamHI	T3	Kobayashi et al. (1998)
sox2	EcoRI	T7	Adolf et al. (2006)
sox3	SalI	SP6	RZPD clone (IRBOp991G0461D)
pCRII-*sox11a*	EcoRV	Sp6	this thesis
pCRII-*sox14*	EcoRV	Sp6	this thesis
pCRII-*sp8*	EcoRV	Sp6	this thesis
pCRII-*spon1b*	NotI	Sp6	this thesis
pCRII-*tbx2b*	BamHI	T7	this thesis
pCRII-*tcfcp2l1*	NotI	Sp6	this thesis
pCRII-*tcf7l1a*	BamHI	T7	this thesis
pCRII-*vgll2b*	EcoRV	Sp6	this thesis
pCRII-*zgc:103720*	EcoRV	Sp6	this thesis
pCRII-*zic2a*	BamHI	T7	this thesis
pCRII-*znf536*	EcoRV	Sp6	this thesis
pBSSK-*zp50* (*pou50*)	SpeI	T7	Hauptmann and Gerster (1996)

Table 6.11: Transcription factor probes

Constructs for neuropeptide probes:

Gene	**Enzyme**	**RNA Pol.**	**Reference**
pCRII-*aanat1*	BamHI	T7	this thesis
pCRII-*adra2a*	NotI	Sp6	this thesis
pCRII-*agouti*	BamHI	T7	this thesis
pCR-BluntII-*agt*	EcoRV	Sp6	this thesis
pCRII-*ak5l*	NotI	Sp6	this thesis
pCRII-*apelin*	NotI	Sp6	this thesis
pCRII-*ar*	NotI	Sp6	this thesis
zVasotocin (*avpl*)	EcoRI	Sp6	Eaton et al. (2008)
pCRII-*bombesin*	NotI	Sp6	this thesis
pCR-BluntII-*cck1* (now *wu:fq26c12-201*)	EcoRV	Sp6	this thesis
pCR-BluntII-*cck*	EcoRV	Sp6	this thesis
pCRII-*chat*	NotI	Sp6	this thesis
pCRII-*CRH*	NotI	Sp6	Löhr et al. (2009)
pCRII-*cyp19a1a*	BamHI	T7	this thesis
pCRII-*gad1*	BamHI	T7	this thesis
pCRII-*gastrin*	NotI	Sp6	this thesis
pCRII-*hdc*	BamHI	T7	this thesis
pCRII-*lin7a*	NotI	Sp6	this thesis
pCRII-*mmp23al*	BamHI	T7	this thesis
pCR-BluntII-*nts*	EcoRV	Sp6	this thesis
zIsotocin (*oxtl*)	SalI	Sp6	Eaton and Glasgow (2006)
pCRII-*pDYN*	BamHI	T7	this thesis
pCR-BluntII-*penk*	BamHI	T7	this thesis
pCR-BluntII-*penkl*	EcoRV	Sp6	this thesis
pCRII-*plcxd3*	NotI	Sp6	this thesis
pCRII-*relaxin3c*	BamHI	T7	this thesis
pCRII-*rhbdf1*	NotI	Sp6	this thesis
pCRII-*slc6a3*	NotI	Sp6	this thesis

pCRII-*slc18a2*	BamHI	T7	this thesis
pCRII-*slit2*	BamHI	T7	this thesis
pCMVSport-*sst1*	SalI	Sp6	Löhr et al. (2009)
pCRII-*tafa5*	NotI	Sp6	this thesis
pBS-z*TH*	XhoI	T3	Holzschuh et al. (2001)
pCRII-z*TRH*	BamHI	T7	Löhr et al. (2009)
pCRII-*Uts1*	NotI	Sp6	this thesis
pCR-BluntII-*vip*	SacI	T7	this thesis

Table 6.12: Neuropeptide probes

6.1.13 Primers

6.1.13.1 Primers for genotyping

Primer	Sequence
*net1a*_4F	ACCATTCAGAGCTGGACAGAA
*net1a*_4R	ATGTATCTGAAACGTGACGCC
MD_WT4F	GTAGCGGTCAACAGTAAGGATCAATA
MD_MUT3F	GTAGCGGTCAACAGTAAGGATCAACG
geno_2A_B	GTCACCGCATGTTAGCAGACTTCC
geno_2A_2B	CTTCCTCTGCCCTCTCCACTGC
geno_*otpa*_F	CTCTCCGGCCCCACCAATGT
geno_*otpa*_2F	CAGCTGTGTAGCTCCCCGGAC
geno_*sim1a*_F	CCTTGGGTGCTCTGGTTCCTCTC
geno_*otpb*_F	CGGTCTTCAGTCGCATCTCTATCAAC
geno_*fezf2*_F	CGGGAAAGGCTTTTGCAGGAAC
geno_*fezf2*_2F	CACATACGGAAATTGCACGATAACG

Table 6.13: Primers for genotyping

6.1.13.2 Primers for Probe synthesis constructs

Primers for probes detecting transcription factors:

Primer	Sequence
*a2bp1*_F	GCAACAGCACGGGTAATGACGAAT
*a2bp1*_B	GCGCGAAACGACTGTATCCACCT
*ascl1a*_F	CAAGATGGAAATAAGCGTAAACCAGCAG
*ascl1a*_R	GTTCTCTCTCGTTCCGTCTGGCC
*barhl1.1*_F	CAGCAGTGTGGAGATGGAAGCGT
*barhl1.1*_B	ACCTGCGGGCTGTCTCTGCTG
*creb1*_F	CTCCACTCCAGCCATAACAACCGTT
*creb1*_B	GGGCAGTGCTGGGGAAGAGG
*dact1*_2F	GAGACACCATGGTCATCACCGACA
*dact1*_2B	CTGTGCTGACGTCATTCCATGGG
*dbx1a*_F	TCATCTTTTAATAGCCGGGACCATG
*dbx1a*_B	AATGGGCACGACCGATGAAGG
*dbx1b*_F	GCCACCTGCCATGTATCCGAG
*dbx1b*_B	CCTCGGCTTCCCTCGTGCTG
*dlx1a*_F	TGACCATGTCTACAATACCAGAGAGTCTAAACA
*dlx1a*_B	TCTGACTCCGGTGCTGGGTCC
*dmrt3a*_F	CCCACTGGAGGTCCTGAAGAAGAT
*dmrt3a*_B	CGATGCTCCTCACTGGGTCTGC
*dnmt4*_F	AGTCGACTGAAATAGTGATGCCATCCA
*dnmt4*_R	TCAGTAGTGGAGGTGGATGTGGTGC
*erm*_F	CTCCAAATAAGTCTCACATGCAGGAACC
*erm*_R	CGATGGAACCTGTGGTCTGTGCTG
*esr2b*_F	TGGACTCCAGCAAGGCCGATC
*esr2b*_B	CTCCTTTACCCACAATGCTCGCG
*fezf2*_F	ACAGTGATGTCCTGCCCGAGACTAGAC
*fezf2*_B	TGTGGTTCTCCCGCACGATTTG
*foxa1*_F	CCAGGCCTGTTCTCAATACATCGTAGTC
*foxa1*_B	ATACATATTTTATTCACAATGCATTTCCACC
*foxb1.1*_F	TACCTCCAACAACAAGCAAAACTCCG

*foxb1.1*_B	TCTTGGGCAGTGCACGGCG
*foxp2*_F	GGAGTCGGCCAATGAGACAATAAGC
*foxp2*_R	CCAGCTGGCGGCAGAGTCTGT
*grhl1*_F	TGCCCAATGATCCTGCGTACAAC
*grhl1*_B	GGTTGTGGGGCAGCTGGCG
*gsh1*_F	CATTTGGTACACGAGCGAGACTGA
*gsh1*_B	GCTGCTCTGAAGTTGACTGCTGCTAT
*her8a*_F	GCCCTCTCCCTCTGTCCCCG
*her8a*_B	TTGGTGTCATCTGTGTTCATGCCC
*hmx3*_F	CGAATATCAAGCCAACATGCCC
*hmx3*_B	TCGTCTTCATCGTCCTTGGCG
*id3*_beg_F	AGACCCAGAATGAAGGCGATCAGTC
*id3*_beg_R	CTCGGAAGGACTCTTGCAGCGG
*id3*_end_F	GATAGCGCTGGAAAACGAGACAGACA
*id3*_end_R	CATTCATCCATCCTAATGGCACATAGCT
*lcorl*_F	CGACCCAGTGCCGGAGCTC
*lcorl*_B	ACAGTGAGGTCCAGAGGCCCATC
*lef1*_F	CAGGATGGTCTGCCAGAGACAATTATG
*lef1*_B	GCAAGTGAGCGAGTGGACATAGTGAAC
*lhx1*_F	TGGAGATTATCAAAGCGAGTACTACGGC
*lhx1*_B	CATGCTGCCCTCTACTGCTCTCTCC
*lhx5*_F	GCTCAGTGCTCTCGGTGCTCG
*lhx5*_B	AAGGTAGACTGGTTAGGTTGGAATCCC
*lhx6*_F	GACTCAAGCAACTGCATCCCAAAAG
*lhx6*_B	GCGCACACGTTCTTCCCGGT
*lhx7*_F	CTCTTCAGCTGCACCAGTGTCATCA
*lhx7*_B	ATCTTTGCAAATCCTTCAGTAAGTGCC
*lhx9*_F	CTTCAGTTTTGCCTTATATCATCCGGA
*lhx9*_B	AGGCTTCTCTGGGCTCATGGAG
*lmo1*_F	GATTTCTTGAATATAGTTACAGTTTCGGAGCA
*lmo1*_B	TCGGCACACCCTCTTCCTTGTCTA
*lmx1b*_F	GGAGATGAGTTTGTACTGAAGGACGGAC
*lmx1b*_B	CACCTCGAACGACGCCTTGAAG

*mbd3b*_F	ACAGACAGAGACCCAGATACGACAACAAC
*mbd3b*_B	AACATGTCAGCCATCAAAGCCTCCT
*neurod4*_2F	TTTCAGAAGTTCTGGAGAGCGGTCA
*neurod4*_2B	TTCTGCTTCACGAGGTGATGGCT
*nkx2.1b*_F	GCGCACTCATACGTTTCCATCCT
*nkx2.1b*_B	CCTTTACCAACATCCGCCAACG
*nkx2.2a*_F	GCTCCAGAACATGTCGTTGACCAAC
*nkx2.2a*_R	GCTTCTTACCAGAGTCGCTGCCG
*nr2e1*_F	AGCACGAGAGGGGACCGAGGA
*nr2e1*_R	ATTCGCACACAGACTCGGTCGC
*nr2f1a*_F	AATTTATTTTGCACAGCACTTTGGATC
*nr2f1a*_B	GCTGACGCCGCCTGCTGT
*nr2f1b*_F	GAGTAAAAGTGCGGTCATGTACATCTCC
*nr2f1b*_B	TGGTTTTGCGCCGCGATC
*nr4a2b*_F	TGTGCGGAGCCATGCCCTG
*nr4a2b*_R	CTGCTTGAACGAGAATAACCGAGACATC
*nr5a1a*_F	GAGGCTGTGCGTGCAGACCG
*nr5a1a*_B	TTTCCTCTGCCGCTCTGCTCCT
*nr5a2*_2F	CCCAGCAGACCTCACCATTACCTC
*nr5a2*_2B	TCGACTGGCCTGTTCCTGCTG
*nr6a1b*_F	CGGGATGCCAGGAGGAAGGAA
*nr6a1b*_B	CTCCGACTGAGTCACGCTGTACCC
*otx1l*_F	GCAGCAGAGCAGCACCAACTCAA
*otx1l*_B	GCCTCCGAAATAAGAAGCAGCGTT
*pax3b*_F	ATGCCATGTCGAGTCTGTCCCC
*pax3b*_B	GAGGAGGATGAAGTATGCTGTCAGTAGTAAT
*pax6a*_F	ACAAGCCAGTAATTCCTCAAGTCACATAC
*pax6a*_B	TGTTCTCTTTTATTCTTCAAGTCTTTGTGTCC
*pax6b*_F	CTTCACGCAGGAGCAGATCGAG
*pax6b*_B	ACATGTCAGGCTCAGTTCCGGG
*rfx2*_F	TTCGTCTGAAGCCGGACTCGC
*rfx2*_B	TCTGATGGCTTGAGTGAGGGTGCT
*roraa*_F	AAGCTTGACATTCCCCACGGTGT

*roraa*_B	ATCGCCCTGTTCTCCGCCTTC
*sall1a*_F	CCTAGCCGAGCACAATGGGGAC
*sall1a*_B	AGAAGAATCTGGTGACGAATCTGCTCTATG
*satb2*_F	GATGATTCCTGTCTTCTGCGTGGTG
*satb2*_B	TCTTTGAGGGCGTTGCGTACTGTT
*sb:cb306*_F	CTGGGGTTTGTAGAAACGTCTGAATGC
*sb:cb306*_B	GCATTAAAACAGAAGTACCTCAACTCACTGATTAC
*sb:cb382*_F	CTGAATTCTTATAATCTCTCACCAAACCTACTTTTC
*sb:cb382*_B	TAAGTTTGTGGATTTGAAAGGTCTGTTATGAG
*sb:cb458*_F	TGTGTCTCCAGAGCCCAGTCCAG
*sb:cb458*_B	AAGGTTTTCATTTGAAGGAGACTTTACACTC
*sb:eu863*_F	AGTCCACCTGCTTTACATCCCCATG
*sb:eu863*_B	GCCATGTTCCTCTGCTCTGAATGAG
*sox11a*_F	CGAAGAAGAAACCAAAGCTGGACAGTT
*sox11a*_B	CGAAGTTCAAACTGAAGTCAAAAAGCAAG
*sox14*_F	CCTGGGCGACACGGATCACC
*sox14*_B	TGGAGTCACATAGCAGCGGCGT
*sp8*_F	AGCTGCTCTCTCTCCCCGTTCG
*sp8*_B	TGAGTGAGGGAGGGATAGTCTGAGTTATAGC
*spon1b*_F	TAATACACATACACACACATACACATGCCAG
*spon1b*_B	CCGGATTAGAAACTGAAATGCTGACA
*tbx2b*_F	GCTGACCCTTCCATCATTACGCA
*tbx2b*_B	GAATTGTTGACTGTGTAGGCCGGAG
*tcfcp2l1*_F	TGTGTTGACTGAGTGCTCCCCTTG
*tcfcp2l1*_B	CTGATAGTGAAGCTTGCCTCCTCTGTAA
*tcf7l1a*_F	TCAGCAAGGCCAGCATATGTACTCC
*tcf7l1a*_B	TCTTCCTCTTTCTTCACGGGCACC
*vgll2b*_F	CCTCAAGCAGTCGGCGCAGTT
*vgll2b*_B	CGTTCAGAGCGTGTGCAGAGCC
*zgc:103720*_2F	GCCTGTTCCACCAGTGCTCTATCTG
*zgc:103720*_2B	CATATTATATCCAGAAAATGAACAGTGCACAATA
*zic2a*_F	TGTTACTGGACGCAGGGCATCA
*zic2a*_B	CAGCTGGGCGGCCGAGTAA

*znf536*_F	GCATCTCAACAAGCTGGCCATCA
*znf536*_B	CAGCTGCATTGGCAAGTTCATCTTC

Table 6.14: TF probe primers

Primers for probes detecting neuropeptides:

Primer	Sequences
*aanat1*_F	CAGGGTCCTTCTGAAATCATTGTAGGG
*aanat1*_B	GGAAGGTACATTAAAAGTTTGTCACATTGG
*adra2a*_F	GCCGGAGAGATAAGAGGAGAGTGGTG
*adra2a*_B	GACATCGACATGCACGTTGAGGC
*agouti*_F	AAGCTGTCCACCTGCAGAGAAG
*agouti*_B	ACACCTTAAAACCGCAGCCAAT
*agt*_fwd	GATTACCCGACAAGATGAAGATGTTCCTC
*agt*_rev	AAATAGAGGGTCACCAATGCACCGAAT
*ak5l*_F	ATGCCGATCGAGAAGAGGATGACAT
*ak5l*_B	CACTATTTCTGGCTTGAGCTCCAAATATAC
*apelin*_F	CATAGCACCTGCTGGTTCTTCA
*apelin*_B	CTGGGCTTGAGCATTTAGCTGT
*ar*_F	ATATGGACGGACCGCGAGCG
*ar*_B	CCTCCCTCCGTCAAACCTGCC
*bombesin*_F	GGGGAAGAAAAGTGTCGTGGAC
*bombesin*_B	GACGTTCGGGCGGATGTG
*cck1*_fwd	TCACAAAACTGAATAAGGATGAACAGCGG
*cck1*_rev	AGGAGCCGCATTTCTGTATTTATATGGTTTA
*cck2*_fwd	ATGAACGCTGGACTCTGTGTATGTGCC
*cck2*_2_rev	TATTCCTCGGCGCTTCGTCGG
*chat*_F	CACTGAGGAGATGTTCTGCTGCTATG
*chat*_B	GACTTGCTTCCATTCTTCATTACCTTAGG
*cyp19a1a*_F2	ATCCGTTCTTATGGCAGGTGATCTG
*cyp19a1a*_B2	AATGTGTGATTTGTGTGGTCGATGG
*gad1*_F	CGGAGACGAGCGAAGGGAGAAG
*gad1*_B	CCCACCACTGGTTTACACACAGACC

*gastrin*_F	CTGTGTGGTTTCGCTCACAGAC
*gastrin*_B	AGCTGCACATGAGGTTTTCTCC
*hdc*_F	GCGACGCAGGAGCTACTGAGGAA
*hdc*_B	GCTGGCACGGCTGGTGATCA
*kctd7*_F2	GGAGGACACCCAGCCGCTG
*kctd7*_B2	CAGACGCCGATGCATTGCTG
*lin7a*_F	CTGGAGGAAATGGAGGCGAGATT
*lin7a*_B	CAGAGACACCAGCACCAAAAGAACC
*mmp23al*_F	TGCCTCGTGGAAAAGTTGTGGG
*mmp23al*_B	GACACGTGTGACTGTGAGCGGC
*nts*_fwd	CAAACAACATGCAGATGCAGCTGACC
*nts*_rev	CAGTAAATTACGCTTCTCTTGAGAATGTACGG
*pDYN*_F	CGGAGAAACAAACGCGTTAAACAC
*pDYN*_B	ACTTCATTATCGTAAACTCCCGTTTCGT
*penk*_fwd	CAGTAATGAAGACCTTGACAGTTCCCATAAG
*penk*_rev	ATCATCTTAATATCCCATAAATCCACCATACCTCT
*penkl*_fwd	ATGGCGTTAATGATGAACTCCTGGTG
*penkl*_rev	TCTAATCCATGAATCCTCCGTATCTCTTCTG
*plcxd3*_F	GGTCTTCTATCACAATCCAATGGCTCTG
*plcxd3*_B	GTATATGTGGAGGTTCAGGTGGCG
*relaxin3c*_F	GAATCTCCAAAACTGGCTGGAT
*relaxin3c*_B	TTCATGGTGCATCCAGAAGTACA
*rhbdf1*_F	AGAGGCAAGGTTTCCACCGCAG
*rhbdf1*_B	CCCTTGACCAGAGCTGCCGC
*slc6a3*_F	GAGAGGCAGACCGGCGGTC
*slc6a3*_B	ACCTCCAGACGTTGGCCAGATC
*slc18a2*_F	AGGCACGTGTCGGTGTATGGAAG
*slc18a2*_B	GACATACATGGCCGGAGGTTAGGAG
*slit2*_F	CTTTGACACACTACATTCGCTCTCCACA
*slit2*_B	ATTACCATGGAGAGACAACAAGCGCA
*tafa5*_F	TTGTGCCACGGAGAGCATACTA
*tafa5*_B	CGTTTCAGTGTTCCATGTCAGC
Uts1 Probe F	TCCCATTGGTCCTGCTCA

Uts1 Probe B	GTGTGAATGTCGGATGCGTTAGC
*vip*_fwd	AATGCTTGTGCGGAACGGCTCTC
*vip*_rev	TGATTTCTTGCGTTCGGACTGACCTC

Table 6.15: Neuropeptide probe primers

6.1.13.3 Primers for cloning genes

Primers for the generation of the over-expression constructs:

Primer	Sequence
*otpa*_ATG_AscI_F	GGCGCGCCATGCTTTCGCATGCCGACCTGCTG
*otpa*_Stop_SalI_B	GTCGACGGTGAAGCTCATGGACACTGTGTG
*sim1a*_ATG_AscI_F	GGCGCGCCATGAAGGAGAAGTCGAAAAACGCG
sim1a-STOP_SalI_B	GTCGACGCTGCCATTGGTGATGATGACG
*otpb*_ATG_AscI_F	GGCGCGCCATGCTCTCTCATGCCGACCTCTTG
otpb-STOP_BspEI_B	TCCGGAAGTGAAACTCATTGAAACGGTGTGCTC
*foxb1.2*_ATG_AscI_F	GGCGCGCCATGCCCAGACCCGGGAGAAACA
foxb1.2-STOP_salI_B	GTCGACGTGCACCGCGACCGACTGCA
*fezf2*_ATG_AscI_F	GGCGCGCCATGCTCACACCAAGTTGGGTAGAAGT GG
fezf2-STOP_salI_B	GTCGACGTTTTGGTGTCCTCTGGAAGAATCGTTT

Table 6.16: Full lenght primers

6.1.13.4 Sequencing primer

For sequencing reaction the standard primers M13 fwd, M13 rev and T7 have been used.

6.1.13.5 Primers for MO control

Primer	Sequence
*sim1a*_splice_F	CGGCGGGAGAAGGAAAACAG
*sim1a*_splice_B	ACCACCGCACGTCAATCCTG
*otpb*_MO_fwd	ATACTCGCCTCGGTGAGTTATCGCCAC
*otpb*_MO_rev	TGGCGGACCCGCTCCGAGACT
geno_*fezf2*_wt_1F	CGCGGCCGTTCGTGTGTAAAGTAT
*fezf2*_stop_SalI_B	GTCGACGTTTTGGTGTCCTCTGGAAGAATCGTTT

Table 6.17: MO control Primers

6.1.13.6 Primers for constructs generated in this thesis

Primer	Sequence
600uc_fwd	AAGCAGCAGATGAATTCGGGTTCCAT
600uc_rev	GCCGTACCTGTGAGGACTTTCCATTCA
3kb_fwd	TTAGTCAGTTTGATTTGATTGAAGTTGGAATGACT
3kb_rev	CCAGATTAATAAAGGGACTATAAGCCGAAAATAAA
MO_*foxb1.2*_5'	AATTCACGCGATTAGGACAGGATGCCCAGAG
MO_*foxb1.2*_3'	AATTCTCTGGGCATCCTGTCCTAATCGCGTG

Table 6.18: Cloning primers

6.1.13.7 Primers for real time PCR

Primer	Sequence
*ef1alpha*2f	CTGGAGGCCAGCTCAAACGT
*ef1alpha*2r	ATCAAGAAGAGTAGTACCGCTAGCATTAC
*otpa*4f	CTTCGCCAAAACCCACTATC
*otpa*2r	ACACGTTGGTGGTCTTCTTGCG

Table 6.19: Real time PCR primers

The primer were designed and tested by Chen-Min Yeh (Ryu Laboratory).

6.1.13.8 Other primer

Primer	Sequence
*hsp70-4*_NotI_F	GCGGCCGCTCAGGGGTGTCGCTTGGTTATTTC
*hsp70-4*_XhoI_B	CTCGAGCAGGAAAAAAAAAAACAATTAGAATTAATT TTATATTTATAC
BamHI-Lyn_F	CGCGGATCCATGGGCTGCATCAAGAGCAAGC
RV.XbaI.Tomato	TGCTCTAGATTACTTGTACAGCTCGTCCATG

Table 6.20: Other Primers

6.1.14 Primary and secondary Antibodies

Primary Antibody	Source	Used Dilution	Provider
αOtpa	rat	1:500	S. Ryu, Ryu Laboratory (unpublished)
αOXTL	rabbit	1:500	S. Ryu, Ryu Laboratory (unpublished)
αAVPL	rabbit	1:500	S. Ryu, Ryu Laboratory (unpublished)
αGFP	chicken	1:1000	Abcam

Table 6.21: Antibodies

Secondary Antibody	Source	Used Dilution	Provider
Alexa Fluor 488 αchicken	goat	1:1000	Invitrogen
Alexa Fluor 546 αrat	goat	1:1000	Invitrogen

Table 6.22: Secondary Antibodies

6.1.15 Other reagents

Reagent	Company
Agarose (low melting)	Roth
Agarose (standard)	Starlab
Alexa 488 tyramide (TSA Kit #12)	Invitrogen / Molecular Probes
Alexa 555 tyramide (TSA Kit #41)	Invitrogen / Molecular Probes
Alexa 647 tyramide (TSA Kit #16)	Invitrogen / Molecular Probes
Anti-Digoxigenin-AP Fab fragment	Roche
Anti-Digoxigenin-POD Fab fragment	Roche
Anti-DNP-POD	Perkin Elmer
Anti-Fluo-POD, IgG fraction	Invitrogen
Blocking Reagent	Roche
Bovine Serum Albumin	Sigma-Aldrich
Dextran sulfate sodium salt	Sigma-Aldrich

DMSO	Sigma-Aldrich
DNA ladder (1 kb)	Fermentas
DNP-11-UTP	Perkin Elmer
dNTPs (dATP, dTTP, dGTP, dCTP)	Fermentas
DyLight 633 NHS Ester	Thermo Scientific
Ethidiumbromide	Gerbu
Fluorescein-12-UTP	Roche
Glas capillaries TW100F-4	World Precisions Instruments, Inc.
4-Iodophenol	Sigma-Aldrich
Normal sera (goat, sheep)	PAA Laboratories GmbH
NTPs (ATP, TTP, GTP, CTP)	Fermentas
Phenol red	Sigma-Aldrich
PTU	Sigma-Aldrich
RNA loading dye	Ambion
RNA from torula yeast Type VI	Sigma-Aldrich
Tyramine hydrochloride	Sigma-Aldrich
5(6)-FAM, SE	Invitrogen/Molecular Probes
5(6)-TAMRA, SE	Invitrogen/Molecular Probes
Tricaine	Sigma-Aldrich
Tween20	Roth
TritonX 100	Roth
Trizol	Invitrogen

Table 6.23: Reagents

Standard chemicals like salts and solvents were ordered from Roth, Applichem, Merck and Sigma.

6.2 Experimental procedures

6.2.1 Fish maintenance

Zebrafish breeding and maintenance was performed under standard conditions at 28.5 °C according to Westerfield (2000). The Embryos were staged after Kimmel et al. (1995) and fixed at desired developmental stage with 4 % PFA. To avoid pigmentation embryos were kept in egg water with 0.2 mM 1-phenyl-2-thiourea (PTU).

6.2.2 Molecular biological methods

6.2.2.1 Standard techniques

Standard techniques like DNA and RNA quantification, isolation and precipitation, digests and ligation were performed according to Sambrook and Russel (2001) if not indicated differently. The following section describes other methods more in detail.

6.2.2.2 Polymerase chain reaction (PCR)

The amplification of DNA fragments by PCR was carried out according to Sambrook and Russel (2001). Primers were designed using the software Gene Runner and ordered at MWG-Biotech. Taq DNA polymerase was used as standard Polymerase. PCR reactions that required proof reading activity were performed by using Phusion as DNA Polymerase.

Thermal cycler program: standard PCR

1	95 °C, 5 min
2	95 °C, 30 s
3	optimal annealing T, 30 s
4	72 °C, 1 min/kb
5	go to step 2, 30-42x
6	72 °C, 5-10 min
7	10 °C, forever

Table 6.24: Standard PCR conditions

6.2.2.2.1 Real time PCR

The real time PCR was carried out according to the Power SYBR Green RNA-to-CT 1-Step Kit manual. Primers were designed using Primer 3 and ordered at MWG-Biotech.

The primers (see table in 6.1.13.7) used for the real time PCR were designed and tested by Chen-Min Yeh (Ryu Laboratory).

6.2.2.3 Gel electrophoresis

DNA and RNA gel electrophoresis using TBE buffer was performed as described in Sambrook and Russel (2001). For standard low resolution gels 1 % agarose gels were used. To achieve a higher resolution for small PCR products 2 % agarose gels were made. Before hardening of the agarose 0.5 µg/ml ethidium bromide was added to stain the DNA or RNA. For RNA gel electrophoresis RNA loading dye was used and the RNA + loading dye mixture was cooked for 5 min at 98 °C prior to loading.

6.2.2.4 DNA purification

6.2.2.4.1 Plasmids in bacterial cultures

Plasmids from bacterial overnight cultures have been purified following the instructions of the GenElute Plasmid Miniprep Kit or the Qiagen Plasmid Midi Kit manual.

6.2.2.4.2 Gel extraction

DNA fragments from gels have been purified with the QIAquick Gelextraction Kit (Qiagen) according to the manufacturers protocol.

6.2.2.4.3 PCR product purification

For the purification of PDR products the GenElute PCR Clean-Up Kit (Sigma-Aldrich) was used following the Kit instruction.

6.2.2.5 Sequencing

In the beginning of the project sequencing was done in house. The sample preparation was performed following the instruction of the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were analyzed using SeqMan Pro within the Lasergene Software (DNASTAR). In later phases of my project Sequencing was done by a commercial sequencing service (GATC Biotech AG, Konstanz).

6.2.2.6 Isolation of total RNA from embryos

Total RNA was isolated using either Trizol reagent in combination with the PureLink RNA Mini Kit (Ambion) for pure RNA extraction or the RNeasy Mini Kit (Qiagen) for standard RNA isolation according to the manufacturers instruction. RNA was isolated from single embryos or from a pool of 30 embryos. The homogenization of the tissue was

done using Trizol or lysis buffer supplied by the Kit utilizing a 27G needle and a 1 ml syringe (BD Plastipak). The quality and the amount of the isolated RNA was assessed by spectrophotometry. RNA samples were stored at -80 °C.

6.2.2.7 First-strand cDNA synthesis

First-strand cDNA was synthesized from total RNA using Superscript III reverse transcriptase (Invitrogen) following the manufacturers protocol. The RNA amount used for the synthesis varied from 500 ng to 1 µg. Random primer (Invitrogen) were employed to prime the synthesis reaction. The cDNA was stored at -20 °C or directly used for PCR (RT-PCR).

6.2.2.8 In vitro transcription to generate RNA hybridization probes

Labeled RNA probes were generated by in vitro transcription. To incorporate into the probes Dig-11-UTP (Dig labeling mix, Roche), DNP-11-UTP (Perkin Elmer) or Fluorescein-12-UTP (Roche) the RNA polymerases SP6, T7 and T3 were used. For these reactions the nucleotide mix contained 10 mM ATP, CTP, GTP and 6.5 mM UTP combined with 3.5 mM labeled UTP. For the generation of ”run off” transcripts linearized and purified plasmids were used as templates. The in vitro transcription and the subsequent precipitation of the labeled RNA was performed according to the instruction provided with the Dig labeling Mix (Roche). The quality of the RNA probe was monitored by gel electrophoresis. To avoid RNA degradation the probes were resuspended in 50 % Formamide. For the RNA hybridization probes used in this thesis constructs, restriction enzymes and RNA polymerases are listed in the tables in section 6.1.12.

6.2.2.9 In vitro transcription to generate capped mRNA

To generate full length capped mRNA the plasmids pCS2FA-transposase and pCS2+MO*foxb1.2*-EGFP were utilized. For the synthesis of the capped mRNA the plasmids were linearized and purified. The following in vitro transcription was carried out according to the manual of the mMessage mMachine (SP6 or T7 RNA Polymerase, Ambion). The template DNA was removed using 2 U DNase I (15 min, 37 °C) and the RNA was subsequently precipitated using the LiCl solution supplied with the kit and stored at -80 °C. The amount and quality of the synthesized capped mRNA was assessed by spectrophotometry and gel electrophoresis.

6.2.3 Cloning

6.2.3.1 TA cloning of PCR products

Full-length PCR products have been amplified using Phusion DNA Polymerase while PCR products for probe synthesis were amplified using Taq DNA Polymerase. Since the Phusion is not generating PCR product with a 3' A-overhang an A-tailing reaction using Taq was subsequently performed. The generated PCR products were ligated according to the Dual Promoter TA cloning Kit (Invitrogen) instruction into pCRII. Full-length fragments were sequenced after the cloning procedure.

6.2.3.2 Directional cloning into plasmid vectors

For the generation of constructs expressing EGFP under the control of the *otpa* 600 bp and 3 kb enhancer element in combination with the *hsp70* heat shock promoter (Halloran et al., 2000) as basal promoter, the enhancer elements were PCR amplified (primer see section 6.1.13.6) and by blunt end cloning inserted into the Hsp339 (obtained from Ettwiller Lab) containing EGFP and the *hsp70*. For the transgenic lines over-expressing full-length cDNAs of *otpa*, *otpb*, *sim1a*, *fezf2* and *foxb1.2*, the viral 2A peptide (Tang et al., 2009) was placed between the cDNA and EGFP or tdTomato to report the expression of the transgene and placed after the *hsp70* heat shock promoter (Halloran et al., 2000) in a multicistronic gene expression cassette. The full-length cDNAs of *otpa*, *otpb*, *sim1a*, *fezf2* and *foxb1.2* were PCR-amplified and cloned into pCRII vector (Invitrogen) using the primers listed in 6.1.13.3. By using the restriction sites added by the PCR the cDNAs were cloned in frame into pT2G12A and pSceIn-lyntdTomato (A. Gutierrez-Triana, Ryu laboratory) were the *hsp70* heat shock promoter was inserted before (primer see 6.1.13.6). To add the *foxb1.2* morpholino binding site into the pCS2+-EGFP(-ATG) in frame two complementary oligos with an added restriction site were ordered (see 6.1.13.6). The oligos were phosphorylated and subsequently annealed by which as double stranded oligo with stick ends was generated. This oligo was ligated into pCS2+-EGFP(-ATG) to achieve pCS2+-*foxb1.2*MO-EGFP.

6.2.4 Genotyping

To genotype adult fish the fish were anesthetized in 1x Tricaine in system fish water, placed onto a piece of Parafilm and a small piece of the caudal fin was cut using a scalpel. To the fin 100 µl of 1x lysis buffer were added, the fin was heated to 98 °C for 10 min and after cooling down 5 µl of 20 mg/ml Proteinase K solution were added. After an overnight incubation the proteinase K was heat inactivated (98 °C, 10 min). Up to 2 µl of this solution could be used for a PCR reaction. To genotype embryos that underwent

an *in situ* hybridization the tails of the respective embryos were cut by a scalpel and treated in the same way as the fin of an adult fish (50 µl lysis buffer and 2.5 µl Proteinase K). The primer used to genotype adult fish or embryos are listed in 6.1.13.1.

6.2.5 Phenotypical analysis of zebrafish embryos

6.2.5.1 Whole mount in situ hybridization (WISH)

WISH was performed according to (Hauptmann and Gerster, 1994) using the alkaline phosphatase based color reaction. The Proteinase K (1 mg/ml) digestion times differed according to the developmental stage. 1 dpf old embryos were digested for 3 min, 2 dpf embryos were digested for 30 min and 3 dpf old embryo were digested for 75 min. Stained embryos have been stored in 4 % PFA at 4 °C until analysis.

6.2.5.2 Whole mount fluorescent in situ hybridization (FISH)

Two color and three color FISH have been carried out according to (Lauter et al., 2011). In the beginning of my thesis Tyramides from the commercial TSA Kits # 12, 16, 41 (Invitrogen) were used. Later in the project the Tyramides were synthesized as described in (Lauter et al., 2011; Speel et al., 1998) to achieve a higher Tyramide concentration and therefore a better signal amplification. The Proteinase K digest was performed as described in section 6.2.5.1. Stained embryos should not be stored longer than 1 week in PBS before imaging.

6.2.5.3 Whole mount immunohistochemistry (IHC)

Fluorescent immunohistochemistry was performed adapting an existent protocol from (Solnica-Krezel and Driever, 1994). The rehydration, Proteinase K digest and the postfixation were performed as in standard WISH. For blocking normal goat serum (10 % in PBTX) was used for 2 h at room temperature. The incubation with the primary antibody over night at 4 °C was also carried out in 10 % NGS in PBTX. Subsequent washing steps at least 6 - 8 times throughout the whole day were performed in 1 % NGS in PBTX as well as the over night incubation of the fluorescent secondary antibody. The following washing steps were carried out in 0.1 % NGS in BPTX. Stained Embryos could be stored in PBTX for several days before the analysis.

6.2.6 Perturbation of gene expression (in vivo)

6.2.6.1 General procedure of microinjection

Glass capillaries with inner filaments (TW100F-4, World Precisions Instruments, Inc.) were used to make the microinjection needles using a micropipette puller (Flaming / Brown). The DNA and RNA samples or the morpholinos were diluted in water with 0.05 % Phenol red and loaded into the needle. Grooves in 1 % Agarose in 0.3x Danieau's were used to immobilize the eggs for injection at 1 cell stage. To determine the injection volume a scale in the objective was used to estimate the diameter of the injected drop.

6.2.6.2 Morpholino injection

Morpholinos used in this study were synthesized at Gene Tools LLC. The *fezf2* MO(Jeong et al., 2006), the *otpb* MO (Ryu et al., 2007) and the *sim1a* MO (Rupp et al., 1994) have been published recently and the indicated amounts of the MO's were used. To control if these MO's work in my hands I tested them by RT-PCR by using cDNA from MO injected embryos (primer listed in 6.1.13.5). Since no *foxb1.2* MO was published so far and the *foxb1.2* gene has no introns I ordered an ATG MO at Gene Tools LLC. To confirm the efficiency of the *foxb1.2* MO, it was tested by using capped mRNA of a construct containing the *foxb1.2* MO binding site in frame with EGFP as reporter (the construct is listed in 6.1.11). No EGFP expression in MO-injected embryos was detected.

6.2.6.3 mRNA injection

30 pg of capped mRNA of pCS2+-*foxb1.2*MO-EGFP were injected either with 1, 2, 4 and 8 ng of the respective MO or without MO to examine the function of the *foxb1.2* MO. After injection the quality of the mRNA in the injection mix was verified by gel electrophoresis.

6.2.6.4 Injection for the generation of transgenes

For the generation of stable transgenic lines constructs containing two flanking Tol2 transposase binding sites were co-injected (20 pg) with capped mRNA (100 pg) from pCS2FA-transposase (Kwan et al., 2007) while constructs containing a I-SceI restriction site were injected (17-30 pg) after the performance of a I-SceI digest.

6.2.6.5 Heat shock treatment

If not indicated differently heat shocks for embryos fixed at 2 dpf and at 3 dpf were given starting at the evening before fixation for over night. Embryos dedicated for the

heat shock experiment were cooled to RT and most of the egg water was removed before prewarmed (42 °C) egg water with 1x PTU was added and the embryos were incubated at 37 °C. After heat shock the embryos were sorted by color and fixed at the desired developmental stage.

6.2.7 Microscopic analysis

6.2.7.1 Embryo embedding procedure

Fixed embryos that were analyzed under the DIC microscope were embedded in 100 % glycerol while fixed embryos analyzed under the confocal microscope were embedded in 80 % buffered glycerol. Living embryos were anesthetized in 1x Tricaine in egg water or analysis under the fluorescence microscope and for confocal imaging embedded in 0.8 % low melting Agarose in 0.3x Danieau's with 1x Tricaine.

6.2.7.2 Imaging

For DIC light images of fixed WISH embryos the Leica DM5500B automated upright microscope system equipped with the Leica DFC500 camera has been used. To screen for stable transgenic lines and to determine the quality of FISH and of fluorescent IHC the Leica MZ16 was used. Confocal stacks of FISH and fluorescent IHC embryos were recorded at the Leica SP5 laser scanning confocal microscope

6.2.8 Quantitative and statistical analysis

To analyze changes in cell number fixed and stained embryos were analyzed at the DM5500 DIC microscope. The cell numbers of the embryos were analyzed and significance of difference was evaluated by using Prism5 GraphPad.

Abbreviations

A-P	anterior-posterior
ACTH	adrenocorticotropic hormone
AD	anterior-dorsal
Amp	ampicillin
aPV	anterior periventricular nucleus
ARC	arcuate nucleus
AVP	arginine-vasopressin
Avpl	arginine-vasopressin like
bHLH	basic helix-loop-helix
Bmp	bone morphogenetic protein
bp	base pairs
°C	degree Celsius
Cck	cholecystokinin
CNS	central nervous system
CRH	corticotropin-releasing hormone
D-V	dorsal-ventral
DA	dopamine
Di	diencephalon
DNA	deoxyribonucleic acid
dpf	days post fertilization
Fezf2	forebrain embryonic zinc finger-like 2
Fgf	fibroblast growth factor
Fig.	figure
FISH	whole-mount fluorescence in situ hybridization
GFP	green fluorescent protein
GHRH	growth hormone-releasing hormone
GnIH	gonadotropin-inhibiting hormone
GnRH	gonadotropin-releasing hormone
GOF	gain of function
Hb	hindbrain
HD	homeodomain

Hdc	histidine decarboxylase
Hh	hedgehog
HPA	hypothalamic-pituitary-adrenal gland
hpf	hours post fertilization
hsp	heat shock promoter
KAN	kanamycin
kb	kilo base pairs
lacZ	β-galactosidase from lac-operon
LB	Luria Bertani
LHA	lateral hypothalamic area
LOF	loss of function
M	molar
mM	millimolar
MA	mammillary area
MB	mammillary body
MDO	mid-diencephalic organizer
MHB	mid-hindbrain boundary
MO	morpholino
mtg	mammillotegmental tract
mth	mammillothalamic tract
n.s.	non significant
ng	nano gram
Ngn1	neurogenin 1
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NPO	neurosecretory preoptic area
Nr5a2	nuclear receptor subfamily 5, group A, member 2
OE	over-expression
ON	over night
OTP	orthopedia
OXT	oxytocin
Oxtl	oxytocin-like
PCR	polymerase chain reaction
Pdyn	pdynorphin
Penka	proenkephalin a
pHyp	posterior hypothalamus
pm	principal mammillary tract

PMd	dorsal premammillary nucleus
PO	preoptic area
Pol.	Polymerase
PT	posterior tuberculum
PTU	phenylthiourea
PV	posterior-ventral
PVN	paraventricular nucleus
RNA	ribonucleic acid
RT	room temperature
SCN	suprachiasmatic nucleus
SF1	steroidogenic factor 1
Shh	sonic hedgehog
SON	supraoptic nucleus
SS	somatostatin
SuM	supramammillary area
Tel	telencephalon
TF	transcription factor
th	tyrosine hydroxylase
TM	tuberomammillary nucleus
TRH	thyrotropin-releasing hormone
UCN1	urocortin 1
µm	micrometer
Uts1	urotensin 1
vHyp	ventral hypothalamus
VIP	vasoactive intestinal peptide
VMH	ventromedial nucleus
VMH	ventromedial hypothalamus
WISH	whole-mount in situ hybridization
Wnt	combination of Wg for Wingless and Int-1 for integration 1
wt	wild-type
ZLI	zona limitans intrathalamica

Bibliography

Acampora, D., Postiglione, M. P., Avantaggiato, V., Di Bonito, M., Vaccarino, F. M., Michaud, J. and Simeone, A. (1999). Progressive impairment of developing neuroendocrine cell lineages in the hypothalamus of mice lacking the orthopedia gene. *Genes Dev.* **13**, 2787–800.

Adolf, B., Chapouton, P., Lam, C. S., Topp, S., Tannhauser, B., Strahle, U., Gotz, M. and Bally-Cuif, L. (2006). Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon. *Dev. Biol.* **295**, 278–93.

Aguiar, D. C. and Guimaraes, F. S. (2011). Blockade of nmda or no in the dorsal premammillary nucleus attenuates defensive behaviors. *Physiol. Behav.* **103**, 279–83.

Akimenko, M. A., Ekker, M., Wegner, J., Lin, W. and Westerfield, M. (1994). Combinatorial expression of three zebrafish genes related to distal-less: part of a homeobox gene code for the head. *J. Neurosci.* **14**, 3475–86.

Alderman, S. L. and Bernier, N. J. (2007). Localization of corticotropin-releasing factor, urotensin i, and crf-binding protein gene expression in the brain of the zebrafish, danio rerio. *J. Comp. Neurol.* **502**, 783–93.

Allen, G. V. and Hopkins, D. A. (1988). Mamillary body in the rat: a cytoarchitectonic, golgi, and ultrastructural study. *J. Comp. Neurol.* **275**, 39–64.

Alvarez-Bolado, G., Zhou, X., Cecconi, F. and Gruss, P. (2000a). Expression of foxb1 reveals two strategies for the formation of nuclei in the developing ventral diencephalon. *Dev. Neurosci.* **22**, 197–206.

Alvarez-Bolado, G., Zhou, X., Voss, A. K., Thomas, T. and Gruss, P. (2000b). Winged helix transcription factor foxb1 is essential for access of mammillothalamic axons to the thalamus. *Development* **127**, 1029–38.

Babu, M. M., Luscombe, N. M., Aravind, L., Gerstein, M. and Teichmann, S. A. (2004). Structure and evolution of transcriptional regulatory networks. *Curr. Opin. Struct. Biol.* **14**, 283–91.

Barresi, M. J., Hutson, L. D., Chien, C. B. and Karlstrom, R. O. (2005). Hedgehog regulated slit expression determines commissure and glial cell position in the zebrafish forebrain. *Development* **132**, 3643–56.

Bassett, J. P., Tullman, M. L. and Taube, J. S. (2007). Lesions of the tegmentomammillary circuit in the head direction system disrupt the head direction signal in the anterior thalamus. *J. Neurosci.* **27**, 7564–77.

Baumgardt, M., Karlsson, D., Terriente, J., Diaz-Benjumea, F. J. and Thor, S. (2009). Neuronal subtype specification within a lineage by opposing temporal feed-forward loops. *Cell* **139**, 969–82.

Baumgardt, M., Miguel-Aliaga, I., Karlsson, D., Ekman, H. and Thor, S. (2007). Specification of neuronal identities by feedforward combinatorial coding. *PLoS Biol.* **5**, e37.

Beracochea, D. (2005). Interaction between emotion and memory: importance of mammillary bodies damage in a mouse model of the alcoholic korsakoff syndrome. *Neural Plast.* **12**, 275–87.

Bittencourt, J. C., Vaughan, J., Arias, C., Rissman, R. A., Vale, W. W. and Sawchenko, P. E. (1999). Urocortin expression in rat brain: evidence against a pervasive relationship of urocortin-containing projections with targets bearing type 2 crf receptors. *J. Comp. Neurol.* **415**, 285–312.

Blackshaw, S., Scholpp, S., Placzek, M., Ingraham, H., Simerly, R. and Shimogori, T. (2010). Molecular pathways controlling development of thalamus and hypothalamus: from neural specification to circuit formation. *J. Neurosci.* **30**, 14925–30.

Blader, P., Fischer, N., Gradwohl, G., Guillemot, F. and Strähle, U. (1997). The activity of neurogenin1 is controlled by local cues in the zebrafish embryo. *Development* **124**, 4557–4569.

Blair, H., Cho, J. and Sharp, P. (1999). The anterior thalamic head-direction signal is abolished by bilateral but not unilateral lesions of the lateral mammillary nucleus. *J. Neurosci.* **19**, 6673–83.

Blair, H. T., Cho, J. and Sharp, P. E. (1998). Role of the lateral mammillary nucleus in the rat head direction circuit: a combined single unit recording and lesion study. *Neuron* **21**, 1387–97.

Blechman, J., Borodovsky, N., Eisenberg, M., Nabel-Rosen, H., Grimm, J. and Levkowitz, G. (2007). Specification of hypothalamic neurons by dual regulation of the homeodomain protein orthopedia. *Development* **134**, 4417–26.

Borodovsky, N., Ponomaryov, T., Frenkel, S. and Levkowitz, G. (2009). Neural protein olig2 acts upstream of the transcriptional regulator sim1 to specify diencephalic dopaminergic neurons. *Dev. Dyn.* **238**, 826–34.

Bradford, Y., Conlin, T., Dunn, N., Fashena, D., Frazer, K., Howe, D., Knight, J., Mani, P., Martin, R., Moxon, S., Paddock, H., Pich, C., Ramachandran, S., Ruef, B., Ruzicka, L., Bauer Schaper, H., Schaper, K., Shao, X., Singer, A., Sprague, J., Sprunger, B., Van Slyke, C. and Westerfield, M. (2011). Zfin: enhancements and updates to the zebrafish model organism database. *Nucleic Acids Res.* **39(suppl 1)**, D822–D829.

Briscoe, J. and Novitch, B. G. (2008). Regulatory pathways linking progenitor patterning, cell fates and neurogenesis in the ventral neural tube. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **363**, 57–70.

Brivanlou, A. H. and Darnell, J. E., J. (2002). Signal transduction and the control of gene expression. *Science* **295**, 813–8.

Brobeck, J. R. (1946). Mechanism of the development of obesity in animals with hypothalamic lesions. *Physiol. Rev.* **26**, 541–59.

Canteras, N. S., Kroon, J. A., Do-Monte, F. H., Pavesi, E. and Carobrez, A. P. (2008). Sensing danger through the olfactory system: the role of the hypothalamic dorsal premammillary nucleus. *Neurosci. Biobehav. Rev.* **32**, 1228–35.

Canteras, N. S., Ribeiro-Barbosa, E. R. and Comoli, E. (2001). Tracing from the dorsal premammillary nucleus prosencephalic systems involved in the organization of innate fear responses. *Neurosci. Biobehav. Rev.* **25**, 661–8.

Canteras, N. S. and Swanson, L. W. (1992). The dorsal premammillary nucleus: an unusal component of the mammillary body. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 10089–10093.

Caqueret, A., Yang, C., Duplan, S., Boucher, F. and Michaud, J. L. (2005). Looking for trouble: a search for developmental defects of the hypothalamus. *Horm. Res.* **64**, 222–30.

Cavodeassi, F. and Houart, C. (2012). Brain regionalization: of signaling centers and boundaries. *Dev. Neurobiol.* **72**, 218–33.

Cayouette, M., Poggi, L. and Harris, W. A. (2006). Lineage in the vertebrate retina. *Trends Neurosci.* **29**, 563–70.

Ceccatelli, S., Eriksson, M. and Hokfelt, T. (1989). Distribution and coexistence of corticotropin-releasing factor-, neurotensin-, enkephalin-, cholecystokin-, galanin- and vasoactive intestinal polypeptide/peptide histidine isoleucine-like peptides in the parvocellular part of the paraventricular nucleus. *Neuroendocrinology* **49**, 309–323.

Chen, B., Schaevitz, L. and McConnell, S. K. (2005a). Fezl regulates the differentiation and axon targeting of layer 5 subcortical projection neurons in cerebral cortex. *Proc. Natl. Acad. Sci. U. S. A.* **102**.

Chen, J., Rasin, M., Kwan, K. and Sestan, N. (2005b). Zfp312 is required for subcortical axonal projections and dendritic morphology of deep-layer pyramidal neurons of the cerebral cortex. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 17792–7.

Chen, L., Zheng, J., Yang, N., Li, H. and Guo, S. (2011). Genomic selection identifies vertebrate transcription factor fezf2 binding sites and target genes. *J. Biol. Chem.* **286**, 18641–9.

Chuang, J. C., Mathers, P. H. and Raymond, P. A. (1999). Expression of three rx homeobox genes in embryonic and adult zebrafish. *Mech. Dev.* **84**, 195–198.

Cleveland, D. and Davis, L. (1936). Further studies on the effect of hypothalamic lesions upon carbohydrate metabolism. *Brain* **59**, 459–465.

Colombo, A., Reig, G., Mione, M. and Concha, M. L. (2006). Zebrafish barh-like genes define discrete neural domains in the early embryo. *Gene Expr. Patterns* **6**, 347–52.

Dale, J. K., Vesque, C., Lints, T. J., Sampath, T. K., Furley, A., Dodd, J. and Placzek, M. (1997). Cooperation of bmp7 and shh in the induction of forebrain ventral midline cells by prechordal mesoderm. *Cell* **90**, 257–69.

Del Giacco, L., Sordino, P., Pistocchi, A., Andreakis, N., Tarallo, R., Di Benedetto, B. and Cotelli, F. (2006). Differential regulation of the zebrafish orthopedia 1 gene during fate determination of diencephalic neurons. *BMC Dev. Biol.* **6**, 50.

Duplan, S. M., Boucher, F., Alexandrov, L. and Michaud, J. L. (2009). Impact of sim1 gene dosage on the development of the paraventricular and supraoptic nuclei of the hypothalamus. *Eur. J. Neurosci.* **30**, 2239–49.

Dutta, S., Dietrich, J. E., Aspock, G., Burdine, R. D., Schier, A., Westerfield, M. and Varga, Z. M. (2005). pitx3 defines an equivalence domain for lens and anterior pituitary placode. *Development* **132**, 1579–90.

Eaton, J. L. and Glasgow, E. (2006). The zebrafish bhlh pas transcriptional regulator, single-minded 1 (sim1), is required for isotocin cell development. *Dev. Dyn.* **235**, 2071–82.

Eaton, J. L. and Glasgow, E. (2007). Zebrafish orthopedia (otp) is required for isotocin cell development. *Dev. Genes Evol.* **217**, 149–58.

Eaton, J. L., Holmqvist, B. and Glasgow, E. (2008). Ontogeny of vasotocin-expressing cells in zebrafish: Selective requirement for the transcriptional regulators orthopedia and single-minded 1 in the preoptic area. *Dev. Dyn.* **237**, 995–1005.

Elsalini, O. A., von Gartzen, J., Cramer, M. and Rohr, K. B. (2003). Zebrafish hhex, nk2.1a, and pax2.1 regulate thyroid growth and differentiation downstream of nodal-dependent transcription factors. *Dev. Biol.* **263**, 67–80.

Filippi, A., Durr, K., Ryu, S., Willaredt, M., Holzschuh, J. and Driever, W. (2007). Expression and function of nr4a2, lmx1b, and pitx3 in zebrafish dopaminergic and noradrenergic neuronal development. *BMC Dev. Biol.* **7**, 135.

Fink, G. (2000). *Encyclopedia of Stress*. Academic Press.

Forlano, P. M. and Cone, R. D. (2007). Conserved neurochemical pathways involved in hypothalamic control of energy homeostasis. *J. Comp. Neurol.* **505**, 235–48.

Gainer, H. and Wray, S. (1994). *The physiology of reproduction: Cellular and molecular biology of oxytocin and vasopressin*. New York: Raven Press.

Givens, B. (1995). Low doses of ethanol impair spatial working memory and reduce hippocampal theta activity. *Alcohol. Clin. Exp. Res.* **19**, 763–7.

Glasgow, E. and Tomarev, S. I. (1998). Restricted expression of the homeobox gene prox 1 in developing zebrafish. *Mech. Dev.* **76**, 175–178.

Goossens, N., Dierickx, K. and Vandesande, F. (1977). Immunocytochemical localization of vasotocin and isotocin in the preopticohypophysial neurosecretory system of teleosts. *Gen. Comp. Endocrinol.* **32**, 371–5.

Goshu, E., Jin, H., Lovejoy, J., Marion, J. F., Michaud, J. L. and Fan, C. M. (2004). Sim2 contributes to neuroendocrine hormone gene expression in the anterior hypothalamus. *Mol. Endocrinol.* **18**, 1251–62.

Guillemot, F. (2007). Spatial and temporal specification of neural fates by transcription factor codes. *Development* **134**, 3771–80.

Guo, S., Wilson, S. W., Cooke, S., Chitnis, A. B., Driever, W. and Rosenthal, A. (1999). Mutations in the zebrafish unmask shared regulatory pathways controlling the development of catecholaminergic neurons. *Dev. Biol.* **208**, 473–87.

Gurskaya, N. G., Verkhusha, V. V., Shcheglov, A. S., Staroverov, D. B., Chepurnykh, T. V., Fradkov, A. F., Lukyanov, S. and Lukyanov, K. A. (2006). Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. *Nat. Biotechnol.* **24**, 461–5.

Haas, H. and Panula, P. (2003). The role of histamine and the tuberomamillary nucleus in the nervous system. *Nat. Rev. Neurosci.* **4**, 121–30.

Halloran, M. C., Sato-Maeda, M., Warren, J. T., Su, F., Lele, Z., Krone, P. H., Kuwada, J. Y. and Shoji, W. (2000). Laser-induced gene expression in specific cells of transgenic zebrafish. *Development* **127**, 1953–60.

Hashimoto, H., Yabe, T., Hirata, T., Shimizu, T., Bae, Y., Yamanaka, Y., Hirano, T. and Hibi, M. (2000). Expression of the zinc finger gene fez-like in zebrafish forebrain. *Mech. Dev.* **97**, 191–5.

Hauptmann, G. and Gerster, T. (1994). Two-color whole-mount in situ hybridization to vertebrate and drosophila embryos. *Trends Genet.* **10**, 266.

Hauptmann, G. and Gerster, T. (1996). Complex expression of the zp-50 pou gene in the embryonic zebrafish brain is altered by overexpression of sonic hedgehog. *Development* **122**, 1769–80.

Hill, A. J., Heiden, T. C., Heideman, W. and Peterson, R. E. (2009). Potential roles of arnt2 in zebrafish larval development. *Zebrafish* **6**, 79–91.

Hirata, T., Nakazawa, M., Muraoka, O., Nakayama, R., Suda, Y. and Hibi, M. (2006). Zinc-finger genes fez and fez-like function in the establishment of diencephalon subdivisions. *Development* **133**, 3993–4004.

Hirata, T., Suda, Y., Nakao, K., Narimatsu, M., Hirano, T. and Hibi, M. (2004). Zinc finger gene fez-like functions in the formation of subplate neurons and thalamocortical axons. *Dev. Dyn.* **230**, 546–56.

Hobert, O. (2008). Regulatory logic of neuronal diversity: terminal selector genes and selector motifs. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 20067–71.

Hokfelt, T., Fahrenkrug, J., Ju, G., Ceccatelli, S., Tsuro, Y., Meister, B., Mutt, V., Rundgren, M., Brodin, E., Terenius, L., Hulting, A., Werner, S., Bjorklund, H. and Vale, W. (1987). Analysis of peptide histidine isoleucine/vasoactive intestinal polypeptide-immunoreactive neurons in the central nervous system with special reference to their relation to corticotropin releasing factor- and enkephalin-like immunoreactivities in the paraventricular hypothalamic nucleus. *Neuroscience* **23**, 827–857.

Holzschuh, J., Ryu, S., Aberger, F. and Driever, W. (2001). Dopamine transporter expression distinguishes dopaminergic neurons from other catecholaminergic neurons in the developing zebrafish embryo. *Mech. Dev.* **101**, 237–43.

Hsu, S. Y. and Hsueh, A. J. (2001). Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor. *Nat. Med.* **7**, 605–11.

Inoue, A., Takahashi, M., Hatta, K., Hotta, Y. and Okamoto, H. (1994). Developmental regulation of islet-1 mrna expression during neuronal differentiation in embryonic zebrafish. *Dev. Dyn.* **199**, 1–11.

Jain, S., Mathur, R., Sharma, R. and Nayar, U. (1999). Neural tissue transplant in the lateral hypothalamic lesioned rats: functional recovery pattern. *Neurobiology* **7**, 421–430.

Jeong, J.-Y., Einhorn, Z., Mathur, P., Chen, L., Lee, S., Kawakami, K. and Guo, S. (2007). Patterning the zebrafish diencephalon by the conserved zinc-finger protein fezl. *Development* **134**, 127–136.

Jeong, J.-Y., Einhorn, Z., Mercurio, S., Lee, S., Lau, B., Mione, M., Wilson, S. W. and Guo, S. (2006). Neurogenin1 is a determinant of zebrafish basal forebrain dopaminergic neurons and is regulated by the conserved zinc finger protein tof/fezl. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 5143–8.

Kapsimali, M., Caneparo, L., Houart, C. and Wilson, S. W. (2004). Inhibition of wnt/axin/beta-catenin pathway activity promotes ventral cns midline tissue to adopt hypothalamic rather than floorplate identity. *Development* **131**, 5923–33.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310.

Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C. H., Ward, J. M. and Gonzalez, F. J. (1996). The t/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev.* **10**, 60–9.

Kobayashi, M., Toyama, R., Takeda, H., Dawid, I. B. and Kawakami, K. (1998). Overexpression of the forebrain-specific homeobox gene six3 induces rostral forebrain enlargement in zebrafish. *Development* **125**, 2973–82.

Koudijs, M. J., den Broeder, M. J., Keijser, A., Wienholds, E., Houwing, S., van Rooijen, E. M., Geisler, R. and van Eeden, F. J. (2005). The zebrafish mutants dre, uki, and lep encode negative regulators of the hedgehog signaling pathway. *PLoS Genet.* **1**, e19.

Krauss, S., Concordet, J. P. and Ingham, P. W. (1993). A functionally conserved homolog of the drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**, 1431–44.

Kurrasch, D. M., Cheung, C. C., Lee, F. Y., Tran, P. V., Hata, K. and Ingraham, H. A. (2007). The neonatal ventromedial hypothalamus transcriptome reveals novel markers with spatially distinct patterning. *J. Neurosci.* **27**, 13624–34.

Kwan, K. M., Fujimoto, E., Grabher, C., Mangum, B. D., Hardy, M. E., Campbell, D. S., Parant, J. M., Yost, H. J., Kanki, J. P. and Chien, C. B. (2007). The tol2kit: a multisite gateway-based construction kit for tol2 transposon transgenesis constructs. *Dev. Dyn.* **236**, 3088–99.

Lako, M., Lindsay, S., Bullen, P., Wilson, D. I., Robson, S. C. and Strachan, T. (1998). A novel mammalian wnt gene, wnt8b, shows brain-restricted expression in early development, with sharply delimited expression boundaries in the developing forebrain. *Hum. Mol. Genet.* **7**, 813–22.

Latchman, D. S. (1997). Transcription factors: an overview. *Int. J. Biochem. Cell Biol.* **29**, 1305–12.

Lauter, G., Soll, I. and Hauptmann, G. (2011). Multicolor fluorescent in situ hybridization to define abutting and overlapping gene expression in the embryonic zebrafish brain. *Neural Dev.* **6**, 10.

Lee, J. E., Wu, S. F., Goering, L. M. and Dorsky, R. I. (2006). Canonical wnt signaling through lef1 is required for hypothalamic neurogenesis. *Development* **133**, 4451–61.

Lee, S., Lee, B., Joshi, K., Pfaff, S. L., Lee, J. W. and Lee, S. K. (2008). A regulatory network to segregate the identity of neuronal subtypes. *Dev. Cell* **14**, 877–89.

Leibowitz, S. F., Hammer, N. J. and Chang, K. (1981). Hypothalamic paraventricular nucleus lesions produce overeating and obesity in the rat. *Physiol. Behav.* **27**, 1031–40.

Levkowitz, G., Zeller, J., Sirotkin, H. I., French, D., Schilbach, S., Hashimoto, H., Hibi, M., Talbot, W. S. and Rosenthal, A. (2003). Zinc finger protein too few controls the development of monoaminergic neurons. *Nat. Neurosci.* **6**, 28–33.

Lewis, K., Li, C., Perrin, M., Blount, A., Kunitake, K., Donaldson, C., Vaughan, J., Reyes, T., Gulyas, J., Fischer, W., Bilezikjian, L., Rivier, J., Sawchenko, P. and Vale, W. (2001). Identification of urocortin iii, an additional member of the corticotropin-releasing factor (crf) family with high affinity for the crf2 receptor. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 7570–7575.

Li, Y., Allende, M. L., Finkelstein, R. and Weinberg, E. S. (1994). Expression of two zebrafish orthodenticle-related genes in the embryonic brain. *Mech. Dev.* **48**, 229–244.

Löhr, H. (2009). *A genetic screen for mutations affecting development of catecholaminergic systems in zebrafish reveals a novel role for the transcription factors Arnt2 and Sim1*. Ph.D. thesis, University of Freiburg.

Löhr, H. and Hammerschmidt, M. (2011). Zebrafish in endocrine systems: recent advances and implications for human disease. *Annu. Rev. Physiol.* **73**, 183–211.

Löhr, H., Ryu, S. and Driever, W. (2009). Zebrafish diencephalic a11-related dopaminergic neurons share a conserved transcriptional network with neuroendocrine cell lineages. *Development* **136**, 1007–17.

Machluf, Y., Gutnick, A. and Levkowitz, G. (2011). Development of the zebrafish hypothalamus. *Ann. N. Y. Acad. Sci.* **1220**, 93–105.

Manning, L., Ohyama, K., Saeger, B., Hatano, O., Wilson, S. A., Logan, M. and Placzek, M. (2006). Regional morphogenesis in the hypothalamus: a bmp-tbx2 pathway coordinates fate and proliferation through shh downregulation. *Dev. Cell* **11**, 873–85.

Marion, J. F., Yang, C., Caqueret, A., Boucher, F. and Michaud, J. L. (2005). Sim1 and sim2 are required for the correct targeting of mammillary body axons. *Development* **132**, 5527–37.

Mathieu, J., Barth, A., Rosa, F. M., Wilson, S. W. and Peyrieras, N. (2002). Distinct and cooperative roles for nodal and hedgehog signals during hypothalamic development. *Development* **129**, 3055–65.

Mattes, B., Weber, S., Peres, J., Chen, Q., Davidson, G., Houart, C. and Scholpp, S. (2012). Wnt3 and wnt3a are required for induction of the mid-diencephalic organizer in the caudal forebrain. *Neural Dev.* **7**, 12.

McNay, D. E., Pelling, M., Claxton, S., Guillemot, F. and Ang, S. L. (2006). Mash1 is required for generic and subtype differentiation of hypothalamic neuroendocrine cells. *Mol. Endocrinol.* **20**, 1623–32.

Michaud, J. L., DeRossi, C., May, N. R., Holdener, B. C. and Fan, C. M. (2000). Arnt2 acts as the dimerization partner of sim1 for the development of the hypothalamus. *Mech. Dev.* **90**, 253–61.

Michaud, J. L., Rosenquist, T., May, N. R. and Fan, C. M. (1998). Development of neuroendocrine lineages requires the bhlh-pas transcription factor sim1. *Genes Dev.* **12**, 3264–75.

Miura, H., Yanazawa, M., Kato, K. and Kitamura, K. (1997). Expression of a novel aristaless related homeobox gene 'arx' in the vertebrate telencephalon, diencephalon and floor plate. *Mech. Dev.* **65**, 99–109.

Molyneaux, B. J., Arlotta, P., Hirata, T., Hibi, M. and Macklis, J. D. (2005). Fezl is required for the birth and specification of corticospinal motor neurons. *Neuron* **47**, 817–31.

Molyneaux, B. J., Arlotta, P., Menezes, J. R. and Macklis, J. D. (2007). Neuronal subtype specification in the cerebral cortex. *Nat. Rev. Neurosci.* **8**, 427–37.

Mori, H., Miyazaki, Y., Morita, T., Nitta, H. and Mishina, M. (1994). Different spatio-temporal expressions of three otx homeoprotein transcripts during zebrafish embryogenesis. *Brain Res. Mol. Brain Res.* **27**, 221–231.

Morita, T., Nitta, H., Kiyama, Y., Mori, H. and Mishina, M. (1995). Differential expression of two zebrafish emx homeoprotein mrnas in the developing brain. *Neurosci. Lett.* **198**, 131–4.

Nakai, S., Kawano, H., Yudate, T., Nishi, M., Kuno, J., Nagata, A., Jishage, K., Hamada, H., Fujii, H., Kawamura, K. and et al. (1995). The pou domain transcription factor brn-2 is required for the determination of specific neuronal lineages in the hypothalamus of the mouse. *Genes Dev.* **9**, 3109–21.

Nieuwenhuys, R., Donkelaar, H. J. and Nicholson, C. (1998). *The Central Nervous System of Vertebrates*, volume 1. Berlin: Springer.

Nussdorfer, G. G. and Malendowicz, L. K. (1998). Role of vip, pacap, and related peptides in the regulation of the hypothalamo-pituitary-adrenal axis. *Peptides* **19**, 1443–67.

Odenthal, J. and Nüsslein-Volhard, C. (1998). fork head domain genes in zebrafish. *Dev. Genes Evol.* **208**, 245–258.

Oki, Y. and Sasano, H. (2004). Localization and physiological roles of urocortin. *Peptides* **25**, 1745–9.

Pabst, O., Herbrand, H., Takuma, N. and Arnold, H. H. (2000). Nkx2 gene expression in neuroectoderm but not in mesendodermally derived structures depends on sonic hedgehog in mouse embryos. *Dev. Genes Evol.* **210**, 47–50.

Pan, W. X. and McNaughton, N. (2004). The supramammillary area: its organization, functions and relationship to the hippocampus. *Prog. Neurobiol.* **74**, 127–66.

Park, H.-C., Mehta, A., Richardson, J. S. and Appel, B. (2002). olig2 is required for zebrafish primary motor neuron and oligodendrocyte development. *Dev. Biol.* **248**, 356–368.

Passemard, S., Sokolowska, P., Schwendimann, L. and Gressens, P. (2011). Vip-induced neuroprotection of the developing brain. *Curr. Pharm. Des.* **17**, 1036–9.

Pepels, P. P., Meek, J., Wendelaar Bonga, S. E. and Balm, P. H. (2002). Distribution and quantification of corticotropin-releasing hormone (crh) in the brain of the teleost fish oreochromis mossambicus (tilapia). *J. Comp. Neurol.* **453**, 247–68.

Peter, P. (1977). Preoptic nucleus in fishes - comparative discussion of function-activity relationships. *Am. Zool.* **17**, 775–785.

Pistocchi, A., Gaudenzi, G., Carra, S., Bresciani, E., Del Giacco, L. and Cotelli, F. (2008). Crucial role of zebrafish prox1 in hypothalamic catecholaminergic neurons development. *BMC Dev. Biol.* **8**, 27.

Radyushkin, K., Anokhin, K., Meyer, B. I., Jiang, Q., Alvarez-Bolado, G. and Gruss, P. (2005). Genetic ablation of the mammillary bodies in the foxb1 mutant mouse leads to selective deficit of spatial working memory. *Eur. J. Neurosci.* **21**, 219–29.

Reyes, T., Lewis, K., Perrin, M., Kunitake, K., Vaughan, J., Arias, C., Hogenesch, J., Gulyas, J., Rivier, J., Vale, W. and Sawchenko, P. (2000). Urocortin ii: A member of the corticotropin-releasing factor (crf) neuropeptide family that is selectively bound by type 2 crf receptors. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 2843–2848.

Rohr, K. B., Barth, K. A., Varga, Z. M. and Wilson, S. W. (2001). The nodal pathway acts upstream of hedgehog signaling to specify ventral telencephalic identity. *Neuron* **29**, 341–51.

Rohr, K. B. and Concha, M. L. (2000). Expression of nk2.1a during early development of the thyroid gland in zebrafish. *Mech. Dev.* **95**, 267–70.

Rupp, R. A., Snider, L. and Weintraub, H. (1994). Xenopus embryos regulate the nuclear localization of xmyod. *Genes Dev.* **8**, 1311–23.

Russek-Blum, N., Gutnick, A., Nabel-Rosen, H., Blechman, J., Staudt, N., Dorsky, R. I., Houart, C. and Levkowitz, G. (2008). Dopaminergic neuronal cluster size is determined during early forebrain patterning. *Development* **135**, 3401–13.

Ryu, S., Holzschuh, J., Erhardt, S., Ettl, A.-K. and Driever, W. (2005). Depletion of minichromosome maintenance protein 5 in the zebrafish retina causes cell-cycle defect and apoptosis. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 18467–18472.

Ryu, S., Mahler, J., Acampora, D., Holzschuh, J., Erhardt, S., Omodei, D., Simeone, A. and Driever, W. (2007). Orthopedia homeodomain protein is essential for diencephalic dopaminergic neuron development. *Curr. Biol.* **17**, 873–80.

Saito, D., Komatsuda, M. and Urano, A. (2004). Functional organization of preoptic vasotocin and isotocin neurons in the brain of rainbow trout: central and neurohypophysial projections of single neurons. *Neuroscience* **124**, 973–84.

Sambrook, J. and Russel, D. (2001). *Molecular Cloning: A Laboratory Manual*, volume 3. Cold Spring Harbor: Cold Spring Harbor Laboratory.

Saper, C. (2004). *Hypothalamus*, volume 4, chapter 17. Elsevier, 2 edition.

Saper, C. B. (2000). Hypothalamic connections with the cerebral cortex. *Prog. Brain Res.* **126**, 39–48.

Sarrieau, A., Najimi, M., Chigr, F., Kopp, N., Jordan, D. and Rostene, W. (1994). Localization and developmental pattern of vasoactive intestinal polypeptide binding sites in the human hypothalamus. *Synapse* **17**, 129–40.

Scholpp, S. and Lumsden, A. (2010). Building a bridal chamber: development of the thalamus. *Trends Neurosci.* **33**, 373–80.

Schonemann, M. D., Ryan, A. K., McEvilly, R. J., O'Connell, S. M., Arias, C. A., Kalla, K. A., Li, P., Sawchenko, P. E. and Rosenfeld, M. G. (1995). Development and survival of the endocrine hypothalamus and posterior pituitary gland requires the neuronal pou domain factor brn-2. *Genes Dev.* **9**, 3122–35.

Sharp, P. E. and Turner-Williams, S. (2005). Movement-related correlates of single-cell activity in the medial mammillary nucleus of the rat during a pellet-chasing task. *J. Neurophysiol.* **94**, 1920–7.

Shen, Q., Wang, Y., Dimos, J. T., Fasano, C. A., Phoenix, T. N., Lemischka, I. R., Ivanova, N. B., Stifani, S., Morrisey, E. E. and Temple, S. (2006). The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells. *Nat. Neurosci.* **9**, 743–51.

Shimizu, T. and Hibi, M. (2009). Formation and patterning of the forebrain and olfactory system by zinc-finger genes fezf1 and fezf2. *Dev. Growth Differ.* **51**, 221–31.

Shimogori, T., Lee, D. A., Miranda-Angulo, A., Yang, Y., Wang, H., Jiang, L., Yoshida, A. C., Kataoka, A., Mashiko, H., Avetisyan, M., Qi, L., Qian, J. and Blackshaw, S. (2010). A genomic atlas of mouse hypothalamic development. *Nat. Neurosci.* **13**, 767–75.

Shirasaki, R. and Pfaff, S. L. (2002). Transcriptional codes and the control of neuronal identity. *Annu. Rev. Neurosci.* **25**, 251–81.

Simeone, A., D'Apice, M. R., Nigro, V., Casanova, J., Graziani, F., Acampora, D. and Avantaggiato, V. (1994). Orthopedia, a novel homeobox-containing gene expressed in the developing cns of both mouse and drosophila. *Neuron* **13**, 83–101.

Simerly, R. B. (2004). *Anatomical substrates of hypothalamic integration*. Amsterdam: Elsevier, pages 335–368.

Skidmore, J. M., Waite, M. R., Alvarez-Bolado, G., Puelles, L. and Martin, D. M. (2012). A novel taulacz allele reveals a requirement for pitx2 in formation of the mammillothalamic tract. *Genesis* **50**, 67–73.

Solnica-Krezel, L. and Driever, W. (1994). Microtubule arrays of the zebrafish yolk cell: organization and function during epiboly. *Development* **120**, 2443–55.

Spaniol, P., Bornmann, C., Hauptmann, G. and Gerster, T. (1996). Class iii pou genes of zebrafish are predominantly expressed in the central nervous system. *Nucleic Acids Res.* **24**, 4874–4881.

Speel, E. J., Saremaslani, P., Roth, J., Hopman, A. H. and Komminoth, P. (1998). Improved mrna in situ hybridization on formaldehyde-fixed and paraffin-embedded tissue using signal amplification with different haptenized tyramides. *Histochem. Cell Biol.* **110**, 571–7.

Stackman, R. W. and Taube, J. S. (1998). Firing properties of rat lateral mammillary single units: head direction, head pitch, and angular head velocity. *J. Neurosci.* **18**, 9020–37.

Staudt, N. and Houart, C. (2007). The prethalamus is established during gastrulation and influences diencephalic regionalization. *PLoS Biol.* **5**, e69.

Sundvik, M., Kudo, H., Toivonen, P., Rozov, S., Chen, Y. C. and Panula, P. (2011). The histaminergic system regulates wakefulness and orexin/hypocretin neuron development via histamine receptor h1 in zebrafish. *FASEB J.* **25**, 4338–47.

Swanson, L. W. (1987). *The Hypothalamus*. Amsterdam: Elsevier, pages 1–124.

Sylvester, J. B., Rich, C. A., Loh, Y. H., van Staaden, M. J., Fraser, G. J. and Streelman, J. T. (2010). Brain diversity evolves via differences in patterning. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 9718–23.

Szabo, N. E., Zhao, T., Cankaya, M., Stoykova, A., Zhou, X. and Alvarez-Bolado, G. (2011). Interaction between axons and specific populations of surrounding cells is indispensable for collateral formation in the mammillary system. *PLoS One* **6**, e20315.

Szarek, E., Cheah, P. S., Schwartz, J. and Thomas, P. (2010). Molecular genetics of the developing neuroendocrine hypothalamus. *Mol. Cell. Endocrinol.* **323**, 115–23.

Takeuchi, Y., Allen, G. V. and Hopkins, D. A. (1985). Transnuclear transport and axon collateral projections of the mamillary nuclei in the rat. *Brain Res. Bull.* **14**, 453–68.

Tang, W., Ehrlich, I., Wolff, S. B., Michalski, A. M., Wolfl, S., Hasan, M. T., Luthi, A. and Sprengel, R. (2009). Faithful expression of multiple proteins via 2a-peptide self-processing: a versatile and reliable method for manipulating brain circuits. *J. Neurosci.* **29**, 8621–9.

Thisse, B., Pflumio, S., Fürthauer, M., Loppin, B., Heyer, V., Degrave, A., Woehl, R., Lux, A., Steffan, T., Charbonnier, X. and Thisse, C. (2001). Expression of the zebrafish genome during embryogenesis. zfin direct data submission.

Tomasini, A. J., Schuler, A. D., Zebala, J. A. and Mayer, A. N. (2009). Photomorphs: a novel light-activated reagent for controlling gene expression in zebrafish. *Genesis* **47**, 736–43.

Toro, S. and Varga, Z. M. (2007). Equivalent progenitor cells in the zebrafish anterior preplacodal field give rise to adenohypophysis, lens, and olfactory placodes. *Semin. Cell Dev. Biol.* **18**, 534–42.

Tyurina, O. V., Guner, B., Popova, E., Feng, J., Schier, A. F., Kohtz, J. D. and Karlstrom, R. O. (2005). Zebrafish gli3 functions as both an activator and a repressor in hedgehog signaling. *Dev. Biol.* **277**, 537–56.

VanDunk, C., Hunter, L. A. and Gray, P. A. (2011). Development, maturation, and necessity of transcription factors in the mouse suprachiasmatic nucleus. *J. Neurosci.* **31**, 6457–67.

Vann, S. D. (2010). Re-evaluating the role of the mammillary bodies in memory. *Neuropsychologia* **48**, 2316–27.

Vann, S. D. and Aggleton, J. P. (2004). The mammillary bodies: two memory systems in one? *Nat. Rev. Neurosci.* **5**, 35–44.

Varga, Z. M., Wegner, J. and Westerfield, M. (1999). Anterior movement of ventral diencephalic precursors separates the primordial eye field in the neural plate and requires cyclops. *Development* **126**, 5533–46.

Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M., Lewis, K., Sutton, S., Chan, R., Turnbull, A., Lovejoy, D., Rivier, C., Rivier, J., Sawchenko, P. and Vale, W. (1995). Urocortin, a mammalian neuropeptide related to fish urotensin i and to corticotropin-releasing factor. *Nature* **378**, 287–292.

Veazey, R. B., Amaral, D. G. and Cowan, W. M. (1982). The morphology and connections of the posterior hypothalamus in the cynomolgus monkey (macaca fascicularis). i. cytoarchitectonic organization. *J. Comp. Neurol.* **207**, 114–34.

Visel, A., Minovitsky, S., Dubchak, I. and Pennacchio, L. (2007). Vista enhancer browser-a database of tissue-specific human enhancers. **35**, D88–92.

Vosko, A. M., Schroeder, A., Loh, D. H. and Colwell, C. S. (2007). Vasoactive intestinal peptide and the mammalian circadian system. *Gen. Comp. Endocrinol.* **152**, 165–75.

Wang, W., Grimmer, J. F., Van De Water, T. R. and Lufkin, T. (2004). Hmx2 and hmx3 homeobox genes direct development of the murine inner ear and hypothalamus and can be functionally replaced by drosophila hmx. *Dev. Cell* **7**, 439–53.

Wang, W. and Lufkin, T. (2000). The murine otp homeobox gene plays an essential role in the specification of neuronal cell lineages in the developing hypothalamus. *Dev. Biol.* **227**, 432–49.

Wang, X., Chu, L. T., He, J., Emelyanov, A., Korzh, V. and Gong, Z. (2001). A novel zebrafish bhlh gene, neurogenin3, is expressed in the hypothalamus. *Gene* **275**, 47–55.

Wehr, R., Mansouri, A., de Maeyer, T. and Gruss, P. (1997). Fkh5-deficient mice show dysgenesis in the caudal midbrain and hypothalamic mammillary body. *Development* **124**, 4447–4456.

Westerfield, M. (2000). *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio).*. Eugene: Univ. of Oregon Press, 4 edition.

Wilson, S. W. and Houart, C. (2004). Early steps in the development of the forebrain. *Dev. Cell* **6**, 167–81.

Winter, S. S., Wagner, S. J., McMillin, J. L. and Wallace, D. G. (2011). Mammillothalamic tract lesions disrupt dead reckoning in the rat. *Eur. J. Neurosci.* **33**, 371–381.

Xi, D., Gandhi, N., Lai, M. and Kublaoui, B. M. (2012). Ablation of sim1 neurons causes obesity through hyperphagia and reduced energy expenditure. *PLoS One* **7**, e36453.

Yulis, C. R., Lederis, K., Wong, K. L. and Fisher, A. W. (1986). Localization of urotensin i- and corticotropin-releasing factor-like immunoreactivity in the central nervous system of catostomus commersoni. *Peptides* **7**, 79–86.

Zhao, T., Szabo, N., Ma, J., Luo, L., Zhou, X. and Alvarez-Bolado, G. (2008). Genetic mapping of foxb1-cell lineage shows migration from caudal diencephalon to telencephalon and lateral hypothalamus. *Eur. J. Neurosci.* **28**, 1941–55.

Acknowledgements

First of all I would like to thank Professor Dr. Detlev Arendt for his willingness to review my dissertation, our paper and for all his helpful comments, which exceedingly contributed to the success of this work.

I am truly indebted and thankful to Dr. Soojin Ryu to accept me as her first PhD student in her research group "Developmental Genetics of Nervous System" at the Max-Planck-Institute for Medical Research. I am especially thankful for her supervision of my work, for her faith in me to always improve myself and for her willingness and availability to always discuss my research when necessary.

I am sincerely and heartily grateful to the present and the past members of the Ryu lab spending this important time with me. Particularly indebted, I am to the "Mädels" Chen-Min Yeh, Dr. Colette Maurer and Antonia Groneberg who were always willing to discuss and to support me in all my problems no matter if personal or scientific.

I owe sincere and earnest thankfulness to Regina Singer, who took care of all kind of consumables (from buffers, tips and fish, to cake and strawberries) and for doing so many boring midis and washing steps for me that I can not count them anymore.
I want to thank Klaus Schott for making me smile, for keeping our fish facility running and for fixing all these broken things in the lab.
I also want to thank Gabi Shoeman, Angelika Schoell and Christiane Brandel for excellent fish care and for the preparation of the media and the selective plates.

The time of being a PhD representative together with Regina Schweizer not only gave me several lessons to learn for my entire life but also gave me a wonderful friendship hopefully lasting as long.

I am deeply grateful to my family and yes my fish are well.

This dissertation would not have been possible without my new family, my best friend, my partner, my bridge over troubled water, my personal IT: my husband Markus. There are no words to express how grateful I am.

Eidesstattliche Erklärung

Erklärungen gemäß § 8 (3) der Promotionsordnung der Universität Heidelberg für die Naturwissenschaftlich-Mathematische Gesamtfakultät vom 22. September 2006 erkläre ich hiermit, dass ich die vorgelegte Dissertation selbst verfasst und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Ferner erkläre ich, dass ich an keiner anderen Stelle ein Prüfungsverfahren beantragt bzw. die Dissertation in dieser oder anderer Form bereits anderweitig als Prüfungsarbeit verwendet oder einer anderen Fakultät als Dissertation vorgelegt habe.

Andrea Wolf

Heidelberg, den 13.08.2012