Zebrafish diencephalic A11-related dopaminergic neurons share a conserved transcriptional network with neuroendocrine cell lineages

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Vertebrate dopaminergic neurons develop in distinct neural territories to constitute one of the major neuromodulatory systems. We have identified a zebrafish mutation in the bHLH-PAS family member *arnt2*, based on a strong reduction in cell number of specific dopaminergic neuron groups in the hypothalamus and posterior tuberculum. Knockdown of *sim1* causes a dopaminergic phenotype similar to *arnt2* mutants, suggesting that Sim1 acts as a binding partner of Arnt2, similar to their role in hypothalamic neuroendocrine cell specification. *sim1*, *arnt2* and *otp* are co-expressed in dopaminergic neurons, and combined overexpression of Sim1 and Otp leads to formation of supernumerary dopaminergic neurons in the ventral diencephalon. Arnt2, Sim1 and Otp thus are core components of a conserved transcriptional network, which specifies neuroendocrine as well as A11-related dopaminergic neurons in the fish hypothalamus and posterior tuberculum. Our data suggest a common evolutionary origin of specific hypothalamic neuroendocrine and dopaminergic systems.

KEY WORDS: Diencephalon, Hypothalamus, Dopaminergic neurons, Neurosecretory cells, *otp*, *arnt2*, *sim1*, Neural differentiation

INTRODUCTION

Dopaminergic (DA) neurons are involved in control of mood regulation, cognitive function, physiological homeostasis and motor coordination. In mammals, DA neurons are formed at stereotypical locations, including cell groups in caudal forebrain and ventral midbrain (Puelles and Verney, 1998; Marín et al., 2005; Prakash and Wurst, 2006; Björklund and Dunnett, 2007). Mammalian diencephalic DA cell groups are classified A11 through A15 from caudal to rostral. In zebrafish (*Danio rerio*), DA neurons are exclusively located in the forebrain, with the majority of groups in the ventral diencephalon located in ventral thalamus, posterior tuberculum and hypothalamus. However, no DA neurons can be detected in the midbrain (Holzschuh et al., 2001; Kaslin and Panula, 2001; Rink and Wullimann, 2002; McLean and Fetcho, 2004). Despite the large evolutionary distance between teleosts and mammals, and the differences in neuroanatomy, some anatomical and functional homologies between zebrafish DA neuron groups and their mammalian counterparts have been established (Smeets and Gonzalez, 2000). Only a few signaling and transcription factors involved in zebrafish DA neuron development are known, and a detailed understanding how specific DA neuronal groups are formed is lacking (reviewed by Ryu et al., 2006) However, recent data suggest that distinct DA neuron groups located in the ventral diencephalon of zebrafish share requirement for the homeobox protein Orthopedia (Otp) with neuroendocrine cells in the anterior hypothalamus of mammals (Ryu et al., 2007).

The neurosecretory system of the hypothalamus consists of two major types of neurons: magnocellular neurons in the paraventricular nuclei (PVN) and supraoptic nuclei (SON). These neurons synthesize oxytocin (OT) and arginine vasopressin (VP), and project their axons to the posterior lobe of the pituitary. Parvocellular neurons of the PVN and anterior periventricular nuclei (APV), conversely, project to the vasculature of the median eminence where they release their peptides, including corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH) and somatostatin (SST). Genetic analysis in mice has provided evidence that a heterodimer of the two bHLH-PAS transcription factors *aryl-hydrocarbon receptor nuclear translocator 2* (*arnt2*) and *single-minded 1* (*sim1*) is required to specify magnocellular neurons of the PVN as well as parvocellular CRH, TRH, SST neurons of the APV, PVN and SON (Michaud et al., 1998; Michaud et al., 2000; Hosoya et al., 2001; Keith et al., 2001). bHLH-PAS transcription factors are important modulators of a wide range of physiological and developmental processes, including control of aspects of neural development (reviewed by Crews and Fan, 1999; Gu et al., 2000; Kewley, 2004). Members of the bHLH-PAS protein family are characterized by an N-terminal bHLH domain required for DNA binding and dimerization, as well as by a PAS domain that acts as a secondary dimerization interface (Crews and Fan, 1999; Taylor and Zhulin, 1999). Transcriptional control of target genes requires heterodimerization of class I and class II bHLH-PAS transcription factors (Ema et al., 1996). The bHLH-PAS class I factors of the Sim type exclusively form heterodimers with class II members Arnt and Arnt2.

In addition to Arnt2 and Sim1, the Otp transcription factor is essential for development of the same neurosecretory cell types through a parallel pathway (Acampora et al., 2000; Wang and Lufkin, 2000). While Arnt2/Sim1 act during late differentiation of postmitotic progenitors, Otp is involved both in proliferation and differentiation of neuroendocrine progenitors. In case of AVP, CRH and OT cells, Arnt2/Sim1 and Otp act upstream of the POU-domain protein Pou3f2/Brn2. Consistent

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with this, mice mutant for *pou3f2* show defects in terminal differentiation of AVP, CRH and OT cell lineages (Schonemann et al., 1995; Sharp and Morgan, 1996; Treier and Rosenfeld, 1996).

For lower vertebrates, including zebrafish, the contributions of Arnt2, Sim1 and Otp to diencephalic development are less well understood. We and others have previously demonstrated that development of a specific subset of DA neurons in the hypothalamus and posterior tuberculum is dependent on Otp function (Blechman et al., 2007; Del Giacco et al., 2006; Ryu et al., 2007). *Otp* mutant mice are deficient in A11 DA neurons of the pretectum and hypothalamus, revealing an evolutionary relationship between these groups in mammals and teleost (Ryu et al., 2007). Otp is essential for specification of *crh*-, *sst*- and *isotocin*- (*itnp*; the zebrafish ortholog of mammalian OT) expressing cells in the preoptic region of the hypothalamus (PO) (Blechman et al., 2007; Ryu et al., 2007). Knockdown of zebrafish *sim1* leads to reduction or loss of *itnp*- and *vasotocin*- (*vsnp*; the zebrafish ortholog of mammalian VP) expressing cells in the zebrafish preoptic region (Eaton and Glasgow, 2007; Eaton et al., 2008). Thus, Otp and Sim1 appear to be involved in specification of various neuronal subtypes in the zebrafish diencephalon.

In order to identify novel determinants for DA neuron development in the zebrafish forebrain, we screened mutations with abnormal DA systems, and isolated a zebrafish mutant line in which the bHLH-PAS transcription factor Arnt2 is disrupted. Here, we explore the function of Arnt2, Sim1 and Otp in DA neuron and neuroendocrine specification in zebrafish.

MATERIALS AND METHODS
Fish maintenance, strains and genetics
Zebrafish breeding and maintenance was carried out under standard conditions at 28.5°C (Westerfield, 2000). To inhibit pigmentation, embryos were incubated in 0.2 mM phenylthiourea.

The *arnt2*^{m1055} mutant allele (TU allele number *t30256*) was isolated from an ENU mutagenesis screen (www.zf-models.org). Other strains used are *arnt2*^{hi2639c} (Golling et al., 2002) and *otpa*^{m866} (Ryu et al., 2007). For the *Tg(hsp70otpa;hsp70YFP)* line *otpa*, full-length coding sequence from pCS2-*otpa* (Ryu et al., 2007) was inserted into a dual heat shock promoter expression plasmid (Dirk Meyer, Innsbruck, unpublished) to obtain *hsp70otpa;hsp70YFP*. A single G0 founder (allele designation *m1072*) was used to create a stable F1 family. In this study, transgenic F2 fish were used exclusively. Analysis of F3 *hsp70otpa;hsp70YFP* embryos showed that the *otpa* and *YFP* cassettes segregate independently (potentially caused by recombination over the dual *hsp70* or polyA sequences in independent transgenes). We therefore could not use YFP expression as a reporter for Otpa overexpression. We refer to the established line as *hs:otpa*.

For heatshock experiments, only F3 embryos derived from incrosses of *hs:otpa* transgenic fish were used for which it had been demonstrated that all embryos overexpressed *otpa*. For verification, embryo clutches were staged and fixed at desired time points. In one half of the fixed embryos, overexpression of *otpa* mRNA was confirmed by whole-mount in situ hybridization, the other half was used for analysis of experimental gene expression. All heatshocks were performed by transferring embryos to 39°C preheated water for 75 minutes starting at 9 hpf. To facilitate detection of otherwise low levels of ectopic *otpa* mRNA in embryos fixed later than 25 hpf, a second heatshock at 39°C for 45 minutes was conducted 2-3 hours before fixation.

Mapping and cloning of *arnt2*^{m1055}
Chromosomal landing of *arnt2*^{m1055} was performed using SSLP marker (Knapik et al., 1998; Geisler et al., 2007). Fine mapping was carried out using DNA from single embryos. Information on relevant SSLP markers in the critical interval was obtained from the MGH and T51 panels. Full-length

arnt2-coding sequence was amplified from cDNA of 3 dpf *arnt2*^{m1055} mutant embryos and wild-type siblings (forward, 5′-GGCAATATGGCAACA-CCAGCCGCTG-3′; reverse, 5′-TTCATGTGCGTCAGGTGTGAGATGA-AGG-3′) and sequenced.

arnt2^{m1055} embryos derived from TÜ×WIK map-crosses were genotyped using the closest SSLP marker sc1024.8_2 (forward, 5′-CTGCACG-CAGAACAAATGAT-3′; reverse, 5′-CACCTGCCAAAACACTCTCA-3′). *arnt2*^{m1055} embryos in TÜ background were genotyped using a common reverse primer in combination with mismatch forward primers whose 3′ ends preferentially hybridize with either mutant or wild-type sequences (mutant forward, 5′-TCGGCTCCAGTCACAGCTGA-3′; wild-type forward, 5′-TCGGCTCCAGTCACAGCTGT-3′; common reverse, 5′-TTGCGATTGGCGAGGTTGGAGTAG-3′). Genotyping of *otpa*^{m866} embryos was performed as described (Ryu et al., 2007). *arnt2*^{hi2639c} embryos were genotyped using one primer pair that crosses the viral insertion to detect the mutant allele (forward, 5′-ATACTGAGGGTGAACGCAGACG-3′; reverse, 5′TCGCTTCTCGCTTCTGTTCG-3′; A. Nasiadka, ZIRC, personal communication) and a second primer pair flanking the viral insertion to detect the wild-type allele (forward, 5′-GAACTGAGTT-TGCGCGTTTGAGAC-3′; reverse, 5′-CGGAAATGTCGCTGTTGTTAG-TTGTG-3′) (Golling et al., 2002).

Plasmids and probes
References for published probes used for expression analysis can be found at www.zfin.org. For preparation of *ddc* (IRBOp991B1037D) and *sim2* (IRBOp991C0138D) probes, cDNA clones from imaGenes/RZPD were used. For generation of the *crh*, *sim1a*, *trh* probes, parts of the coding sequences were PCR amplified and cloned (primer sequences are available on request).

Full-length *sim1a* (ZFIN ID: ZDB-GENE-020829-1) and *sim1b* (*si:ch211-152c2.1*; ZFIN ID: ZDB-GENE-041210-5) were PCR amplified and cloned into pCRII using the following primers: *sim1a* forward, 5′-GCCGTAGAGCATGAAGGAGAAGT-3′ and *sim1a* reverse, 5′-TCAG-CTGCCATTGGTGATGA-3′; *sim1b* forward, 5′-GACGGGATTTTAA-CGCGATTAACG-3′ and *sim1b* reverse, 5′-AAAGCGCGAGTGTT-TCTCCGTCAG -3′.

sim1a was subcloned using the Gateway system (Kwan et al., 2007) to yield pDestTol2pA2;CMV/Sp6:*sim1a*-pA. *sim1b* was subcloned into pCS2+ vector.

Whole-mount in situ hybridization, and fluorescent immunohistochemistry were performed as described (Filippi et al., 2007). TUNEL staining was carried out on whole mounts as for whole-mount in situ hybridization. The TdT reaction (ApopTag Kit, Chemicon) and detection with Anti-Dig-AP antibody (Roche) were carried out according to the manufacturer's instructions.

Morpholino and mRNA injections
The following morpholinos were used (Gene Tools LLC): *arnt2* (ATG), 5′-GGTTTACAGCGGCTGGTGTTGCCAT-3′; *arnt2* (e2i2), 5′-TTCT-CAGAAGAATTGCTCACCCCGC-3′; *pou47* (ATG), 5′-ATGATTGG-ATGCTGTAGTCGCCATG-3′: *sim1a* (e2i2), 5′-TGTGATTGTGTAC-CTGAAGCAGATG-3′; *sim1b* (ATG), 5′-CTCCTTCATCGTGCCGTT-AATCGCG-3′; *sim1b* (e1i1), 5′-TATATATCTGACCTTGCGGGAACAC-3′; *sim1b* (e2i2), 5′-GCATCTTGCTGATTATTACCTGGAG-3′; *sim1b* (e4i4), 5′-TTTTCTATAAAGTGACTCACCCTGG-3′. The standard control, *p53* ATG, *otpb* splice and *sim1a* ATG morpholinos have been described previously (Langheinrich et al., 2002; Eaton and Glasgow, 2006; Ryu et al., 2007). The published *sim1a* ATG morpholino did not work in our hands and was substituted by a newly designed *sim1a* splice morpholino. Efficiencies and function of unpublished splice morpholinos were confirmed by PCR on cDNA derived from morpholino-injected embryos using the following primers: *arnt2* splice forward, 5′-ACAGAAACCGAGCAAA-ATCGCATC-3′; *arnt2* splice reverse, 5′-GAAAATTTGCTGGGTCCC-TCGC-3′; *sim1a* splice forward, 5′-CGGCGGGAGAAGGAAAACAG-3′; *sim1a* splice reverse, 5′-ACCACCGCACGTCAATCCTG-3′; forward, 5′-GAAGGAGAAATCAAAGACTGCGGC-3′; *sim1b* splice 1-3 reverse, 5′-TCAGTCCGGCGTTTCGCTTC-3′. Full-length capped mRNA was synthesized from pDestTol2pA2;CMV/Sp6:*sim1a*-pA (Sp6 mMessage
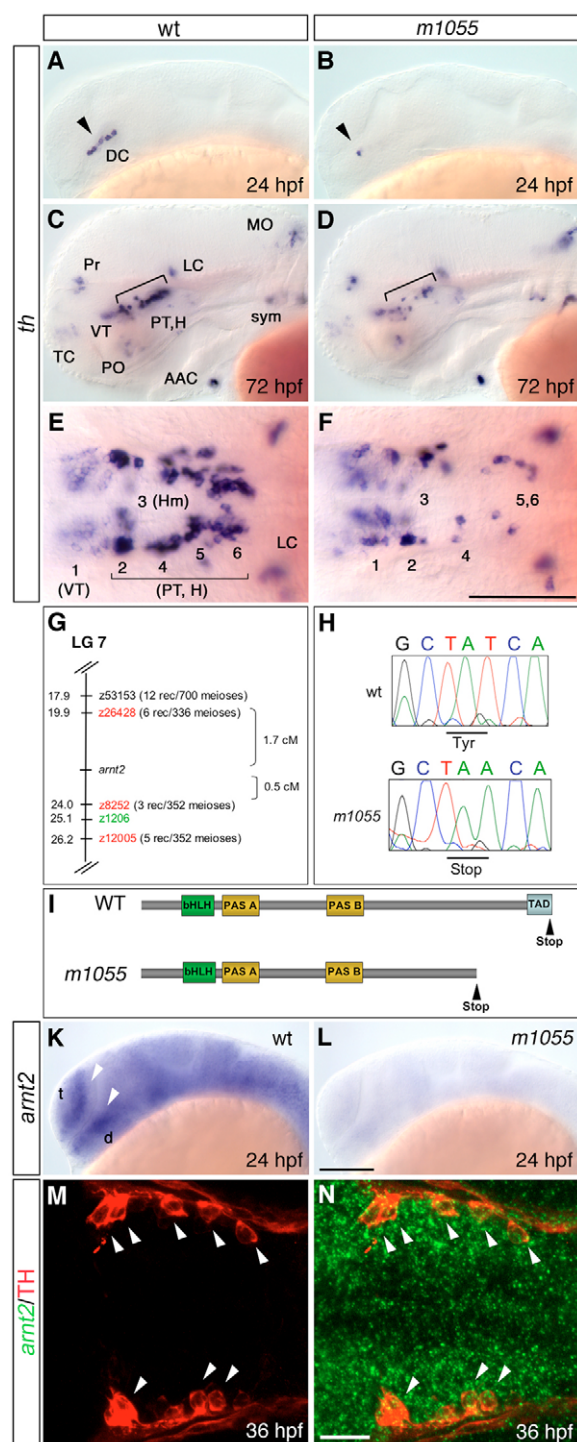
Fig. 1. A mutation in *arnt2* affects DA neuron groups in the ventral diencephalon of zebrafish. (**A-F**) Reduction of *th*-expressing DA neuron groups in the ventral diencephalon of *m1055* mutants (B,D,F) compared with wild-type siblings (A,C,E) at 24 hpf (A,B) and 72 hpf (C-F). (**G**) Genetic mapping of the *m1055* allele. SSLP markers (MGH panel black and T51 panel red) and genetic distances are shown. (**H**) Sequencing of *arnt2* in *m1055* mutants reveals a T to A exchange at position 1869. (**I**) Protein structure of the PAS family member *arnt2*. The premature stop codon in the *m1055* allele disrupts the essential transactivation domain (TAD). (**K,L**) *arnt2* is broadly expressed in wild type (K) at 24 hpf, but almost absent in *m1055* mutants (L). (**M,N**) Fluorescent whole-mount in situ hybridization for *arnt2* (green) combined with immunohistochemistry for TH (red) at 36 hpf. Arrowheads indicate co-expression. (A-D,K-L) Lateral views, (E,F,M,N) dorsal views, anterior towards the left. Scale bars: in F, 100 μm for E,F; in L, 100 μm for A-D,K,L; in N, 25 μm for M,N. AAC, arch associated cluster; d, diencephalon; DC, diencephalic cluster; H, hypothalamus; Hm, medial hypothalamus; LC, locus coeruleus; MO, medulla oblongata; PO, preoptic region; Pr, pretectum; PT, posterior tuberculum; sym, sympathetic CA neurons; t, telencephalon; TC, telencephalic cluster; VT, ventral thalamic cluster. Numbers in E,F indicate DA clusters in VT (1) and PT/H (2-6) according to Rink and Wullimann (Rink and Wullimann, 2002).

mMachine Kit Ambion). *sim1a* mRNA (100-200 pg) was injected per embryo. Both morpholinos and mRNAs were diluted in H$_2$O containing 0.05% Phenol Red and 0.05% rhodamine dextran and injected at the one-cell stage.

RESULTS
m1055 mutants form fewer DA neurons in the ventral diencephalon
In a forward genetic screen for mutations affecting the development of catecholaminergic (CA) neurons, we isolated *m1055*, which causes a reduction of DA cells in the diencephalon.
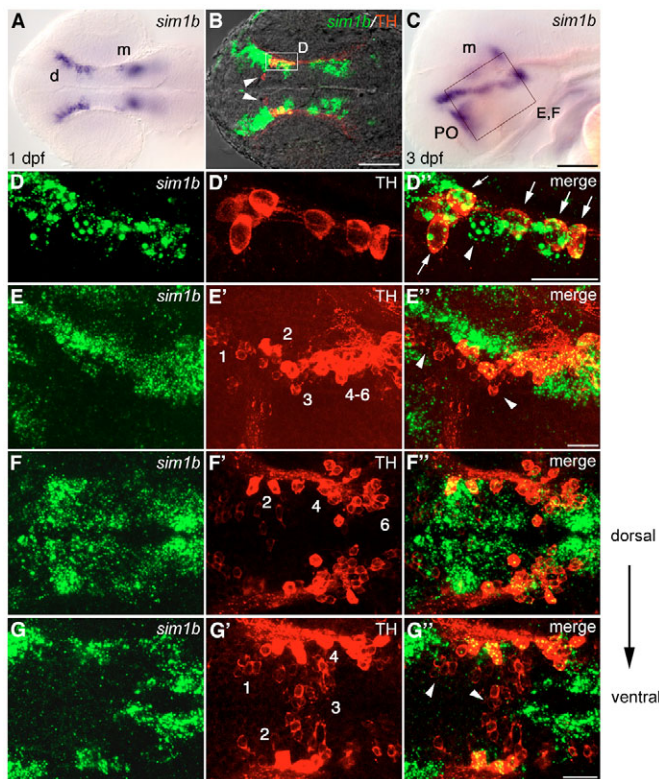
The *m1055* mutant phenotype was first detectable at 24 hpf when the number of earliest forming DA neurons in the ventral diencephalon was reduced from an average of 6.2 cells in *m1055* heterozygous fish or wild type (*n*=32) to an average of 1.8 cells in *m1055* homozygous mutants (*n*=12) (Fig. 1A,B). At 72 hpf, when most embryonic CA groups have formed, only a subset of DA cells in the ventral diencephalon located in hypothalamus and posterior tuberculum are affected, whereas other DA neuron groups in the telencephalon, pretectum and the preoptic region, as well as all noradrenergic groups, develop normally (Fig. 1C-F; see Fig. S1 in the supplementary material). Based on cell position and morphology, DA neuron groups 1-4 and 6 in the posterior tuberculum, and 5 and 7 in the hypothalamus have been distinguished in the ventral diencephalon at 3-4 dpf (Rink and Wullimann, 2002). However, we consider group 1 to be prethalamic, groups 2, 4, 5 and 6 to be *otp*-dependent posterior tubercular/hypothalamic (Ryu et al., 2007), and groups 3, 7 and preoptic DA neurons to be hypothalamic, based on molecular marker expression (A. Filippi and W.D., unpublished). To quantify reduction of DA cells in the ventral diencephalons, we performed cell counts of distinct groups in *m1055* mutants and heterozygous siblings. As DA neuron groups 4 to 6 are located in direct proximity to each other, we combined these groups for analysis. In *m1055* mutants, reduced DA cell numbers were observed only for groups 2 (82.0% reduction) and 4-6 (81.3% reduction), whereas groups 1 and 3 were not affected (Fig. 1E-F; see Fig. S1E in the supplementary material). For group 7 neurons, no obvious differences were apparent between wild-type and mutant embryos, and cells were not counted, as these neurons just form at 3 dpf. In *m1055* mutants, the number of both *dopa decarboxylase* and *dopamine transporter/slc6a3*-expressing cells was selectively reduced in those ventral diencephalic groups in which *th* expression was also affected (see Fig. S2A-D in the supplementary material). Thus, *arnt2*^{m1055} affects not only *th* expression, but multiple aspects of DA neuron differentiation.

Fig. 2. *sim1b* is expressed in a subset of DA neurons in the ventral diencephalon. (**A-G″**) Expression of *sim1b* (A,C) and co-expression of *sim1b* (fluorescent whole-mount in situ hybridization, green) and TH (B,D-G″; immunohistochemistry red). (A,B) 1 dpf. (C) *sim1b* expression at 3 dpf. (D-D″) Higher magnification of area framed in B. All TH-positive cells in this region co-express *sim1b* (arrows), whereas some *sim1b*-expressing cells are negative for TH (arrowheads). (E-E″) Higher magnification of area framed in C. DA neuron groups 2, 4-6 colocalize with *sim1b*; DA neuron groups 1 and 3 do not co-express *sim1b* (arrowheads). (F-G″) Dorsal views at 3 dpf in more dorsal (F-F″) and more ventral (G-G″) coronal optical sections. (A,B,F-G″) Dorsal views, (C-E″) lateral views, anterior towards the left. Scale bars: in C, 100 μm for A,C; in B, 100 μm; in D″,E″,G″, 50 μm for D-G″. Images in B,D-G″ are *z*-projections of multiple adjacent focal planes, depths (B,D) 12 μm, (E) 30 μm and (F,G) 20 μm. d, diencephalon; m, mesencephalon; PO, preoptic region.

The *m1055* mutation disrupts the bHLH-PAS family protein Arnt2

We mapped *m1055* close to the marker z1206 located at 25.1 cM of linkage group seven and defined a critical interval between z26428 on the distal side and z8252 on the proximal side (Fig. 1G). This interval contains a number of candidate genes, including *arnt2*. Complementation analysis with the previously published *arnt2[hi2639c]* retroviral insertion allele (Golling et al., 2002) did not result in complementation of the *m1055* DA phenotype (data not shown), suggesting that Arnt2 function is disrupted in *m1055*. We sequenced the complete *arnt2* ORF in *m1055* homozygous mutants and wild-type siblings, and detected a point mutation (T to A) at nucleotide position 1869 of the *arnt2* transcript isoform *arnt2b* ORF (Tanguay et al., 2000), which leads to a premature stop after 622 amino acids (Fig. 1H,I). The truncated Arnt2 protein in *arnt2[m1055]* of both major *arnt2* transcript isoforms (*arnt2b* and *arnt2c*) therefore still contains the conserved bHLH domain, as well as the PAS domains, but lacks

the essential transactivation domain at the C terminus (Jain et al., 1994). *arnt2[hi2639c]* contains a retroviral insertion 26 bp upstream of the *arnt2* translation start site, which leads to a complete absence of the *arnt2* transcripts (Golling et al., 2002) (data not shown) and thus it can be considered as a null allele. DA cell counts at 3 dpf in both *arnt2[m1055]* and *arnt2[hi2639c]* alleles showed similar phenotypic strength, suggesting that *arnt2[m1055]* also behaves as a null allele (see Fig. S1A-E in the supplementary material). At 24 hpf, *arnt2* transcript can be detected in the entire brain (Andreasen et al., 2002) with an enhanced expression in parts of the telencephalon and ventral diencephalon (Fig. 1K). In *m1055* mutants, *arnt2* is strongly downregulated, suggesting a positive autoregulatory loop (Fig. 1L). Co-expression analysis of *arnt2* and TH clearly indicated that *arnt2* is expressed in all TH immunoreactive DA cells affected by the *arnt2[m1055]* mutation at 36 hpf (Fig. 1M,N).

We next injected translation blocking (ATG MO) and splice blocking antisense morpholinos (splice MO) targeting *arnt2* (see Fig. S5A,C in the supplementary material). Injection of both morpholinos reduced the number of DA cells in the ventral diencephalon in a similar way to *m1055* (Table 1; Fig. 3B,C,F,G; and data not shown). It is known that *p53*-dependent cell death is induced by some morpholinos through off-target effects (Robu et al., 2007). Co-injection of *p53* and *arnt2* morpholinos did not attenuate the reduction of DA cells in *arnt2* ATG or in splice MO-injected embryos, suggesting that the reduction of DA cells is due to a specific knockdown of *arnt2* (Table 1; and data not shown). We next investigated the neural phenotypes of *arnt2[m1055]* mutant embryos in more detail. As the reduction of DA neurons in *arnt2[m1055]* could be due to elevated cell death, we investigated apoptosis in the mutants by TUNEL. We did not observe increased apoptosis in *arnt2[m1055]* embryos between 24 and 72 hpf (see Fig. S2E-H in the supplementary material; data not shown). We also detected no significant changes in cell proliferation by analyzing *pcna* and *mcm5* expression in mutant embryos at 36 and 60 hpf (data not shown). At 32 hpf, we further analyzed the expression of the following genes important for early forebrain regionalization and patterning: *dlx2*, *dlx4*, *fezl*, *foxa2*, *lim1*, *nkx2.1a*, *nkx2.2a*, *nkx5.1*, *pou50* and *shh* (see Table S1 and Fig. S2I-Q in the supplementary material). As we did not detect any significant changes, we conclude that lack of Arnt2 function does not affect establishment of early forebrain patterning.

Sim1 may cooperate with Arnt2 to specify ventral diencephalic DA neurons

Similar to their role in neuroendocrine development (Michaud et al., 1998), Arnt2 may also require Sim1 as dimerization partner during the development of DA neurons in the diencephalon of zebrafish. Searching the zebrafish genome (Ensembl version Zv6), we noticed that two *sim1* paralogs exist. One is located on chromosome 16 (termed *sim1* in Ensembl) and a second one is located on chromosome 4 (termed *si:ch211-152c2.1* in Ensembl). ClustalW alignment of Sim1 proteins from several species show that the sequence of the zebrafish predicted Sim1 protein shares 73% amino acid identity with human SIM1 (see Fig. S3 in the supplementary material). We will further refer to this *sim1* gene as *sim1a*. Sim1a shares a total of 54% identical amino acids with the second zebrafish Sim1 predicted from *si:ch211-152c2.1*. The ORF of *si:ch211-152c2.1* has only 53% amino acid identity with human SIM1 protein, and will be termed *sim1b*. *sim1a* and *sim1b* are nearly identically expressed in the embryonic diencephalon (see Fig. S4A-H″ in the supplementary material). As the *sim1b* probe produced a stronger signal in fluorescent in situ hybridization than did the *sim1a*
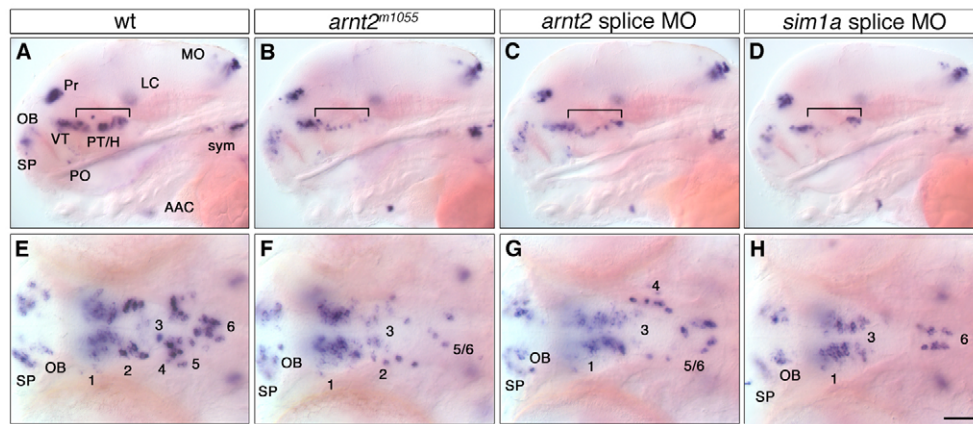
Fig. 3. Similar reduction of DA cells in embryos injected with *arnt2* or *sim1a* antisense morpholinos. (**A-H**) Expression of *th* at 4 dpf in uninjected wild-type embryos (A,E), *arnt2^{m1055}* mutants (B,F), embryos injected with 2.8 ng *arnt2* splice morpholino (C,G) and embryos injected with 1.1 ng *sim1a* splice morpholino (D,H). (A-D) Affected groups are marked by a bracket. (E-H) Magnification of ventral diencephalic groups 1-6. (A-D) Lateral views; (E-H) dorsal views. Anterior towards the left. Scale bar in H: 100 μm for A-D, 50 μm for E-H. Images in E-H represent *z*-projections from several adjacent focal planes. AAC, arch associated cluster; LC, locus coeruleus; MO, medulla oblongata; OB, olfactory bulb; PO, preoptic region; Pr, pretectum; PT, posterior tuberculum; SP, subpallium; sym, sympathetic CA neurons; VT, ventral thalamic cluster.

probe, we decided to use *sim1b* for co-expression analyses. At 1 dpf, most of the TH immunoreactive (THir) cells belong to DA neuron group 2, whereas DA neurons of group 3 are located closer to the midline (Fig. 2B, arrowheads). At 1 dpf, *sim1b* is expressed in two major domains in the diencephalon and midbrain (Fig. 2A,B). All THir cells of group 2 expressed *sim1b*, whereas the THir cells of group 3 were located outside the *sim1b*-expressing domain (Fig. 2B,D-D″). In most embryos, we detected *sim1b*-positive non-THir cells (Fig. 2D-D″, arrowhead) intermingled with *sim1b*-positive/THir cells (arrows). Those cells might either represent DA neuron precursors that are not fully differentiated and have not yet started to express TH, or cells of another neuronal identity. At 3 dpf, the expression domain of *sim1b* in the diencephalon had separated into a smaller ventral domain in the preoptic region and a larger rostrocaudally elongated domain slightly dorsal to it (Fig. 2C). At this time point, colocalization of *sim1b* and TH was clearly detectable in DA neuron groups 2, 4, 5 and 6, whereas no colocalization was apparent in DA neuron groups 1 and 3 (Fig. 2E-G″, arrowheads in E″,G″). Thus, *sim1* is expressed in the same DA neurons, which are reduced in *arnt2^{m1055}* mutants.

To test whether *sim1* is also required for DA neuron specification, we injected an antisense morpholino targeting *sim1a*. The *sim1a* splice MO caused a complete loss of exon 2, leading to a frameshift and a truncated Sim1 protein (see Fig. S5B,D in the supplementary material). Injection of *sim1a* splice morpholino alone, or in combination with *p53* morpholino, into wild-type embryos resulted in a phenocopy of the DA neuron phenotype observed in *arnt2^{m1055}* (Table 1; Fig. 3D,H; see Fig. S6B,C in the supplementary material). Morpholino knockdown of *sim1b* did not have an effect on DA cell number, albeit two out of four morpholinos used reduced the amount of *sim1b* transcript to only ~50% (data not shown). In summary, our results demonstrate that *sim1a* is required for specification of *sim1*-expressing DA neurons, whereas *sim1* non-expressing DA neurons are not affected in *sim1a* morphants. Furthermore, the DA neuron reduction in *sim1a* morphants is very similar to *arnt2^{m1055}* mutants, suggesting that Arnt2/Sim1 acts as a heterodimer to specify DA neurons in the diencephalon of zebrafish.

arnt2^{m1055} mutants and *sim1a* morphants reveal a common function in neurosecretory hypothalamus

To characterize the defects in differentiation of other neuronal subtypes in the hypothalamus of embryos lacking Arnt2 or Sim1 function, we analyzed the expression of neuroendocrine hormones at 76 hpf (Fig. 4; see also Table S1 in the supplementary material). *crh* is expressed in various parts of the embryonic zebrafish brain, including telencephalon, hypothalamus, posterior tuberculum, thalamus, retina and hindbrain (Fig. 4A). *crh* neurons in posterior tuberculum and hypothalamus are intermingled with DA neurons of the ventral diencephalic groups (Fig. 4Q,R) (Chandrasekar et al., 2007). *crh* expression in *arnt2^{m1055}* mutants and *sim1a* morphants was strongly reduced or completely absent for a small group of *crh* cells in the preoptic region. CRH neurons adjacent to DA neurons in the posterior tuberculum/hypothalamus are reduced in number, although we never observed a complete loss of these cells (Fig. 4A-C, arrowheads). The expression of *trh* in zebrafish has only been analyzed in adult brain (Diaz et al., 2002). In 76 hpf forebrain, *trh* is expressed in the preoptic region in a domain close to the anterior commissure. In *arnt2^{m1055}* mutants and *sim1a* morphants, *trh* expression in the preoptic area was lost, whereas a domain in the hindbrain was unchanged (Fig. 4D-F). A requirement for *sim1* in specification of zebrafish *itnp*- and *vsnp*-expressing neurons has already been suggested (Eaton and Glasgow, 2006; Eaton and Glasgow, 2007). In *arnt2^{m1055}* mutants, as in *sim1a* morphants, *itnp* and *vsnp* neurons in the preoptic region were strongly reduced or absent (Fig. 4G-M). *vsnp* expression in the ventral hypothalamus was not affected in the *arnt2* mutants or *sim1a* morphants (Fig. 4K-M). We frequently observed *itnp*-expressing cells at ectopic locations posterior to the domain in the anterior hypothalamus in *sim1a* morphants (Fig. 4I, arrow), but not in *arnt2^{m1055}* mutants. *sst1* transcript was detectable in diencephalon, midbrain and hindbrain (Devos et al., 2002). Within the diencephalons, *sst1* cells are located in four distinct groups: two in the preoptic region, one in the hypothalamus close to the postoptic commissure, and one in the dorsal diencephalon. In both *arnt2^{m1055}* mutants and *sim1a* morphants, only the more dorsally positioned cell group in the
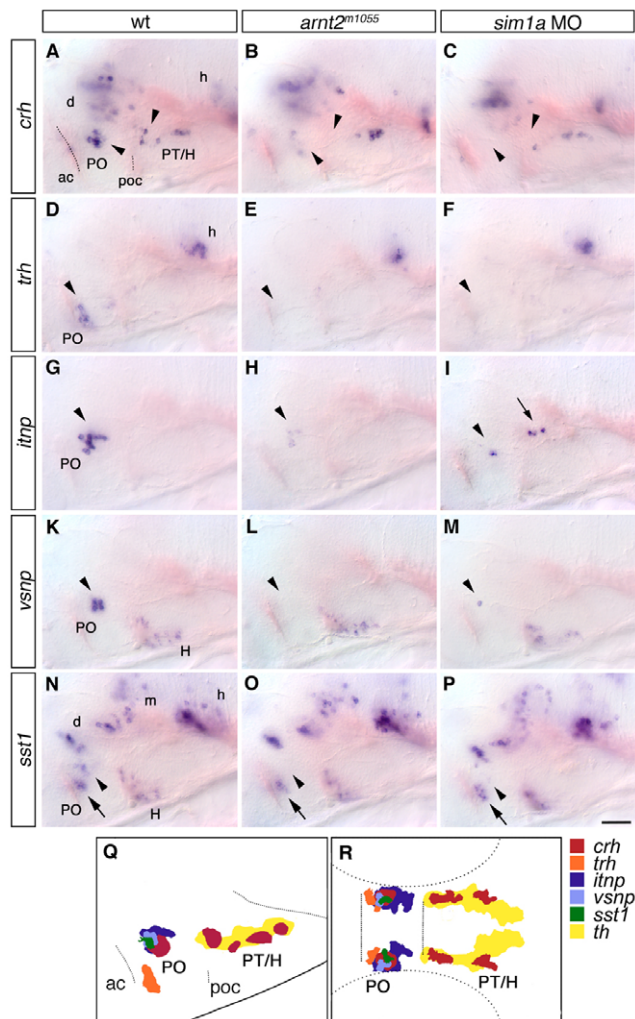
Fig. 4. Reduction of neurohormone-producing cells in the hypothalamus of *arnt2^{m1055}* mutants and *sim1a* morphants. (A-P) Expression of *crh* (A-C), *trh* (D-F), *itnp* (G-I), *vsnp* (K-M) and *sst1* (N-P) at 76 hpf in wild-type embryos (A,D,G,K,N), *arnt2*^{m1055} mutants (B,E,H,L,O) and embryos injected with 1 ng *sim1a* morpholino (C,F,I,M,P). (**A-C**) *crh* neurons in the PO (lower arrowheads) and in the posterior tuberculum and hypothalamus (upper arrowheads) are reduced. (**D-F**) *trh*-expressing cells in the PO (arrowheads) are strongly reduced. (**G-I**) *itnp*-expressing cells in the PO (arrowheads) are strongly reduced or absent. In *sim1a* morphants, *itnp*-expressing cells are detected at ectopic locations within the diencephalon (arrow). (**K-M**) *vsnp*-expressing cells in the PO (arrowheads) are strongly reduced or absent. (**N-P**) A group of *sst1*-expressing cells in the PO (arrowheads) is strongly reduced or absent, whereas all other *sst1*-positive domains, including a second group in the PO (arrow) are not affected. (**Q,R**) Camera lucida drawing showing the relative position of all analyzed neuronal groups reduced in *arnt2* mutants or *sim1a* morphants in lateral (Q) or dorsal (R) views; non-affected neuronal groups are not included. (A-P) Whole-mount in situ hybridization, lateral views; anterior is towards the left. Scale bar in P: 50 μm for A-P. Images represent z-projections from several consecutive focal planes. ac, anterior commissure; d, diencephalon; h, hindbrain; H, hypothalamus; m, mesencephalon; PO, preoptic region; poc, post optic commissure; PT, posterior tuberculum.

preoptic region was strongly reduced, whereas all other *sst1* groups formed normally (Fig. 4N-P). We demonstrated that the reduction of neurosecretory cell types in *sim1a* morphants is not caused by
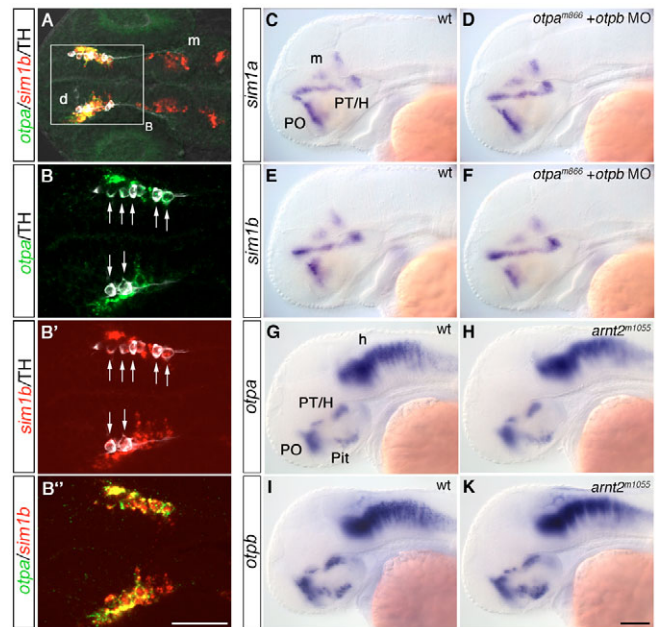

Fig. 5. Mutually independent expression of *sim1* and *otp* genes in DA neurons. (A-B″) Detection of *otpa* (green) and *sim1b* (red) expression by fluorescent whole-mount in situ hybridization combined with immunohistochemistry for TH (white) at 1 dpf. (**A**) THir cells are located within an *otpa*- and *sim1b*-expressing domain in the diencephalon (z-projection, 16 μm). (**B-B″**) Higher magnification of area framed in A (z-projection 10 μm). All THir cells co-express both *otpa* and *sim1b* (arrows in B,B′). (**C-F**) Expression analysis of *sim1a* (C,D) and *sim1b* (E,F) in wild-type embryos (C,E) and in *otpa*^{m866} homozygous mutants injected with 2 ng *otpb* morpholino (D,F) at 2 dpf. (**G-K**) Expression analysis of *otpa* (G,H) and *otpb* (I-K) in wild-type (G,I) and in *arnt2*^{m1055} (H,K) embryos at 2 dpf. (A-B″) Dorsal, (C-K) lateral views. Anterior towards the left. Scale bars: in B″, 100 μm for A, 50 μm for B-B″; in K, 100 μm for C-K. d, diencephalon; h, hindbrain; H, hypothalamus; m, mesencephalon; Pit, pituitary; PO, preoptic region; PT, posterior tuberculum.

non-specific morpholino induced cell death using co-injection of *p53* MO to suppress non-specific apoptosis (see Fig. S6 and Table S2 in the supplementary material).

Sim1 and Otp act in parallel during terminal differentiation of DA neurons

We analyzed co-expression of *sim1b* and *otpa* in the region where early Arnt2-, Sim1-dependent DA neurons are specified, and found expression of both transcription factors in all THir cells affected in *arnt*^{m1055} mutants (Fig. 5A-B″, arrows in Fig. 5B,B′), indicating a tight co-regulation of these two transcription factors. To determine whether expression of *sim1b* and *otpa* are independent of each other, we analyzed the expression of *sim1a* (Fig. 5C,D) and *sim1b* (Fig. 5E,F) in *otpa*^{m866} mutants injected with *otpb* splice morpholino. In the complementary experiment, we analyzed the expression of *otpa* (Fig. 5G,H) and *otpb* (Fig. 5I,K) in *arnt2*^{m1055}. As *arnt2*^{m1055} embryos are expected to lack functional Arnt2/Sim1 heterodimers, this mimics Sim1 loss-of-function. We did not detect changes in *sim1a* and *sim1b* expression in the Otp deficient embryos, or of *otpa* and *otpb* in Arnt2/Sim1-deficient embryos. Therefore, the initiation and maintenance of *otpa(b)* and *sim1a(b)* expression are indeed independent of each other. We observed that *sim1b* but not *sim1a*
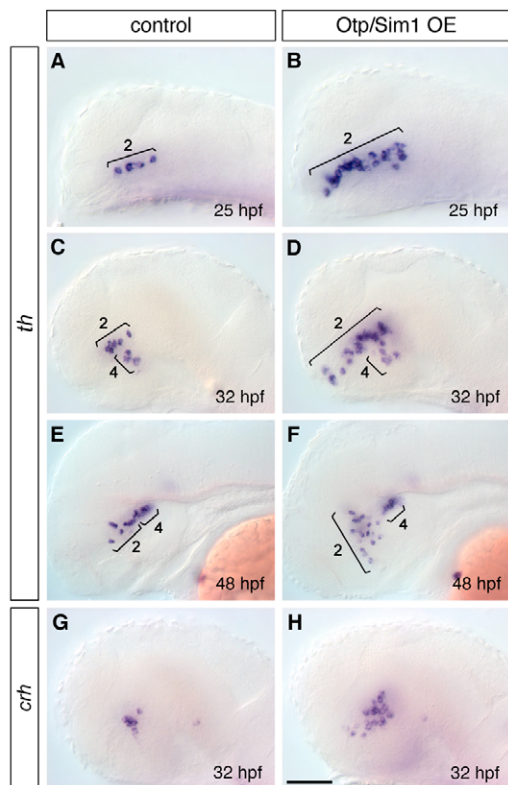
Fig. 6. Combined overexpression of Otpa and Sim1a leads to supernumerary DA and CRH cells in the ventral diencephalon. (**A-H**) Whole-mount in situ hybridization. Expression of *th* at 24 hpf (A,B), 32 hpf (C,D) and 48 hpf (E,F), and *crh* at 32 hpf (G,H) in wild-type controls (A,C,E,G) compared with transgenic embryos expressing *otpa* from a hsp70 promoter, and injected with *sim1a* mRNA (Otp/Sim1OE; B,D,F,H). Only the number of group 2 cells is strongly increased in Otp/Sim1OE embryos, whereas groups 4/6 are not affected (A-F). The number of CRH cells is increased in Otp/Sim1OE embryos (G,H). Lateral views, anterior towards the left. Scale bar: 50 μm for A,B; 62.5 μm for C,D,G,H; 80 μm for E,F.

expression was strongly downregulated in *arnt2^{m1055}* mutants, indicating that maintenance of *sim1b* but not *sim1a* depends on Sim1/Arnt2 function (data not shown).

In mammals, Sim1 is required during differentiation of postmitotic neurons whereas Otp additionally controls specification and proliferation of neuronal precursors (Michaud et al., 1998; Acampora et al., 1999; Ryu et al., 2007). *Ngn1* is involved in neurogenesis of diencephalic DA neurons (Jeong et al., 2006) and *elavl3* (*huC*) is a marker of differentiated neurons (Kim et al., 1997). Early-born *sim1b*/THir DA neurons were located adjacent to a diencephalic *ngn1* expression domain, suggesting that *ngn1* expression is already shut off in prospective DA neurons before *sim1a* expression is turned on (see Fig. S7A-A‴ in the supplementary material, arrowheads). Furthermore, all *sim1b*/TH-positive cells co-express *elavl3*, indicating that Sim1 acts late during DA neuron differentiation only (see Fig. S7B-B‴ in the supplementary material). During specification of a subset of neurosecretory cell types in the mammalian hypothalamus, parallel Sim1 and Otp regulatory input converges at the level of the common downstream target *pou3f2/brn2* (Wang and Lufkin, 2000). In zebrafish embryos lacking either Arnt2/Sim1 function or Otp function, or both, we detected significant reduction of *pou47* (zebrafish homolog of mammalian *pou3f2*) only in the preoptic area, whereas expression in the posterior tuberculum/hypothalamus is only slightly reduced, if at all (see Fig. S8 in the supplementary material). We attempted to analyze the role of *pou47* by morpholino knockdown; however, knockdown of *pou47* did lead to severe brain malformations, preventing evaluation of *pou47*-specific defects (data not shown). In summary, Arnt2 and Sim1 appear to act late in neuronal differentiation.

Combined overexpression of Sim1 and Otp induces differentiation of supernumerary ectopic DA and CRH cells in the posterior tuberculum/hypothalamus

We next analyzed whether Sim1a/Arnt2 and Otpa are sufficient to induce ectopic DA or neurosecretory cell types. It has already been shown that ectopic Otpa can induce some aspects of DA cell identity

Table 1. Effect of *arnt2* and *sim1a* morpholino knockdown on development of DA neurons

Morpholino	Class I	Class II	Class III	Small head/eyes	Total number
arnt2 ATG (3.5 ng)	1	7	8	0	16
arnt2 ATG (4.5 ng)	0	2	29	5	31
arnt2 ATG (4.5 ng) + p53 (7 ng)	0	0	18	10	18
arnt2 ATG (7 ng)	0	1	28	27	29
arnt2 ATG (7 ng) + p53 (7 ng)	0	1	18	19	19
arnt2 splice (2.75 ng)	3	15	4	0	22
arnt2 splice (3.5 ng)	0	0	20	3	20
arnt2 splice (3.5 ng) + p53 (7 ng)	3	15	4	0	22
arnt2 splice (4.5 ng)	0	0	32	27	32
arnt2 splice (4.5 ng) + p53 (7 ng)	0	4	24	20	28
sim1a splice (0.7 ng)	7	10	5	0	22
sim1a splice (1 ng)	0	1	69	1	70
sim1a splice (1 ng) + p53 (7 ng)	0	0	77	0	78
sim1a splice (1.8 ng)	0	1	28	3	29
sim1a splice (1.8 ng) + p53 (7 ng)	0	0	24	1	24
sim1a splice (2.2 ng)	0	0	20	5	20
sim1a splice (2.2 ng) + p53 (7 ng)	0	1	19	9	20

Wild-type embryos were injected with morpholino solution and *th* expression was subsequently analyzed by whole-mount in situ hybridization at 3-4 dpf. Three different phenotypic classes were defined by the level of reduction of *th*-expressing cells: class I, *th* expression comparable with wild type; class II, less than 50% percent reduction in ventral diencephalic DA groups compared with wild type; class III, more than 50% reduction in ventral diencephalic DA groups compared with wild type. For classification of the different classes, only *th*-expressing groups that are affected by the morpholinos were considered (see text). Class III phenotypes are similar to *arnt^{m1055}* mutant embryos. The number of embryos with reduced head and eye size is also shown.
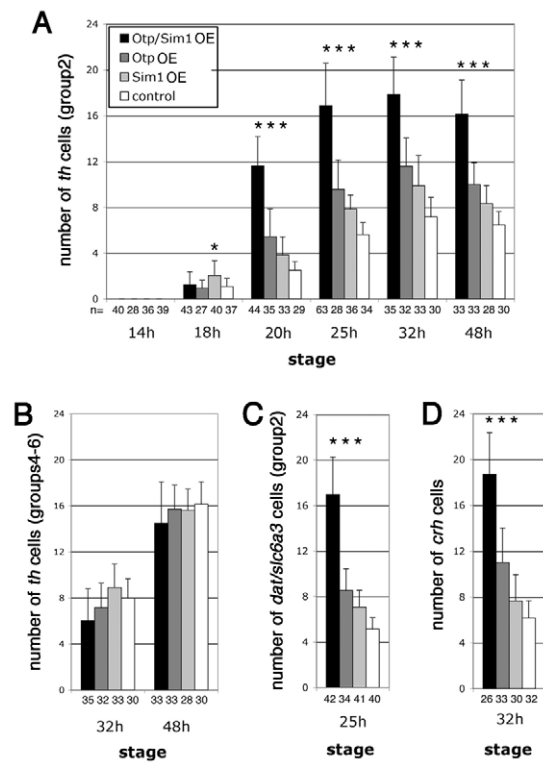
Fig. 7. Quantification of DA and CRH cell numbers in Otpa- and Sim1a-overexpressing embryos. (A-D) Bar charts illustrating the average number of *th*- (A,B), *dat*- (C) and *crh*- (D) positive neurons in hsOtpa transgenic embryos injected with *sim1a* mRNA (Otp/Sim1OE, black bars), uninjected hsOtpa transgenic embryos (OtpOE, dark-gray bars), wild-type embryos injected with *sim1a* mRNA (Sim1OE, light-gray bars) and uninjected wild-type embryos (control, white charts). Error bars indicate standard deviation of the mean. Significance was evaluated by paired Student's *t*-test. Asterisks indicate significant differences compared with the same stage control (*P*<0.001). The developmental stage as well as the number of embryos analyzed are depicted.

upon mosaic expression by plasmid injection under the control of the CMV promoter (Ryu et al., 2007). Global overexpression of *otpa* mRNA instead leads to strong embryonic malformations owing to disturbed gastrulation (Ryu et al., 2007). For late stage-specific overexpression, we created a transgenic line for heatshock inducible Otpa. Activation of *otpa* expression in *hs:otpa* fish directly after gastrulation resulted in morphologically intact embryos, which expressed *otpa* ubiquitously (see Fig. S9 in the supplementary material). In contrast to *otpa*, injection of *sim1a* mRNA did not distort embryonic development and could therefore be used in gain-of-function studies. We did not overexpress *arnt2*, as it appears to be expressed globally during early brain development.

To dissect the roles of Otpa and Sim1a during specification of neuronal cell lineages, we analyzed *hs:otpa* embryos that were also injected with *sim1a* mRNA (termed Otp/Sim1 OE for overexpression) and compared them with uninjected *hs:otpa* embryos (OtpOE), *sim1a* mRNA-injected wild-type embryos (Sim1OE) and uninjected wild-type embryos (control). *hs:otpa* embryos, as well as wild-type embryos, were subjected to a heatshock directly at the end of gastrulation at 9 hpf. We started our analysis at 16 hpf, 2 hours before the first DA neurons appear to investigate whether supernumerary DA cells are formed

prematurely. Not a single neuron had initialized *th* expression in any experimental treatment at 16 hpf. At 18 hpf, when single DA neurons start to express *th*, there was still no obvious difference in cell number observed between Otp/Sim1OE, OtpOE, Sim1OE and control embryos (Fig. 7A). Thus, Otp/Sim1OE does not appear to induce premature differentiation.

However, when we assayed *th* expression at 20 and 25 hpf in Otp/Sim1OE embryos, we detected a threefold increase in cell number compared with control embryos (Fig. 6A,B; Fig. 7A). OtpOE and Sim1OE embryos also showed an elevated number of *th* neurons compared with controls, albeit to a much smaller extent. Consistent with the elevated number of *th* cells the number of *dat/slc6a3*-expressing cells was also increased at 25 hpf in Otp/Sim1OE embryos. Therefore, the supernumerary *th* cells appear to form mature DA neurons (Fig. 7C). In support of this, all supernumerary DA cells expressed both *sim1b* and *otpb*, even if these cells were located far away of the normal *sim1/otp*-positive domains (see Fig. S11 in the supplementary material). The rostrocaudal diencephalic domain in which the DA neurons were formed was elongated in Otp/Sim1OE embryos (see brackets in Fig. 6). We monitored the expression of *dlx2*, *nk2.1*, *pitx3* and *shh* in all four experimental situations at 24 hpf. We did not detect a change in expression pattern of the analyzed patterning genes in OtpOE, Sim1OE or Otp/Sim1OE embryos, showing that early forebrain patterning was not affected (see Fig. S10 in the supplementary material).

At 32 and 48 hpf, additional DA neurons of groups 4-6 form posterior and ventral to group 2. We performed separate cell counts of anterior and posterior DA neuron populations at these stages. At 32 hpf and 48 hpf, the cell number of DA cells of group 2 in Otp/Sim1OE, OtpOE, Sim1OE and control embryos was similar to 25 hpf, arguing that new group 2 cells did not form during this time interval. At 48 hpf, group 2 DA cells were not tightly clustered as in control embryos, but instead were more dispersed and occupied ectopic locations in the ventral diencephalon (Fig. 6E-F). The number of DA cells of groups 4-6 was not altered in Otp/Sim1OE, OtpOE or Sim1OE embryos compared with controls (Fig. 7B). For an additional Arnt2/Sim1- and Otp-dependent neuronal cell type, we analyzed the expression of *crh* at 32 hpf. The first CRH neurons appear only around 25 hpf (Chandrasekar et al., 2007). The number of CRH neurons was more than threefold increased in Otp/Sim1OE embryos compared with control embryos (Fig. 6G-H; Fig. 7D). For CRH neurons, the cell numbers in OtpOE and Sim1OE embryos were also slightly elevated compared with control embryos (Fig. 7D). These observations indicate that Otp and Sim1 overexpression lead to supernumerary ectopic DA and CRH cells in the posterior tuberculum/hypothalamus only within defined diencephalic competence fields. The supernumerary ectopic DA cells display features of mature DA neurons, including the typical caudal axonal projections (see Fig. S11E,E″, arrowheads in the supplementary material).

DISCUSSION
In the present study, we use genetic evidence in zebrafish to demonstrate that specification of the *otpa*-dependent subset of DA neurons in the ventral diencephalon is also dependent on *arnt2* and *sim1a*. Zebrafish *arnt2* expression has been previously described (Andreasen et al., 2002), and an insertional mutation reported (Golling et al., 2002); however, functions during zebrafish development have not been analyzed so far. Genetic knockouts of Arnt2 in mice are perinatally lethal (Hosoya et al., 2001; Keith et al., 2001). Arnt2 functions both in developmental and physiological processes in mammals (Gu et al., 2000; Kewley, 2004). Despite the broad expression of *arnt2*, we did not observe gross abnormal
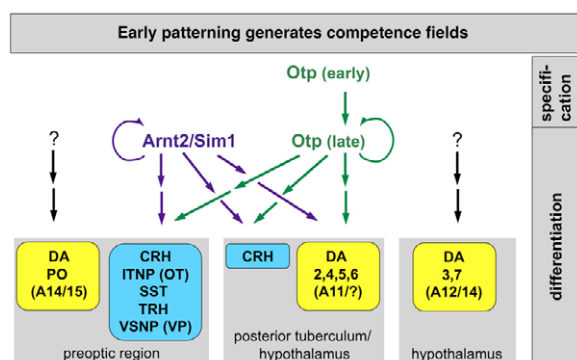
Fig. 8. Model summarizing the roles of Otp, Sim1 and Arnt2 during development of DA and neurosecretory neurons in the hypothalamus. Top to bottom schematic time axis from early patterning through neuronal specification and differentiation. Left to right DA and neurosecretory cell groups of the hypothalamus (for abbreviations, see text). The model is based on analysis in zebrafish. Tentative assignments of homologous mammalian groups are indicated using the mammalian nomenclature. The zebrafish diencephalic group 1 (mammalian A13) is located in the prethalamus and, thus, not included in this scheme.

patterning or morphological malformations during embryonic brain development of *arnt2* mutants. Homozygous *arnt2* zebrafish are viable during embryonic and early larval stages, but die eventually later than 5 dpf. The late lethality of *arnt2* zebrafish mutants makes them a well-suited tool for studying Arnt2 function during vertebrate development and physiology.

In the absence of Arnt2/Sim1 function, the three DA markers analyzed, *th*, *dat/slc6a3* and *ddc*, are expressed in a reduced number of DA cells located in posterior tuberculum and hypothalamus. This argues that Arnt2 may be involved in cell specification or control of multiple aspects of terminal differentiation of ventral diencephalic DA neurons. As *sim1* is co-expressed with the late neuronal differentiation marker *huC* but not with the proneural marker *ngn1*, *sim1* is more likely to be involved in late differentiation. Intriguingly, *arnt2* or *sim1* loss of function affects the same DA neuron groups affected in zebrafish *otpa* mutant embryos (Ryu et al., 2007). Thus, Arnt2, Sim1 and Otp are each required for specification of the same set of DA subgroups in the zebrafish brain. Arnt2-, Sim1- and Otp-dependent neurons in the anterior hypothalamus of mice do not differentiate terminally but instead either die or change fate in the absence of any one of these transcription factors. In *arnt2* mutant the expression domains of *otpa/b* and *sim1a/b* in the precursor territory are not altered at least until 3 dpf. Thus, DA progenitors expressing both Otp and Sim1 may continue to exist until 3 dpf but fail to complete differentiation in the absence of Arnt2. Whether these undifferentiated DA precursor cells subsequently die or adapt other neuronal identities could not be determined.

Arnt2, Sim1 and Otp have been demonstrated to act in genetic pathways that specify distinct neurosecretory cells in the murine hypothalamus (Michaud et al., 1998; Acampora et al., 1999; Michaud et al., 2000; Hosoya et al., 2001; Keith et al., 2001). Similarly, expression of five neurohormones is strongly reduced or absent in the preoptic region (PO) of *arnt2* mutants and *sim1* morphants. Not all the neurons in the PO depend on Arnt2/Sim1 function, as only one of two SST-expressing groups in the PO is reduced and DA neurons form normally in the PO. Combined with

a recent study, which shows the requirement of Sim1 for ITNP- and VSNP-producing cells in the PO (Eaton and Glasgow, 2006; Eaton and Glasgow, 2007), our data establish that the specification of specific neurosecretory cell types of the hypothalamus is largely conserved in zebrafish. The mammalian Otp and Arnt2/Sim1 pathways eventually merge at the level of Pou3f2/Brn2. For CRH differentiation in mouse it is known that Pou3f2/Brn2 can directly bind and activate the *crh* promoter (Nakai et al., 1995). Similarly, in Arnt2/Sim1- and Otp-deficient zebrafish, *pou47* expression in the preoptic region is strongly reduced. We also detected a slight reduction of *pou47* in the hypothalamus, but could not directly evaluate a role in DA differentiation because of widespread defects in *pou47* morphants.

We examined the epistatic relationship between *arnt2*, *otpa/b* and *sim1a/b* in zebrafish. *arnt2*, *otpa/b* and *sim1a* expression appear to be established independently, as expression domains appear unchanged if Arnt2/Sim1 or Otp activity is eliminated. However, the expression of the second *sim1* paralog, *sim1b*, was abolished in *arnt2* mutants. This could reflect subfunctionalization of the two paralogous *sim1* genes and loss of the control mechanisms for initiation of expression in *sim1b*. Regardless of the mechanism of *sim1b* regulation, we were not able to assign a function to Sim1b protein using morpholino knockdown. Given that Sim1a knockdown phenocopies the *arnt2* phenotype, Sim1b protein may have been rendered nonfunctional since the teleostean genome duplication.

We investigated whether Arnt2/Sim1 together with Otpa may be sufficient to direct neuronal differentiation. Global overexpression of Sim1 by mRNA injection together with Otp from a heat-shock inducible transgene induces supernumerary DA and CRH cells in the ventral diencephalon. In case of DA neurons, the field competent to respond to Sim1a/Otpa overexpression is rostrocaudally elongated compared with wild type, whereas in case of CRH it is enlarged both rostrocaudally and dorsoventrally. Thus, Sim1a/Otpa-induced specification of DA and CRH neurons depends on a competence area for differentiation (Fig. 8). Notably, the enlarged area of DA/CRH differentiation is not caused by an altered pre-pattern of the forebrain in Otp/Sim1OE embryos, as judged from normal expression of *dlx2*, *nkx2.1a*, *pitx3* and *shh* at 24 hpf. Supernumerary DA cells in Otp/Sim1OE embryos may contribute only to the anterior DA neuron cluster (group 2), and not to posterior DA neuron groups (groups 4-6), which appear shortly after group 2. This could be caused by a short time period of sufficient Otpa and Sim1a levels during which DA neurons of groups 4-6 might not have reached the proper state to respond to these factors. The supernumerary DA neurons in Otp/Sim1OE embryos express *otpb* as well as *sim1b*. This suggests that the maintenance of *otpb* and *sim1b* expression may be caused by an auto-regulatory mechanism after initiation of *otpa/b* and *sim1a/b* expression. The fact that overexpression of Otpa or Sim1a alone increased the number of DA and CRH neurons in a much less pronounced way implies that each factor has a limited potential to drive DA neuron differentiation by itself. In a previous experiment (Ryu et al., 2007), we used microinjection of large amounts of an overexpression plasmid for mosaic high level ectopic expression of Otpa and detected ectopic TH expression. By contrast, in the experiments here, heat shock-induced Otpa expression from a transgene probably produced only physiological Otpa levels. A further difference is that plasmid-driven mosaic expression provides Otp from early stage on throughout random single cell lineages, whereas heat shock-induced expression generates sufficient amounts of Otp only in a time window following heat shock. Therefore, the different outcome of the experiments is probably due to different

Otpa levels, arguing that the potential of Otpa to drive neuronal differentiation independently or in combination with Sim1a may be concentration and time dependent.

Interestingly, two other bHLH-PAS transcription factors have been previously linked to a DA neuronal phenotype. Two neuronal PAS domain protein members (NPAS1, NPAS3) are linked to schizophrenia-like behavior (Erbel-Sieler et al., 2004), a disease connected to DA dysfunction (reviewed by Sesack and Carr, 2002). Furthermore, the expression of TH is negatively regulated by NPAS1 in a mouse DA neuronal cell line and NPAS1 might repress TH expression by directly binding to the TH promoter (Teh et al., 2007). These data suggest a more modulatory activity of NPAS1 on TH expression levels in existing DA neurons rather than a role during DA specification. Expression of Arnt2 (and Sim1) is also maintained during adulthood in the paraventricular nucleus of mammals, making it possible that these factors also modulate gene expression levels in mature neurons (Michaud, 2001).

The di-mesencephalic DA systems of the substantia nigra/ventral tegmental area are the most prominent DA systems in mammals. However, mesencephalic DA neurons do not exist in zebrafish. Genetic analysis revealed that the largest complement of zebrafish ventral diencephalic DA neurons is specified by Otp (Ryu et al., 2007), whereas, in mouse, dorsal hypothalamic and anterior pretectal A11 group DA neurons depend on Otp function (Ryu et al., 2007). As Arnt2, Sim1 and Otp are essential for specification of the same populations of DA cells in zebrafish, one might suggest that Arnt2/Sim1 could also play a role in A11 specification. Analyses of *sim1* expression domains in murine embryos indicate that *sim1* may also be expressed in the region of A11 (Ema et al., 1996; Fan et al., 1996).

It is interesting to speculate that the A11-type DA system may have co-evolved with neurosecretory hypothalamic systems. A11-type DA neurons in fish and mouse establish the diencephalospinal DA system (Bjorklund and Skagerberg, 1979), but A11 neurons also have ascending projections and local hypothalamic connectivity (Fuxe et al., 1985; Takada et al., 1988). Mammalian A11 DA neurons have been implicated in restless legs syndrome (for a review, see Clemens et al., 2006), a motor disorder that is characterized by abnormal limb sensations and involuntary movement. CRH, OT, VP cells of the PVN and SST cells of the APV also display descending projections to the brain stem and spinal cord (Krisch, 1981; Sawchenko, 1987; Hallbeck and Blomqvist, 1999). Thus, most Otp-specified neurosecretory cell types display diencephalospinal projections in addition to local connectivity. Interestingly, VP circuitry has been implied with sensorimotor function (Rose and Moore, 2002), and VP/OT neural circuits have been postulated to simultaneously regulate somatomotor and autonomic systems (Kerman, 2008). Therefore, we hypothesize that Otp-specified neurons may share a common function in control of motor activity patterns, which may have evolved from an ancient motor control system at the base of vertebrate evolution.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/6/1007/DC1

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