A role for *foxd3* and *sox10* in the differentiation of gonadotropin-releasing hormone (GnRH) cells in the zebrafish *Danio rerio*

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Accepted 11 October 2005

Development 132, 5491-5502
Published by The Company of Biologists 2005
doi:10.1242/dev.02158

Summary

Gonadotropin-releasing hormone (GnRH) is found in a wide range of vertebrate tissues, including the nervous system. In general, GnRH has two functions: endocrine, acting as a releasing hormone; and neuromodulatory, affecting neural activity in the peripheral and central nervous system. The best understood population of GnRH cells is that of the hypothalamus, which is essential for reproduction. Less well understood are the populations of GnRH cells found in the terminal nerve and midbrain, which appear to be neuromodulatory in function. The GnRH-containing cells of the midbrain are proposed to arise from the mesencephalic region of the neural tube. Previously, we showed that neuromodulatory GnRH cells of the terminal nerve arise from cranial neural crest. To test the hypothesis that neuromodulatory GnRH cells of the midbrain also arise from neural crest, we used gene knockdown experiments in zebrafish to disrupt neural crest development. We demonstrate that decrement of the function of *foxd3* and/or *sox10*, two genes important for the development and specification of neural crest, resulted in a reduction and/or loss of GnRH cells of the midbrain, as well as a reduction in the number of terminal nerve GnRH cells. Therefore, our data support a neural crest origin for midbrain GnRH cells. Additionally, we demonstrate that knockdown of *kallmann* gene function resulted in the loss of endocrine GnRH cells of the hypothalamus, but not of neuromodulatory GnRH cells of the midbrain and terminal nerve, thus providing additional evidence for separate pathways controlling the development of neuromodulatory and endocrine GnRH cells.

Key words: Midbrain, GnRH2, Kallmann Syndrome, Neural crest, Morpholinos

Introduction

Gonadotropin-releasing hormone (GnRH) is a functionally diverse decapeptide having both reproductive and neuromodulatory roles (Dubois et al., 2002; Somoza et al., 2002). Vertebrates have three forms of GnRH distinguished on the basis of their amino acid sequence, and these forms have been named: GnRH1, GnRH2 and GnRH3 (Dubois et al., 2002; Fernald and White, 1999). Several species express variants of GnRH1 (Montaner et al., 2002; Somoza et al., 2002), and although GnRH3 is considered to be specific to fishes it has been recently reported in rats (Parhar et al., 2005). These various forms of GnRH have distinct sites of expression in the nervous system. The endocrine form of GnRH, GnRH1, is expressed in hypothalamic cell populations that have been proposed to originate from the olfactory placodes (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989a) and, more recently, from the anterior pituitary (Whitlock, 2005; Whitlock et al., 2003). The neuromodulatory form of GnRH found in the midbrain, called GnRH2, is unique in that its amino acid sequence is conserved in taxa from jawed fish to mammals (Millar, 2003). The GnRH2 neurons of the midbrain have been proposed to modulate behaviors relevant to reproduction (Millar, 2003; Temple et al., 2003), and are currently believed to arise from within the neural tube (Amano et al., 2004; Gonzalez-Martinez et al., 2002). The terminal nerve (a cranial nerve) contains cells that express GnRH (GnRH3), and these have a neuromodulatory role in the forebrain and olfactory epithelium (Abe and Oka, 2002; Eisthen et al., 2000), and modulate olfactory-mediated behaviors (Wirsig-Wiechmann et al., 2002). The terminal nerve GnRH cells are proposed to originate from the olfactory placodes (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989a) and, more recently, from the cranial neural crest (Whitlock, 2004b; Whitlock et al., 2003). Thus the GnRH3 cells of the terminal nerve are functionally more like the GnRH2 cells of the midbrain, in that they appear to modulate behaviors. For this reason, we examined whether GnRH2 cells might originate from neural crest.

The vertebrate head arises through a complex interplay between epithelial and mesenchymal cell populations. Prominent among the latter are cells derived from the cranial neural crest. Depending on the specific structure, neural crest cells may be the sole contributor or may complement contributions from other organs, e.g. mesoderm and placodes (Hall, 1999). Cranial neural crest cells give rise to a myriad of different cell types, including but not limited to: neurons, glia, pigment, and many skeletal elements of the head. Cranial neural crest cells also contribute to the specialized sensory

systems, which have become concentrated and elaborated as the structure of the vertebrate head evolved (Le Douarin and Kalcheim, 1999).

Neural crest development is a complex, multi-step process, involving a myriad of inducing signals and responding transcription factors (Heeg-Truesdell and LaBonne, 2004). Two genes encoding transcription factors important for neural crest differentiation are *foxd3* and *sox10*. A member of the forkhead family of transcription factors, *foxd3* is expressed in neural crest precursor cells. In *Xenopus*, overexpression of *foxd3* results in the induction of neural crest-specific genes and loss of function suppressed the expression of neural crest markers, suggesting a primary role in neural crest cell differentiation (Sasai et al., 2001). Zebrafish *foxd3* is expressed in the pre-migratory neural crest, floorplate, somites and tailbud (Odenthal and Nusslein-Volhard, 1998). Subsequently, it has also been shown to be expressed in glia of the peripheral nervous system (Kelsh et al., 2000). The Sry-related transcription factor gene *sox10* is expressed in pre-migratory neural crest, and loss-of-function studies support its role in promoting the survival of undifferentiated neural crest cells (Honore et al., 2003; Mollaaghababa and Pavan, 2003). In zebrafish, *sox10* is expressed in developing pre-migratory neural crest and plays an important role in specifying non-ectomesenchymal (neurons, glia and pigment) neural crest (Dutton et al., 2001b); in mammals, it is important for the maintenance of multipotency in neural crest stem cells (Kim et al., 2003).

Deficits in GnRH (hypogonadic hypogonadism) underlie some human disease. Kallmann Syndrome in humans is characterized by deficits in GnRH that are associated with anosmia (loss of sense of smell). Previous studies have shown that development of the terminal nerve and hypothalamic GnRH cells is disrupted in a human Kallmann embryo (Schwanzel-Fukuda et al., 1989), and that this phenotype results from mutations in the KAL (*anosmin 1*) gene (Duke et al., 1995; Legouis et al., 1991). Homologues of the *kallmann* gene have yet to be identified in mouse, but, in the zebrafish, there are two *kallmann* genes, *kallmann 1.1* and *kallmann 1.2* (*Kallman syndrome 1a* and *Kallman syndrome 1b* – Zebrafish Information Network). The genes are widely expressed in regions of the developing central and peripheral nervous system, as well as in the reproductive tract and pronephric ducts (Ardouin et al., 2000).

The function of midbrain GnRH2 cells suggested that, like the GnRH cells of the terminal nerve, they may arise from the cranial neural crest. We report that the decrement of *foxd3* and *sox10* gene function results in the loss of midbrain GnRH2 cells, as well as the loss of terminal nerve GnRH3 cells. In addition, we show, using a *sox10*-GFP line, that the GFP signal and *gnrh2* expression co-localize during a discreet time window in early development, thereby supporting a neural crest origin for these cells. Finally, we demonstrate that the decrement of function of the *kallmann 1.1* gene, but not the *kallmann 1.2* gene, results in loss of the hypothalamic endocrine GnRH1 cells, but did not affect the neuromodulatory midbrain or terminal nerve GnRH cells. Together, these data suggest that the neuromodulatory midbrain GnRH2 cells, like the terminal nerve GnRH cells, have a neural crest origin.

Materials and methods

Animals

All zebrafish (*Danio rerio*) used for these experiments were of the wild-type 'Cornell Strain' developed in the Whitlock Laboratory. Animals were maintained on a 14 hour/10 hour light:dark cycle. Embryos were collected on the morning of the day of the experiments from group crosses consisting of three males and three females. The *acerebellar* (*ace*) mutant (Reifers et al., 1998) was provided by the Brand Laboratory, and embryos were collected from single-pair crosses of parents heterozygous for *ace*. All animal procedures were approved by the Cornell University Institutional Animal Use and Care Committee. The *sox10*-GFP line (Wada et al., 2005) was provided by the Kelsh Laboratory and maintained in our fish facility.

Morpholinos

Morpholinos (MOs), modified oligonucleotides that interfere with mRNA translation (Heasman, 2002; Nasevicius and Ekker, 2000), have successfully been used in zebrafish and *Xenopus* to reduce gene function, including that of genes important for neural crest development (Dutton et al., 2001a; Honore et al., 2003; O'Brien et al., 2004). Here, we used MOs to knockdown the function of the *foxd3*, *sox10*, *kal1.1* and *kal1.2* genes. All MOs and corresponding 5-nucleotide mismatch controls (mm; mismatches indicated in lower case in sequence below) were synthesized by the manufacturer (Gene Tools, OR), using existing sequence information as follows:

foxd3 MO, 5′-CACTGGTGCCTCCAGACAGGGTCAT-3′;

foxd3 mm, 5′-CAgTGcTGCCTgCAGACAGcGTgAT-3′ (GenBank #NM131290);

sox10 MO, 5′-ATGCTGTGCTCCTCCGCCGACATCG-3′;

sox10 mm 5′-ATcCTcTGCTCaTCCGaCGAgATCG-3′ (GenBank #AF402677);

kal1.1 MO, 5′-CCGTCGCGCATCTTGAAGAACAGTA-3′;

kal1.1 mm, 5′-CCcTCcCGCATgTTGAAaAACAcTA-3′ (GenBank #AF163310);

kal1.2 MO, 5′-GCAGAGATTCCTCAAAAGCAGCATC-3′;

kal1.2 mm, 5′-GCAGAcATTgCTgAAAAGgAGgATC-3′ (GenBank #AF163311).

The *foxd3* and *kal1.1* MOs were tagged with lissamine (red fluorescence), and the *sox10* and *kal1.2* MOs were tagged with fluorescein (green fluorescence). This allowed for visualization of the MOs in the embryos. The control mismatch MOs were co-injected with rhodamine dextran dye (Molecular Probes), at a final concentration of 0.02%. For each MO and mismatch MO, 1, 5 and 10 nl of MO were tested to determine the most effective concentration. Injections of 10 nl or more of MOs resulted in ~80% mortality, with embryos exhibiting non-specific necrosis; injections of 5-10 nl resulted in a lower mortality with embryos living through the segmentation stages, but they still displayed high levels of necrosis. For all MOs (*foxd3*, *sox10*, *kal1.1*, *kal1.2*) 2-4 nl produced specific defects, including those previously reported for *sox10* (Dutton et al., 2001a), and little general necrosis, and were therefore the volumes injected in the experiments reported here. For all mm MOs, we injected 0.5-1 nl, which produced no observable phenotype; volumes above 2 nl resulted in 80% mortality due to non-specific necrosis. For injections of *foxd3*+*sox10*, equal amounts of MOs to *foxd3* and *sox10* were pre-mixed. Injection of 10 nl or greater of the combined MOs resulted in 80% mortality, as was seen with the individual MOs. Injections were done into one- to two-cell stage embryos (Kimmel et al., 1995). Injection pipettes were pulled using thin walled borosilicate glass tubing with microfilament (OD=1.2 mm, ID=0.94 mm) on a Sutter Puller P-2000 (Sutter Instruments).

In situ hybridization

Zebrafish embryos were staged as described in Kimmel et al. (Kimmel et al., 1995). Embryos were fixed in phosphate-buffered 4% paraformaldehyde. Whole-mount in situ hybridization was performed

as described in Thisse et al. (Thisse et al., 1993), using single-stranded RNA probes labeled with digoxigenin-UTP (Roche), the only modification being that the pronase (Sigma) permeabilization step was shortened to 3 minutes. The GnRH probes were generated after cloning these cDNAs from zebrafish (Gopinath et al., 2004). Briefly, the *gnrh2* gene was initially cloned using heterologous primers to the goldfish *gnrh2* gene [GenBank Accession number U30386 (Bogerd et al., 1994); forward primer, 5′-ATGGTGCACATCTGCAGGCT-3′; reverse primer, 5′-GTCATTTTCTCTTTTGGGAATC-3′] and the *gnrh3* gene, expressed in the terminal nerve, was cloned using primers to the zebrafish *gnrh3* sequence [GenBank Accession number AJ304429 (Torgersen et al., 2002); forward primer, 5′-CAGCACTGGTCATATGGTTGGCTTCCCGG-3′; reverse primer, 5′-CACTCTTCCCCGTCTGTCGG-3′]. *gnrh2* and *gnrh3* cDNA was amplified by RT-PCR, the products were cloned into pGEM-T Easy Vector System I (Promega) and the identity of the resulting clones confirmed by sequencing (Gopinath et al., 2004). Probes to both *gnrh2* and *gnrh3* included the sequences to the GnRH decapeptide and the GnRH associated protein (GAP) of the pre-pro GnRHs, thereby making the probes highly specific to a given form of GnRH. The pattern of in situ hybridization showed that the *gnrh2* and *gnrh3* probes had non-overlapping expression patterns, thereby confirming this specificity (see Results).

For analysis of *gnrh2* and *gnrh3* expression in the *foxd3*, *sox10* and *foxd3*+*sox10* MO-injected (morphant) animals, embryos were fixed at 35±1 hours post-fertilization (hpf) and processed for in situ hybridization using probes to *gnrh2* or *gnrh3*, as described previously (Gopinath et al., 2004). For analysis of *gnrh2* and *gnrh3* expression in the *kal1.1* morphant animals, embryos were fixed at 55±1 hpf.

Immunocytochemistry

To date the molecular form of GnRH expressed in the migrating hypothalamic cells has not been identified in zebrafish, and there is no antibody that specifically recognizes only this group of GnRH cells (see Results). Thus, for our analysis of hypothalamic GnRH (GnRH1), we used the LRH13 antibody (Park and Wakabayashi, 1986), which recognizes both the terminal nerve and hypothalamic GnRH cell populations. Immunocytochemistry was carried out as previously described (Whitlock et al., 2003), except that the embryos were fixed in 4% paraformaldehyde (Westerfield, 1993) with 7% saturated picric acid for two hours at room temperature.

Double labeling for *sox10*-GFP and *gnrh2*

Embryos positive for *sox10*-GFP were fixed at 26-28 hpf and protocol 5 of Schulte-Merker (Schulte-Merker, 2002) for immunocytochemistry/in situ hybridization double labeling was followed. The anti-GFP antibody (Molecular Probes, rabbit polyclonal) was used at a dilution of 1:1000 and the in situ probe for *gnrh2* was that described above.

Statistical analyses

The number of cells expressing *gnrh2* (midbrain) and *gnrh3* (terminal nerve) was counted in whole-mount embryos. Cell counts obtained in different groups of fish were compared using the Wilcoxon Rank Sum Test, comparing the medians of non-normal distributions.

Results

foxd3 and *sox10* morphant phenotypes

In zebrafish, the *foxd3* and *sox10* genes are expressed in the developing pre-migratory neural crest (Fig. 1A-C), and in the majority of these cells this expression is then downregulated as they migrate anteriorly (Fig. 1D) to contribute to elements of the head (Fig. 1E). These genes are later expressed in neural crest-derived structures (Dutton et al., 2001b; Kelsh et al., 2000), but the lack of continuous expression prevented us

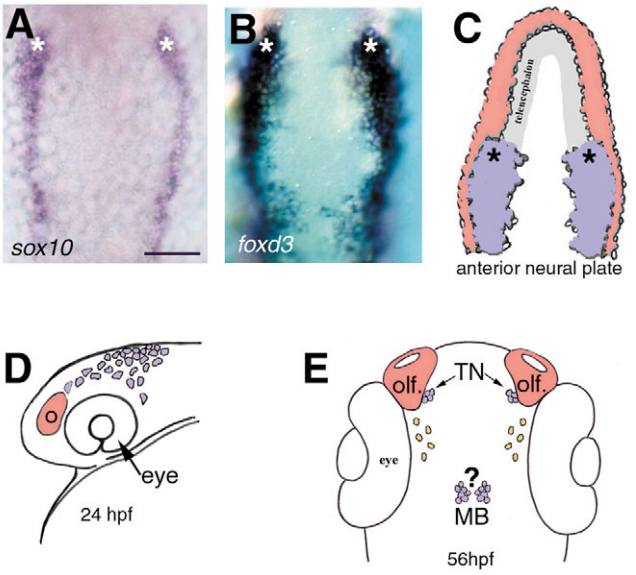

Fig. 1. Cranial neural crest domains in the developing zebrafish embryo. (A,B) Pattern of *sox10* (A) and *foxd3* (B) gene expression in 4- to 6-somite stage embryos (dorsal views). (C) The locations of the pre-migratory neural crest (purple) and placode (red) domains. Asterisks in A, B and C indicate the anterior limit of cranial neural crest domain. (D) Lateral view of a zebrafish head depicting the migratory routes of cranial neural crest (purple). Cranial neural crest cells migrating dorsal to the eye will contribute to the terminal nerve (TN) GnRH cells (E, purple) adjacent to the developing olfactory (olf) organ, and those migrating posterior to the eye may give rise to the midbrain (MB) GnRH cells (E, purple). Hypothalmic GnRH cells are indicated in orange (E). Scale bar in A: 100 μm for A,B.

from using expression as a de facto lineage tracer. In order to disrupt the development of neural crest cells, we used morpholinos (MOs; modified oligonucleotides) (Nasevicius and Ekker, 2000) to knockdown the function of *sox10* and *foxd3*, two genes essential for the differentiation of neural crest (Fig. 1A,B). Embryos injected with MOs to either *foxd3* or *sox10* alone were distinct in their appearance: *foxd3* MO-injected animals (*foxd3* morphants) had pigment but showed a flexed trunk and tail (Fig. 2B), whereas the *sox10* MO-injected embryos (*sox10* morphants) lacked body pigment (Fig. 2C). The *sox10* morphant phenotype that we obtained was the same as that previously reported (Dutton et al., 2001a; Dutton et al., 2001b). The knockdown of both Foxd3 and Sox10 resulted in a combined phenotype, such that the animals had no body pigment and a flexed trunk and tail (Fig. 2D).

Role of *foxd3* and *sox10* in the development of midbrain GnRH (*gnrh2*)

Prior to the migration of neural crest, the cranial neural crest (Fig. 1C, purple) and the region giving rise to the olfactory placodes (Fig. 1C, red) share a common border. We have shown previously, through lineage-tracing techniques, that the neuromodulatory GnRH cells of the terminal nerve (Fig. 1E) come from cranial neural crest (Whitlock et al., 2003) (Fig. 1A-C, asterisks), and that the *gnrh3* gene is expressed in these cells starting at 24-26 hpf (Gopinath et al., 2004). Here, we investigated whether the neuromodulatory *gnrh2* cells of the

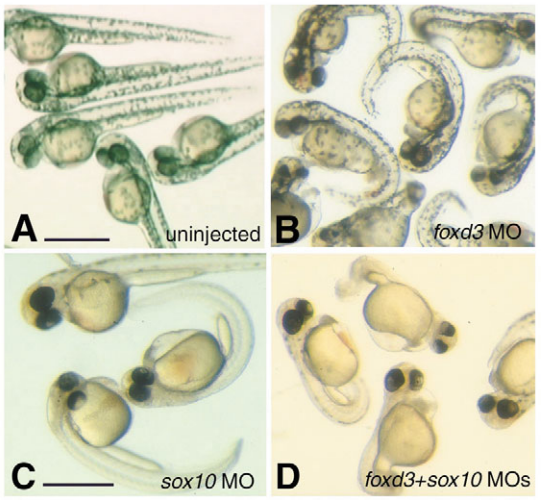

Fig. 2. Morphological phenotypes resulting from the knockdown of genes important in neural crest development. Live zebrafish embryos at 40 hpf showing uninjected controls (A), and morpholino-induced knockdown phenotypes for *foxd3* (B), *sox10* (C) and *foxd3+sox10* (D) genes. (B) *foxd3* morphant animals had pigment, but a slightly smaller head than controls and a ventrally curled tail. (C) *sox10* morphant animals had no pigment [as previously reported (see, Dutton et al., 2001b)], and occasionally a slight ventral curl to the tail. (D) *foxd3+sox10* morphant animals had no pigment and a ventrally curled tail. Scale bar: A-D, 1 mm.

midbrain (Fig. 1E), like the *gnrh3* cells, also arise from neural crest.

Previously, we have shown that *gnrh2* is expressed at 24 hpf in cells lying laterally within the mesencephalon (Gopinath et al., 2004). By in situ hybridization using a mRNA probe specific for *gnrh2*, we found that the reduction of either Foxd3 (Fig. 3B) or Sox10 (Fig. 3C) protein resulted in a significant reduction in the number of *gnrh2*-expressing cells at 35±1 hpf relative to the normal number of cells observed in mismatch MO-injected controls (Fig. 3A, Table 1). The *foxd3* morphants had a median of 12.5 cells expressing *gnrh2* (Fig. 3B, Table 1) compared with 25.0 *gnrh2*-expressing cells in the mismatch controls (Table 1; $P<0.001$). The *sox10* morphants showed a larger reduction in cell number, showing a median of 7.0 *gnrh2*-expressing cells (Fig. 3C, Table 1) compared with 23.0 *gnrh2*-expressing cells in the mismatch controls (Table 1; $P<0.001$). The numbers of *gnrh2*- and *gnrh3*-expressing cells in mismatch controls were the same as we had previously reported for uninjected wild-type animals (Gopinath et al., 2004). Strikingly, injection of both MOs together resulted in an overall loss of *gnrh2*-expressing cells in the midbrain (Fig. 3D, Table 1, $P<0.001$). Thus, knockdown of both Foxd3 and Sox10 proteins had an additive effect on the reduction in *gnrh2* cells of the midbrain, suggesting that *foxd3* and *sox10* may act together in the development of these cells.

Role of *fgf8* in the development of midbrain GnRH (*gnrh2*) cells

The loss of function of *foxd3* and *sox10* resulted in a decrement and/or loss of the midbrain *gnrh2* cells, suggesting a non-neural tube origin for these cells. To further exclude the neural

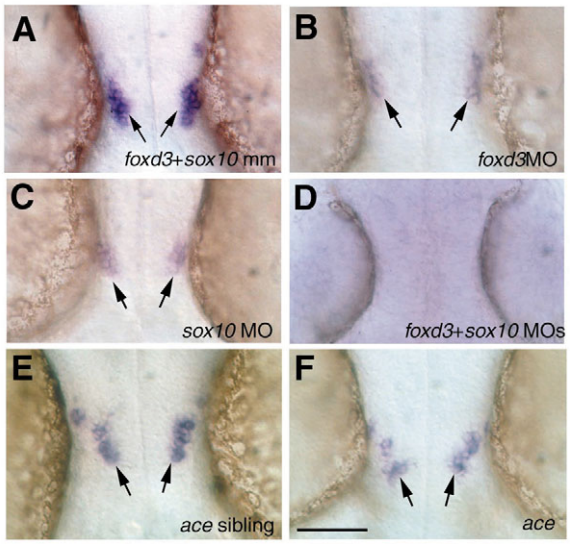

Fig. 3. Role of *foxd3*, *sox10* and *fgf8* in the development of midbrain *gnrh2* cells. Dorsal views of whole-mount preparations, showing *gnrh2* expression in the head of embryos at 35±1 hpf. (A-D) The effects of knockdown of *foxd3* and *sox10*. (A) *foxd3+sox10* mismatch control embryos, showing a normal complement of *gnrh2* cells (arrows) (Gopinath et al., 2004). *foxd3* (B)and *sox10* (C) morphant animals showed significantly fewer *gnrh2* cells than did mismatch control embryos (A), whereas animals injected with both *foxd3* and *sox10* MOs (D) showed a complete loss of *gnrh2* cells (see Table 1). (E,F) Role of *fgf8* in *gnrh2* development. (E) Expression of *gnrh2* in an *ace* non-mutant sibling control. (F) *ace* mutant embryo showing the normal number of *gnrh2*-expressing cells (see Table 1). Scale bar: 100 μm.

Table 1. Number of cells* expressing GnRH in the midbrain (*gnrh2*) and terminal nerve (*gnrh3*) in MO- and mismatch control (mm)-injected embryos

Target gene	*n*	min.	median	max.	*P*-value†
Midbrain (*gnrh2*)					
foxd3 mm	50	14	25.0	40	
foxd3 MO	60	4	12.5	24	<0.001
sox10 mm	50	15	23.0	32	
sox10 MO	60	0	7.0	19	<0.001
foxd3+sox10 mm	53	13	22.0	34	
foxd3+sox10 MOs	60	0	0	7	<0.001
ace siblings	53	8	21	33	
ace mutants	68	10	21	38	0.561‡
Terminal nerve (*gnrh3*)					
foxd3 mm	50	1.0	7.0	13	
foxd3 MO	53	0	1.5	5	<0.001
sox10 mm	50	1.0	6.0	12	
sox10 MO	72	0	0.5	5	<0.001
foxd3+sox10 mm	50	0.5	4.0	9	
foxd3+sox10 MOs	60	0	1.0	3	<0.001

*Total number of cells on both sides of embryo.
†Values were compared with mm controls using the Wilcoxon Rank Sum Test.
‡*ace* mutants were compared with their control siblings.
All embryos were fixed at 35±1 hours post-fertilization.
Mismatch controls were in agreement with wild-type cell counts for GnRH2, $n=24$; GnRH3, $n=5$ (Gopinath et al., 2004).
min., minimum number of cells per animal.
max., maximum number of cells per animal.

tube as a possible origin for these cells, we examined the role of *fgf8* (*fibroblast growth factor 8*) in the development of midbrain *gnrh2* cells. The *fgf8* gene plays a role in the patterning of the midbrain-hindbrain boundary (MHB) region of the neural tube, as well as in the maintenance of gene expression in the developing midbrain (Irving and Mason, 2000; Jaszai et al., 2003; Reifers et al., 1998). We examined *gnrh2* expression in the *acerebellar* (*ace*) mutant, which carries a null mutation in the *fgf8* gene. We found that there was no statistically significant change in the number of cells expressing *gnrh2* in *ace* mutant embryos (Fig. 3F) relative to their sibling controls (Fig. 3E, Table 1, P=0.561). The normal pattern of *gnrh2* expression in these embryos mutant for Fgf8 function provided further support that *gnrh2* cells do not arise from the neural tube.

Role of *foxd3* and *sox10* in the development of terminal nerve GnRH (*gnrh3*) cells

In order to better understand the genetic pathways underlying the development of the neural crest-derived *gnrh3* cells, we examined *foxd3* and *sox10* morphant animals for the presence of *gnrh3*-expressing neurons in the terminal nerve. In *foxd3* morphants, the median number of cells expressing *gnrh3* in the terminal nerves at 35 hpf was reduced from 7.0 (control, Fig. 4A) to 1.5 (morphant, Fig. 4B, Table 1; P<0.001). The number of *gnrh3* cells in *sox10* morphants was also decreased, showing a median number of 0.5 cells (Fig. 4C, Table 1; P<0.001), compared with that in *sox10* mismatch control morphants (median=6.0, Fig. 4A, Table 1). The combined reduction of both Foxd3 and Sox10 proteins also resulted in a decrease in the number of *gnrh3*-expressing cells (Fig. 4D-F, Table 1; P<0.001) relative to the double-mismatch morphant control (Fig. 4A). The phenotype of embryos injected with both MOs ranged from a complete lack of *gnrh3* cells (Fig. 4D) to *gnrh3* cells present in association with only one developing olfactory organ (Fig. 4E, arrow). In a few preparations there were cells associated with both developing placodes, but they were displaced dorsal to the olfactory organs (Fig. 4F, arrows). This displacement was observed only in the double morphant embryos, and may be due to the disrupted timing of neural crest migration and differentiation. These data suggest that both *foxd3* and *sox10* play a role in the development of the *gnrh3*-expressing cells of the terminal nerve.

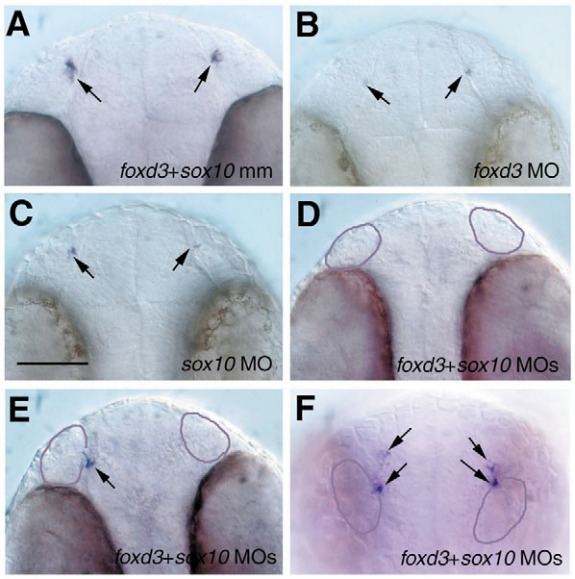

Fig. 4. Role of *foxd3* and *sox10* in the development of *gnrh3* cells in the terminal nerve. Whole mounts showing *gnrh3* expression (arrows) at 35±1 hpf in *foxd3+sox10* mismatch control (A), *foxd3* MO (B), *sox10* MO (C) and *foxd3+sox10* MO (D-F) morphant animals. Knockdown of either gene, or of both genes together, caused a significant decrease in the number of cells expressing GnRH3 (see Table 1). Knockdown of both Foxd3 and Sox10 proteins resulted in a total loss of *gnrh3* cells (D), or a partial loss of *gnrh3* cells either associated with the olfactory placode (E, arrow), or displaced dorsally (F, arrows). Purple outline indicates the olfactory placodes. (A-E) Ventral views with anterior at the top; (F) frontal view with dorsal at the top. Scale bar: 80 μm.

Specificity of MO-induced defects

To determine whether the reductions in *gnrh3* and *gnrh2* cell number caused by the injection of *sox10* and *foxd3* MOs were

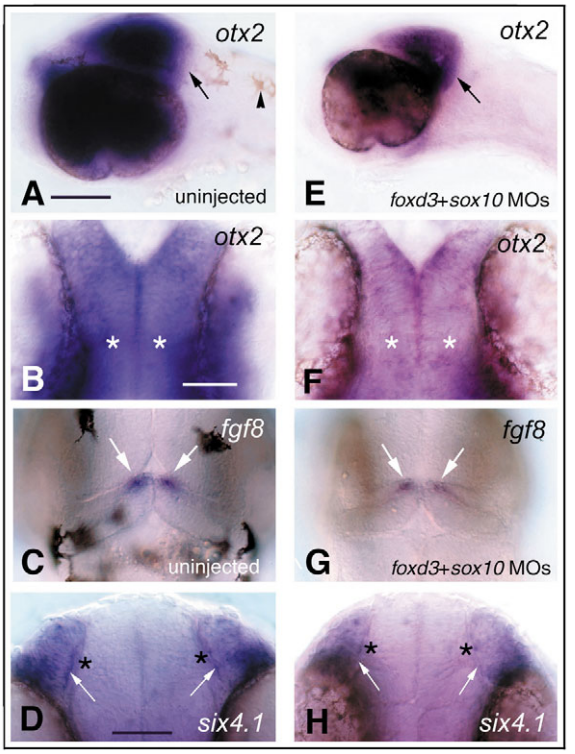

Fig. 5. *otx2*, *fgf8* and *six4.1* genes are expressed in a normal spatio-temporal pattern in the *foxd3+sox10* morphant embryos. (A-D) Uninjected embryos showing the pattern of *otx2* (A,B), *fgf8* (C) and *six 4.1* (D) gene expression at 35±1 hpf. (E-H) Corresponding pattern of gene expression of *otx2* (E,F), *fgf8* (G) and *six 4.1* (H) in *foxd3+sox10* morphant embryos at 35±1 hpf. (A,E) Lateral views with MHB gene expression (arrow), and pigment cells in the uninjected animals (A, arrowhead). (B,F) Dorsal view showing *otx2* expression in the midbrain; asterisks indicate the approximate location of the *gnrh2* cells at this stage of development. (C,G) Dorsal views showing that *fgf8* expression (arrows) is maintained at the MHB. (D,H) Ventral view of olfactory organs (arrows) expressing *six4.1*; asterisks indicate where the *gnrh3* cells would be found at this stage of development. Scale bars: 100 μm in A,E; 50 μm in B,C,F,G; 100 μm in D,H.

due to non-specific effects, we examined the expression of *otx2*, *fgf8* and *six4.1* in MO-injected embryos. The *otx2* gene normally has a border of expression at the midbrain hindbrain boundary (MHB) (Li et al., 1994), and *fgf8* is expressed in a distinct stripe of cells at the MHB (Scholpp et al., 2003). The MHB is an important signaling center in the developing neural tube, and is the region where the midbrain GnRH2 cells are first found (Amano et al., 2004; White and Fernald, 1998). We found no detectable alterations to the pattern of *otx2* or *fgf8* expression in *sox10+foxd3* morphant embryos, the class of MO-treated embryos most likely to express non-specific defects because of their more extreme phenotype (Fig. 3D, Fig. 4D; Table 1). The posterior border of *otx2* expression was unaltered in *sox10+foxd3* morphant animals (Fig. 5E, arrow) when compared with that of wild-type uninjected fish (Fig. 5A, arrow). Likewise, *sox10+foxd3* morphants showed strong *otx2* expression in the midbrain (Fig. 5F), similar to that observed in wild-type embryos (Fig. 5B). Consistent with the maintenance of the MHB, expression of *fgf8* at 36 hpf was present in the *sox10+foxd3* morphant animals (Fig. 5G, arrows) in the same pattern as was observed in wild-type uninjected fish (Fig. 5C, arrows). Thus, both *otx2* and *fgf8* expression patterns are maintained at the midbrain-hindbrain boundary in the *sox10+foxd3* morphants.

The *six4.1* gene encodes a transcription factor that is expressed in the developing olfactory placodes (Kobayashi et al., 2000) (Fig. 5D,H). As shown in Fig. 5H, the *sox10+foxd3* morphant embryos also showed a normal pattern of gene expression (Fig. 5D). Thus, our results suggest that the effects of knocking down *sox10* and *foxd3* on the number of *gnrh3*- and *gnrh2*-expressing cells were not due to a non-specific interference with the development of this region of the head.

Expression of *sox10*-GFP in the *gnrh2* cells of the midbrain

The *sox10* gene is initially expressed in pre-migratory neural crest (12 hpf; Fig. 1A). As the neural crest starts to migrate *sox10* expression is downregulated. At 18-20 hpf, there is a small cluster of cells lying between the neural tube and the backside of the developing optic cup (Fig. 6A, arrows). By 24 hpf, this gene expression is no longer apparent by in situ hybridization. Using the *sox10*-GFP embryos, we were able to visualize this cluster of cells until 28 hpf because of the perdurance of the GFP protein (Fig. 6B, arrows, anti-GFP antibody). Because *gnrh2* is first expressed at 24-26 hpf (Fig. 6C, arrows), as the *sox10*-GFP expression was waning, we were able to double label for *gnrh2* (Fig. 6C, arrows) and the GFP protein (Fig. 6B, arrows). A subset of the *gnrh2*-expressing cells lying at the edge of the neural tube against the eye (Fig. 6D, purple, arrowhead; see also Fig. 6C) were also positive for *sox10*-GFP immunoreactivity (Fig. 6D, brown and purple, arrow; see also Fig. 6B), indicating that these cells still expressed *sox10*-GFP. Not all *gnrh2* cells were positive for GFP (Fig. 6D, arrowhead), presumably because some of the cells had already lost their *sox10*-GFP signal. On average there are 5.33±0.58 *gnrh2*-positive cells at 24 hpf (Gopinath et al., 2004). In our analysis of ten preparations, 2.9±0.69 (±s.e.m.) *gnrh2*-positive cells also contained GFP. Thus, around 54% of the *gnrh2* cells were double labeled at 24-26 hpf.

The expression of *sox10* in the first two days of development is very dynamic. At 36 hpf, *sox10* expression is initiated in the

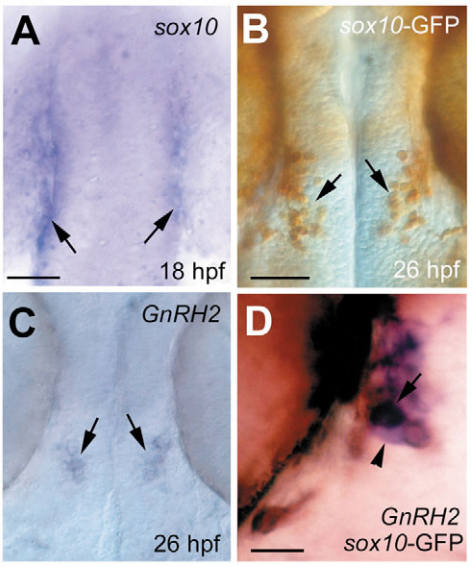

Fig. 6. Co-localization of *sox10*-GFP and *gnrh2* expression in the developing midbrain. (A) The expression *sox10* is maintained in a small cluster of cells (arrows) at the backside of the developing optic cup at 18 hpf. (B) The GFP protein, recognized with an anti-GFP antibody at 26 hpf (arrows), reflects the earlier expression of *sox10*. (C) Differentiating *gnrh2* cells (arrows) are found in the same region as the *sox10*-GFP immunoreactivity (see B). (D) *gnrh2*-expressing cells (purple, arrowhead) lie in the lateral neural tube, and in a subset of these cells the in situ signal (purple) co-localizes with the *sox10*-GFP immunoreactivity (purple+brown, arrowhead). Scale bars: A, 100 μm; B,C, 100 μm; D, 20 μm.

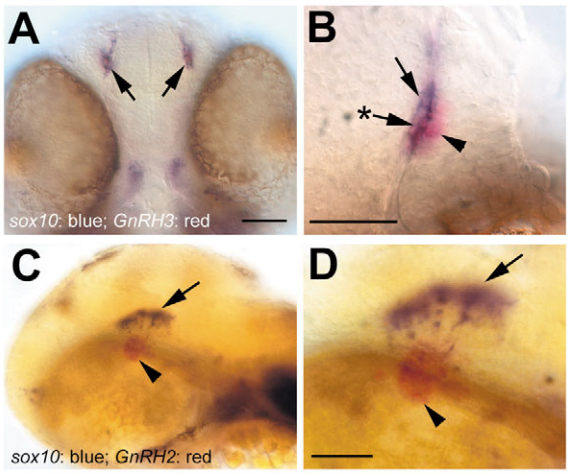

Fig. 7. Onset of *sox10* expression in the terminal nerve and midbrain at 48 hpf. (A) Ventral view showing *sox10* expression in the terminal nerve (blue, arrows). (B) Higher magnification view of the terminal nerve on one side, showing *gnrh3*-positive cells (arrowhead, red), *sox10*-positive cells (arrow, blue), and cells apparently labeled for *sox10* and *gnrh3* (arrow with asterisk). (C) Lateral view showing *sox10* (blue, arrow) and *gnrh2* (red, arrowhead) expression in the midbrain. (D) Higher magnification view of the *gnrh2*-positive cells (arrowhead, red) and *sox10*-positive cells (arrow, blue); there is no co-localization of *sox10* and *gnrh2* at this stage in development. Scale bars: A,C, 50 μm; B,D, 40 μm.

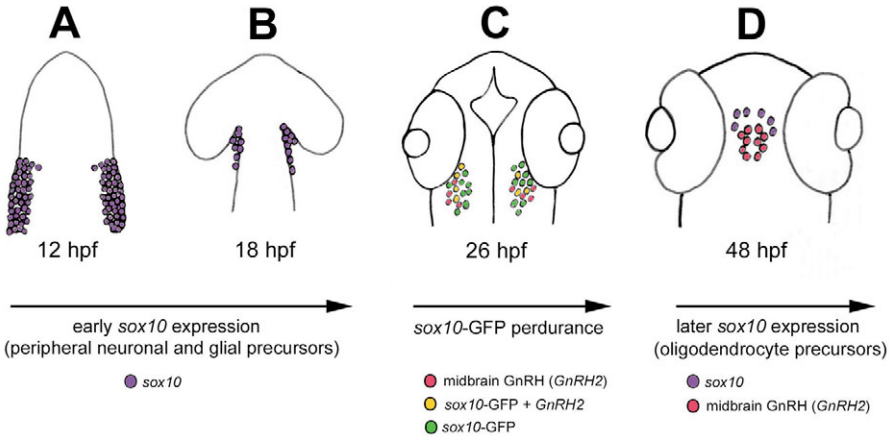

Fig. 8. Schematic of the developmental profile of *sox10* and *gnrh2* expression. (A) Dorsal view of the anterior neural plate at the 4- to 5-somite stage (~12 hpf), depicting *sox10* expression (purple) in the pre-migratory neural crest. (B) At 18-20 hpf, there are small populations of *sox10*-expressing cells on the backside of the forming optic cup (purple). (C) At 26 hpf, the perdurance of GFP allows the visualization of the cells previously expressing *sox10* (green), which lie adjacent to *gnrh2* cells (red) or co-express *gnrh2* (yellow). (D) Within the developing midbrain at 48 hpf, the onset of *sox10* expression (purple) is adjacent to but not overlapping with the *gnrh2* expression (red).

region of the terminal nerve and at 48 hpf is in the cells surrounding the *gnrh3* expression in the terminal nerve (Fig. 7A,B). In general, it appears that the *gnrh3* (Fig. 7B, arrowhead, red) and *sox10* (Fig. 7B, arrow, blue) signals do not overlap, although some preparations showed cells that appeared to co-express *gnrh3* and *sox10* (Fig. 7B, arrow with asterisk). Within the midbrain there was no expression of *sox10* at 36 hpf, and at 48 hpf there were *sox10*-expressing cells in the CNS (Fig. 7C,D, arrows). This *sox10* expression in the midbrain (Fig. 7C,D, arrows, blue) did not co-localize with *gnrh2* expression (Fig. 7C,D, arrowheads, red), and most likely represents oligodendrocyte precursors (Woodruff et al., 2001).

The earliest *sox10* expression (Fig. 8A,B, purple) may play a role in maintaining the pluripotency of the pre-migratory and early migratory neural crest cells. The use of the *sox10*-GFP line allowed us to follow the lineage of the early *sox10*-expressing cells by the perdurance of the GFP cells (Fig. 8C, green, yellow), and to visualize the loss of *sox10* as the *gnrh2* cells were differentiating. By 48 hpf, *sox10* is expressed within the developing brain (Fig. 8D, purple) in cells dorsal to the *gnrh2* cells (Fig. 8D, red).

Role of foxd3 and *sox10* in the development of hypothalamic GnRH cells

Previously, we have shown that the endocrine GnRH cells populating the hypothalamus arise from the anterior pituitary placode, and that these cells are lost in mutants lacking the *gli1* and *gli2* signaling molecules, that disrupt anterior pituitary development (Whitlock et al., 2003). In order to determine whether the development of the endocrine GnRH cells of the hypothalamus was affected by knocking down *foxd3* and *sox10* function, we assayed for the presence of these GnRH cells in *sox10+foxd3* morphant embryos. In zebrafish, the anti-GnRH antibody LRH13 labels both the terminal nerve and the hypothalamic GnRH cells (Fig. 9E, arrowheads), whereas in situ probes to *gnrh3* only label the terminal nerve cells (Fig. 9A). This suggests that hypothalamic cells express a different form of GnRH, as occurs in fish as well as some mammals (Lethimonier et al., 2004; Montaner et al., 2002; Somoza et al., 2002). To date, the gene encoding the form of GnRH expressed

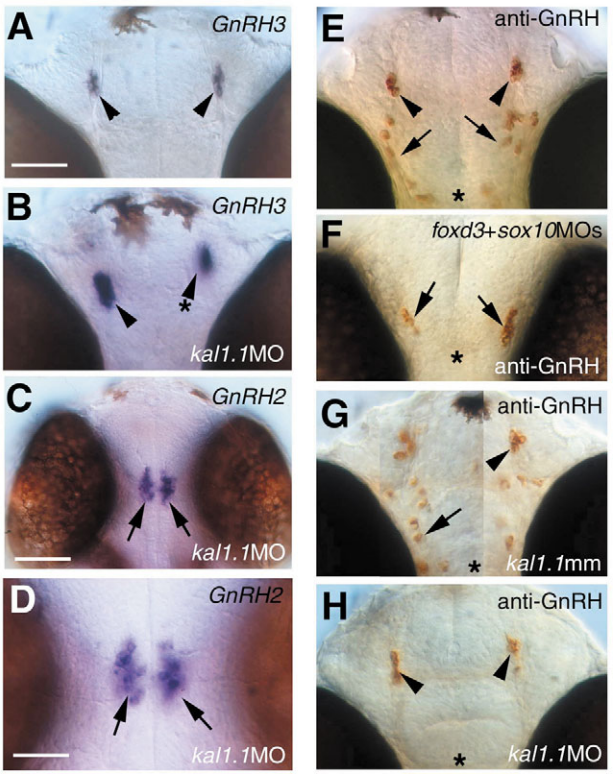

Fig. 9. Role of *foxd3*, *sox10* and *kal1.1* in the development of GnRH cells. (A) Normal pattern of *gnrh3* expression in cells of the terminal nerve (arrowheads). (B) *gnrh3* cells were present in normal numbers in *kal1.1* morphants, but could be disorganized; arrowhead with asterisk indicates *gnrh3* cells displaced dorsally. (C,D) *gnrh2* cells (arrows) were present in normal numbers in *kal1.1* morphants. (E-H) Effects of *foxd3+sox10* and *kal1.1* knockdown on the development of hypothalamic GnRH, visualized using a pantropic anti-GnRH antibody. (E) Normal pattern of GnRH-IR showing hypothalamic GnRH cells (arrows) extending back to the post-optic commissure (asterisk), and terminal nerve GnRH3 cells (arrowheads). (F) Presence of GnRH cells of the hypothalamus (arrows) was not affected by knockdown of *foxd3* and *sox10*, but these cells were clustered near the region of the post-optic commissure (asterisk). (G) Normal pattern of GnRH immunoreactivity obtained in *kal1.1* mismatch controls (arrowhead, terminal nerve cells; arrow, hypothalamic cells). (H) Knockdown of *kal1.1* eliminated the hypothalamic population of cells but did not affect the development of the terminal nerve cells (arrowhead; cf. B). All embryos were 56±1 hpf. Scale bars: 70 μm in A,B,E-H; 100 μm

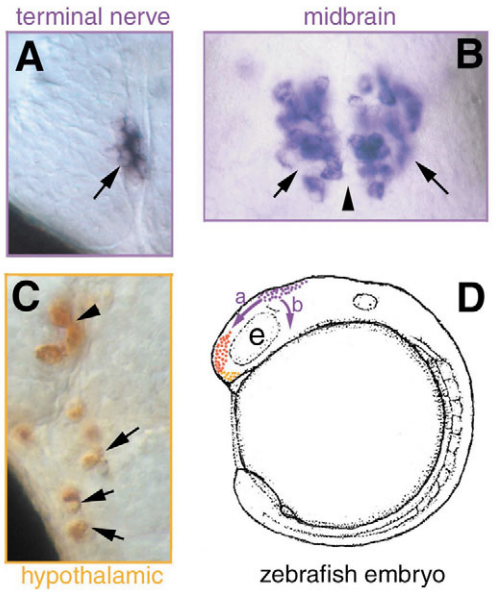

Fig. 10. Summary indicating the proposed route of migration for the *gnrh3* cells of the terminal nerve and the *gnrh2* cells of the midbrain. (A) Terminal nerve *gnrh3*-expressing cells (arrow) arise from cranial neural crest and migrate rostrally dorsal to the eye (D, purple arrow, a) to join the forming olfactory placode (D, red). (B) Midbrain *gnrh2* cells (arrows) lying on either side of the midline (arrowhead) also arise from cranial neural crest and migrate posterior to the developing eye (D, purple arrow, b). (C) Hypothalamic GnRH cells (arrows) arise from the anterior pituitary region [D, orange (see Whitlock et al., 2003)]. (D) Lateral view of an embryo showing the migration routes (purple arrows) of cranial neural crest. e, eye.

in this migratory population of GnRH cells has not been identified in zebrafish. Therefore, we used an antibody recognizing GnRH (Park and Wakabayashi, 1986) to visualize the hypothalamic GnRH cells in *sox10+foxd3* morphant embryos. Previously, we have shown that GnRH-IR is not expressed in hypothalamic GnRH cells until 52-54 hpf. As shown in Fig. 9F, hypothalamic GnRH cells were present in *sox10+foxd3* morphant embryos when scored at 56 hpf. In spite of the altered morphology of the head, the hypothalamic GnRH cells were found in a group (Fig. 9F, arrows) near the post-optic commissure (Fig. 9F, asterisk). Thus, the combined knockdown of the Foxd3 and Sox10 proteins did not result in the loss of the GnRH cells of the hypothalamus, suggesting that these cells do not depend on *foxd3* and *sox10* gene function for their development.

Role of Kallmann gene function in the development of hypothalamic GnRH cells

We used MOs to knockdown *kal1.1* and *kal1.2* gene function, and assayed the effects on the GnRH populations of the hypothalamus (GnRH1), midbrain (GnRH2) and terminal nerve (GnRH3), at 55±1 hpf. We found that injection of *kal1.2* MO had no effect on the number of neuromodulatory GnRH2 or GnRH3 cells, but caused a slight decrease in the number of endocrine GnRH cells (H.K. and K.E.W., unpublished). Surprisingly, we found that the *kal1.1* MO resulted in a complete loss of the endocrine GnRH cells of the hypothalamus (Fig. 9H, *n*=35), an effect not seen in the *kal1.1* mismatch control (Fig. 9G). By contrast, the number of GnRH3 cells of the terminal nerve was unaffected in the *kal1.1* morphants, as judged both by immunocytochemistry (Fig. 9H, arrowheads) and by *gnrh3* gene expression at 55±1 hpf (Fig. 9B, arrowheads). Although the two clusters of terminal nerve GnRH cells could sometimes be found in abnormal positions (Fig. 9B, arrowhead with asterisk), this defect was most likely the result of disrupted development of the olfactory system (H.K. and K.E.W., unpublished). Likewise, the *gnrh2*-expressing cells of the midbrain (Fig. 9C,D, arrows) were unaffected in the *kal1.1* morphants, when scored at 55±1 hpf. Thus, only the endocrine GnRH cells of the hypothalamus were affected by the loss of function of the *kal1.1* gene. These data support a model where the development of the neuromodulatory midbrain and terminal nerve GnRH cells is controlled by signaling pathways separate from those regulating the development of the endocrine GnRH cells.

Discussion

Vertebrate brains contain up to three distinct populations of gonadotropin-releasing hormone (GnRH) cells. Both the GnRH3 cells of the terminal nerve, as well as the GnRH2 cells of the midbrain have a neuromodulatory function in the adult animal (Pawson et al., 2003), whereas the hypothalamic GnRH cells play an endocrine role in the hypothalamic/pituitary axis. The neuromodulatory GnRH3 cells of the terminal nerve arise from cranial neural crest (Von Bartheld and Baker, 2004; Whitlock, 2004b; Whitlock et al., 2003), whereas the endocrine GnRH1 cells of the hypothalamus arise from the anterior pituitary placode (Whitlock and Westerfield, 2000; Whitlock et al., 2003). The data presented here suggest a cranial neural crest origin for the GnRH2 cells of the midbrain, much like the origin of the terminal nerve GnRH3 cells. Cranial neural crest cells follow stereotyped migratory routes (Le Douarin and Kalcheim, 1999): one route passing rostrally over the dorsal prosencephalon (Fig. 10D, purple arrow, a), and another route passing ventrally caudal to the developing eye (Fig. 10D, purple arrow, b). These are the most likely routes followed by the precursors of the *gnrh3* (Fig. 10A) and *gnrh2* (Fig. 10B) cells, respectively. The hypothalamic GnRH1 cells (Fig. 10C) were unaffected by the knockdown of *sox10* and/or *foxd3* function, further supporting an origin independent of neural crest.

Neural crest origin of GnRH2 cells as reflected by *sox10*-GFP expression

In pre-migratory neural crest, the expression patterns of *foxd3* and *sox10* overlap extensively, but not completely (Dutton et al., 2001b; Odenthal and Nusslein-Volhard, 1998). *foxd3* is downregulated upon migration (Kelsh et al., 2000) (K.E.W., unpublished), and *sox10* is maintained in the medial migratory route giving rise to glia of the cranial ganglia (Dutton et al., 2001b). We were able to use *sox10*-GFP embryos to follow the development of the *sox10* cells past 18-20 hpf, when the *sox10* gene was no longer expressed in the clusters of cells between the neural tube and eye. The co-localization of GFP with *gnrh2* at 26 hpf suggests that the *gnrh2* cells arise from this specific group of cranial neural crest-derived cells. The expression of *sox10* has been extensively discussed as being important in the

differentiation of peripheral glia and central nervous system oligodendrocytes (Woodruff et al., 2001). We have shown that *sox10* is expressed in cells clustered around the *gnrh3* cells of the terminal nerve at 36 hpf, and these cells may indeed be peripheral glia. More recently, *sox10* has been shown to play a role in neuronal differentiation in rat neural crest stem cells (NCSCs). Constitutive expression of SOX10 allows NCSCs to maintain their proliferative activity, thus allowing for the induction of the proneural gene MASH1 (Kim et al., 2003). Here, we show that an MO-induced decrement in Sox10 expression results in a loss of *gnrh2* cells, suggesting that, like in rat NCSCs, *sox10* expression may be important for the differentiation of these neurons. Therefore, as previously reported (Kim et al., 2003), *sox10* expression may allow for the maintenance of the proliferative plasticity necessary for GnRH2 neural differentiation and, unlike in glial subtypes, the expression is not maintained after the neurons differentiate.

At 48 hpf, *sox10* expression is maintained in the terminal nerve and is also expressed in a cluster of cells in the midbrain (Fig. 7C,D; Fig. 8D). The midbrain *sox10* expression does not co-localize with the *gnrh2* cells and may represent differentiating oligodendrocytes previously described in the developing spinal cord of the zebrafish (Park and Appel, 2003; Park et al., 2002). In zebrafish, the co-expression of *sox10* and *olig2* within the spinal cord is not evident until 48 hpf (Park et al., 2002), a time well after we scored our morphants (36 hpf). Thus, in our experiments we would not have been able to observe any effects on oligodendrocyte differentiation.

Signaling pathways involved in the development of GnRH cells

The effects of reducing Foxd3 and/or Sox10 on the development of the midbrain and terminal nerve GnRH cells suggest a neural crest origin for both cell types. However, the relative contributions of these gene products to the development of the two populations differed: we observed, on average, a complete loss of *gnrh2* cells in the double morphant, whereas the *gnrh3* cell population was less affected, suggesting that other genes may be involved. This is consistent with previous work showing that the signals patterning cranial neural crest differ, such that the crest cells contributing to the frontal mass express different genes than those contributing to the branchial arches (for a review, see Santagati and Filippo, 2003). The roles of specific transcription factors, such as *sox10* and *foxd3*, in neural crest development are complex. For example, in *Xenopus*, *sox10* is required for early development of the neural crest, and its suppression results in the loss of *foxd3* and *slug* expression (Honore et al., 2003). In zebrafish, loss of *sox10* results in the elimination of melanocytes, as well as in the disruption of enteric ganglia (Dutton et al., 2001a; Dutton et al., 2001b). The *foxd3* gene appears to act as a transcriptional repressor in *Xenopus*, where overexpression can lead to the loss of neural crest (Pohl and Knochel, 2001), and, in chick, it appears to repress melanogenesis (Kos et al., 2001). Both *foxd3* and *sox10* are necessary for the genesis of glia and neurons in the peripheral nervous system of animals ranging from fish to mammals (Britsch et al., 2001; Dutton et al., 2001b; Honore et al., 2003; Southard-Smith et al., 1998). The knockdown of *foxd3* and *sox10* most likely disrupts both the initial genesis of neural crest, as well as the development of neuronal and glial lineages. Future analysis of the role of other transcription factors important for neural crest development, such as AP2 (Hilger-Eversheim et al., 2000; Mitchell et al., 1991), will allow us to further define the signaling pathways involved in segregating the GnRH2 and GnRH3 neuronal populations in zebrafish. Whether the neural crest origins for the GnRH2 and GnRH3 cells, and the underlying genetic pathways reported here for zebrafish, apply to all vertebrates will require further investigations.

Disruption of midbrain-hindbrain patterning: interpretations of the possible phenotypes

The hypothesis that GnRH2 cells originated from the neural tube was based on the location of these cells in developing and adult animals. In the flounder and the zebrafish, GnRH2 cells are found in a region of the midbrain (midbrain tegmentum) in juvenile and adult animals (Amano et al., 2004; Gopinath et al., 2004). In addition, ablation studies support the idea that the GnRH2 cells have an origin independent from that of the GnRH1 and GnRH3 cells (Northcutt and Muske, 1994). It was subsequently proposed that this population arises from the germinal zone around the third ventricle in the mesencephalic region of the neural tube (Amano et al., 2004; Parhar et al., 1998; White and Fernald, 1998). The posterior border of the midbrain and regions immediately adjacent to it are specified by *fgf8*, which is expressed in the midbrain-hindbrain boundary (MHB). Establishment of midbrain and hindbrain progenitors is independent of *fgf8*, but the maintenance of midbrain gene expression appears to be dependent upon *fgf8* (Jaszai et al., 2003; Reifers et al., 1998). We used the zebrafish *ace* mutant, which lacks *fgf8* function, to disrupt patterning of the neural tube. Our data show that the loss of *fgf8* does not result in a measurable change in the number of *gnrh2* cells, suggesting that *gnrh2* cells either do not arise from the neural tube, or arise from a region that is not dependent upon *fgf8* for patterning information.

Neural crest migration into the neural tube

The developing embryo is a study in cell migration, and the neural tube is notable for the migration of the neural crest from its dorsal surface. In addition, there have been reports of ventrally emigrating neural tube cells (VENT) that leave the neural tube and contribute neurons to the peripheral nervous system, as well as neurons and glia to the enteric nervous system (Dickinson et al., 2004). Furthermore, cells associated with the brain vasculature (vascular smooth muscle cells) migrate into the central nervous system and may arise from neural crest-derived head mesenchyme re-entering the developing brain (Korn et al., 2002). Thus, there may be precedents for neural crest-derived non-neuronal cell types re-entering the neural tube during development.

Our results indicate that the *gnrh2*-expressing cells of the midbrain arise from cells whose site of origin and early molecular characteristics are similar to those of neural crest cells. This is not the first report of centrally located neurons arising from neural crest and migrating into the neural tube. For example, the neurons of the mesencephalic nucleus of the trigeminal (Mes5) have been reported to arise from neural crest (Narayanan and Narayanan, 1978) and to then migrate to their adult location in the mesencephalon. However, we first see *gnrh2*-expressing cells at 24 hpf lying laterally within the neural tube, and, by 56 hpf, they are in two clusters lying on

either side of the midline (Gopinath et al., 2004). Thus, they do not appear to be 'unique primitive population along the dorsal midline', as has been described for Mes5 neurons (Sanchez et al., 2002). Furthermore, loss of *fgf8* in the isthmus did not affect the number of *gnrh2* cells, as it does for the Mes5 neurons (Hunter et al., 2001). Our data support the idea that *gnrh2* cells arise from neural crest-derived cells that migrate into the neural tube during development.

Neural crest origin for GnRH3 cells of the terminal nerve

In zebrafish, the terminal nerve is an easily identifiable telencephalic population of GnRH cells in the forebrain that expresses *gnrh3* early in development (Gopinath et al., 2004; Whitlock et al., 2003). In medaka, the differences in timing between the onset of expression of *gnrh3* in the terminal nerve and *gnrh1* in the hypothalamus led to the proposal that these populations do not share a common origin in the olfactory placode (Dubois et al., 2002; Parhar et al., 1998). Consistent with this hypothesis, we have found that, in zebrafish, GnRH3 cells of the terminal nerve have their origin in the cranial neural crest (Whitlock, 2004a; Whitlock, 2004b; Whitlock et al., 2003). Whether the terminal nerve arises from neural crest or the olfactory placode has been the subject of debate for the last century (Von Bartheld and Baker, 2004; Whitlock, 2004b). The data presented here support and extend our previous findings that GnRH3 cells of the terminal nerve arise from cranial neural crest, by defining *foxd3* and *sox10* function as being necessary for the differentiation of the *gnrh3* cells of the terminal nerve.

Endocrine GnRH1 is not affected when neural crest is disrupted

Originally, it was proposed that the endocrine and neuromodulatory GnRH cells of the forebrain of mammals arose from the olfactory placode and migrated to their adult location using routes along the developing olfactory and vomeronasal nerves (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989a; Wray et al., 1989b). Subsequently it was suggested, in both fish and mammals, that there are multiple origins for these populations of GnRH cells (Amano et al., 2004; Dubois et al., 2002; Parhar et al., 1998; Quanbeck et al., 1997). Our results further define the different developmental mechanisms underlying the differentiation of the neuromodulatory GnRH cells and the endocrine GnRH cells. Strikingly, we have shown that the knockdown of the *kal1.1* gene resulted in a loss of the endocrine GnRH1 cells, suggesting a crucial role for one isoform of the *kallmann* gene in the differentiation of the endocrine GnRH1 cells.

The idea of a neural crest origin for GnRH2 cells is in agreement with previous reports of GnRH2 in the neural crest-derived sympathetic ganglia in amphibians (Troskie et al., 1997). *gnrh2* expression has also been reported in non-neuronal, mesodermally derived structures such as bone marrow, kidney and reproductive tissues (for a review, see Millar, 2003), clearly indicating multiple origins for *gnrh2*-expressing cells beyond what we have described here. Recently, it was demonstrated through an analysis of the sequences of reported structural variants of GnRH that the midbrain *gnrh2* form is more closely related to the form found in the terminal nerve of fishes (*gnrh3*) than it is to the hypothalamic forms (*gnrh1*) (Lethimonier et al., 2004). Thus, the neuromodulatory midbrain GnRH2- and terminal nerve GnRH3-containing cells have molecularly similar forms of GnRH (when compared with the endocrine hypothalamic form, GnRH1), and our data support a common embryonic origin for these populations in the cranial neural crest.

We thank members of the Whitlock laboratory for helpful discussions; the Brand and Kobayashi laboratories for providing the *otx2* and *fgf8*, and *six4.1* cDNAs, respectively; Dr Robert Kelsh for providing the *sox10*-GFP line; and Drs D. Noden and J. Ewer for critical reading of the manuscript. This work was supported by NIH/DC0421801, NIH/HD050820, and a New York State Hatch Grant (K.E.W.).

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