Noradrenergic neurons in the zebrafish hindbrain are induced by retinoic acid and require *tfap2a* for expression of the neurotransmitter phenotype

Jochen Holzschuh[1,*], Alejandro Barrallo-Gimeno[2,†], Anne-Kathrin Ettl[1], Katrin Dürr[1], Ela W. Knapik[2] and Wolfgang Driever[1,‡]

[1]Developmental Biology, Institute Biology 1, University of Freiburg, Hauptstrasse 1, D-79104 Freiburg, Germany
[2]GSF, Institute for Mammalian Genetics, Ingolstadter Landstrasse 1, D-85764 Neuherberg, Germany
*Present address: Department of Developmental and Cell Biology, University of California Irvine, 5205 BioSci II, Irvine, CA 92697-2300, USA
†Present address: Ramón y Cajal CSIC, Avenida Dr Arce 37, Madrid 28002, Spain
‡Author for correspondence (e-mail: driever@biologie.uni-freiburg.de)

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Summary

Tfap2a is a transcriptional activator expressed in many different cell types, including neurons, neural crest derivatives and epidermis. We show that mutations at the zebrafish locus previously called *mont blanc* (*mob*) or *lockjaw* (*low*) encode *tfap2a*. The mutant phenotype reveals that *tfap2a* is essential for the development of hindbrain noradrenergic (NA) neurons of the locus coeruleus, medulla and area postrema, as well as for sympathetic NA neurons, epibranchial placode derived visceral sensory ganglia, and craniofacial and trunk crest derivatives. We focus our analysis on the role of *tfap2a* NA differentiation in the CNS. In the locus coeruleus, Phox2a and Tfap2a are co-expressed and are both required for NA development. By contrast, in the medulla Phox2a and Tfap2a are expressed in adjacent overlapping domains, but only *tfap2a* activity is required for NA differentiation, as NA neurons develop normally in *soulless/phox2a* mutant medulla. *phox2a* and *tfap2a* do not appear to affect each others expression. Our studies show that two distinct inductive mechanisms control NA development in the zebrafish hindbrain. For the posterior hindbrain, we identify retinoic acid as an important signal to induce NA differentiation in the medulla oblongata and area postrema, where it expands the *tfap2a* expression domain and thus acts upstream of *tfap2a*. By contrast, previous work revealed Fgf8 to be involved in specification of NA neurons in the locus coeruleus. Thus, although the inductive signals may be distinct, hindbrain NA neurons of the locus coeruleus and the posterior groups both require Tfap2a to establish their noradrenergic identity.

Key words: Norepinephrine, Catecholaminergic system, Zebrafish, *AP-2*, Hindbrain, Mont blanc, Lockjaw, Retinoic acid, Neuronal differentiation

Introduction

Noradrenergic neurons of the brain stem play a major role in modulating sensory, motor and arousal tone in vertebrates. Several evolutionary conserved noradrenergic (NA) centers exist in the vertebrate hindbrain (Smeets and Gonzalez, 2000). In the rostral hindbrain, NA neurons located ventrolaterally in the first rhombomere form the locus coeruleus (LC). In the posterior hindbrain, NA neurons are more loosely distributed in the medulla and area postrema. To adopt a given neurotransmitter phenotype, developing neurons begin to express enzymes to synthesize a particular neurotransmitter, as well as other proteins for neurotransmitter storage, degradation or re-uptake. Noradrenergic (NA) neurons synthesize noradrenaline from tyrosine in a three-step process in which tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase (L-AADC) and dopamine beta-hydroxylase (DBH) act sequentially.

Several factors that control development of NA centers have been identified. Two closely related homeodomain proteins, Phox2a and Phox2b, have been shown to be crucial for the development of the LC (Pattyn et al., 1999; Pattyn et al., 2000b). In contrast to Phox2a, which controls development of NA neurons in the LC, Phox2b is required for differentiation of all NA centers in the brain (Pattyn et al., 2000b). Phox2a and Phox2b are also directly involved in the development of autonomic neurons and hindbrain motoneurons (Valarche et al., 1993; Tiveron et al., 1996; Pattyn et al., 1997; Pattyn et al., 2000a). The proneural protein Mash1 is required for most NA neurons (Hirsch et al., 1998), and has been shown to act upstream of the Phox2 genes (Hirsch et al., 1998; Pattyn et al., 2000b). Recently, another homeodomain-containing protein, Tlx3, has been shown to control the development of nearly all NA neurons in the hindbrain independently of Phox2b (Qian et al., 2001). The transcriptional activator Tfap2a (Williams et al., 1988; Mitchell et al., 1991) is expressed in hindbrain NA neurons. Tfap2a has been shown to bind to the *th* and *dbh* promoters, and in cell culture assays may activate expression of the key enzymes for development of the NA neurotransmitter phenotype, TH and DBH (Kim et al., 2001). Inactivation of the *Tcfap2a* gene in mouse revealed a critical function of Tfap2a for development of the neural tube, craniofacial structures and eyes (Schorle et al., 1996; Zhang et

al., 1996; Nottoli et al., 1998). The defects in neural tube closure in *Tcfap2a* mutant mice prevented adequate assessment of the *Tcfap2a* mutant phenotype during neuronal differentiation. Thus, a potential role of Tfap2a during NA differentiation in vivo has not been previously investigated.

We show that mutations isolated as *mont blanc* alleles [*mob* (Neuhauss et al., 1996); allelic with *lockjaw* (Schilling et al., 1996)] affect zebrafish *tfap2a*. *tfap2a* mutant embryos develop defects in neural crest and epibranchial placode derivatives. Analysis of the development of the sympathetic and central nervous systems in *tfap2a* mutants revealed loss of *th* expression in the LC, medulla and area postrema, and sympathetic ganglia. Thus, neurons in the hindbrain and peripheral nervous system do not express the noradrenergic transmitter phenotype in the absence of *tfap2a* function. Furthermore, we show that retinoic acid (RA) is a necessary inducer of NA cell fate in the hindbrain in vivo. *neckless* mutant embryos, which have a disrupted *raldh2* gene and are thus deficient in RA synthesis (Begemann et al., 2001), lack NA neurons in the medulla oblongata. Moreover, exogenously applied retinoic acid can upregulate *tfap2a* expression and induce ectopic NA neurons in both the hindbrain medulla and the sympathetic nervous system. By contrast, in *tfap2a* mutant embryos RA fails to induce ectopic *th* expression in the hindbrain or sympathetic nervous system. Taken together, our results provide evidence that the neurotransmitter phenotype of hindbrain NA neurons is regulated by *tfap2a*. Our data further reveal distinctive control mechanisms for NA neurons in the LC and medulla: Although LC neurons are specified through a pathway including Fgf8 and *phox2a* (Guo et al., 1999a), neurons of the medulla are induced by RA, which we have identified as acting upstream of *tfap2a*.

Materials and methods

Fish maintenance, strains, mutagenesis and phenotype analysis

Zebrafish maintenance and breeding was under standard conditions at 28.5°C (Westerfield, 1995). Embryos were staged and fixed at the desired time points (hpf or dpf, hours or days post fertilization) (Kimmel et al., 1995). To avoid formation of melanin pigments, embryos were incubated in 0.2 mM 1-phenyl-2-thiourea (Sigma). The following mutant zebrafish strains were used in this study: *neckless* (*nls*^{i26}) (Begemann and Meyer, 2001) and *soulless* (*sou*^{m810}) (Guo et al., 1999a; Guo et al., 1999b). The craniofacial defects in embryos mutant for the *tfap2a* alleles *mob*$^{m610/780}$ and *lockjaw* (*low*^{ts213}) have previously been described (Neuhauss et al., 1996; Schilling et al., 1996). A new *tfap2a* allele, *mob*^{m819}, was isolated during a mutagenesis screen performed in our laboratory. G0 males (AB strain) were mutagenized with ethyl-nitrosourea (ENU) as described (Solnica-Krezel et al., 1994). F1 progeny were grown from G0 males crossed to AB strain females. Individual F1 females were used to generate haploid F2 progeny. F2 embryos were fixed at 24 hpf or 60 hpf and screened by whole-mount in situ hybridization for expression of *tyrosine hydroxylase* (*th*) (Holzschuh et al., 2001). Egg clutches in which about half of the embryos developed abnormalities in the catecholaminergic systems were identified by screening using a dissecting microscope. Strains were established from the corresponding F1 females and the diploid phenotype investigated in F3 embryos. Among several strains characterized during the screen, one contained *mob*^{m819}.

To simplify cross-reference with the mammalian literature, we will use the approved UCL/HGNC/HUGO Human Gene Nomenclature database symbol *tfap2a* for the zebrafish gene, instead of a gene name derived from the allele designations of the initial isolates *mont blanc* *mob*$^{m610/780}$, *mob*^{m819} and *lockjaw* (*low*^{ts213}). The use of the gene name *tfap2a* for the zebrafish gene will also avoid confusion with other genes that have been named AP2 (e.g. adaptor protein 2).

Mapping and cloning of *mob/tfap2a*

We mapped *mob*^{m819} using bulked segregant analysis (Michelmore et al., 1991) with pooled DNA from homozygous mutants and siblings from a *mob*^{m819} AB × India map-cross. We linked *mob*^{m819} to linkage group 24 between SSLP markers Z23011 and Z13695. Linkage with *tfap2a* was determined by RFLP analysis of PCR products from DNA of single *mob*^{m819} mutant and wild-type sibling embryos, obtained using primers flanking exon 5. *Dra*II restriction digest of the amplified *tfap2a* products did not digest the 360 bp PCR product from wild-type embryos, but cleaved that of *mob*^{m819} mutant embryos into two fragments of 123bp and 237bp. Analysis of the *mob*^{m610} and *mob*^{mm780} alleles reported previously (Neuhauss et al., 1996) revealed that they are likely re-isolates of one allele, as both represent the same mutational event: a point mutation in the splice acceptor at the 5′ end of exon 7 resulting in the use of a cryptic splice acceptor 14 bp further 3′, which causes a frame shift in the DNA-binding and dimerization domain, and a truncation at amino acid 395 (A.B. and E.K., unpublished).

Sequencing the EST fc31a07 revealed that it contained the full-length ORF of *tfap2a* isoform 3. Using gene specific primers for this EST, we performed RT-PCR with mRNA from *mob*^{m819} mutant and wild-type embryos to obtain the full-length ORF. Total RNA was isolated using the RNAeasy kit (Qiagen) and RT-PCR was performed with the RevertAid cDNA synthesis kit (MBI Fermentas). PCR products were cloned into pCR4 vector using the TOPO TA kit (Invitrogen). Sequencing was carried out on an automated sequencer (MWG). The full-length sequence of *tfap2a* isoform 3 has been submitted to GenBank (Accession Number AY166856).

Retinoic acid and cycloheximide treatment

Embryos from wild-type or heterozygous *mob*^{m819} parents were exposed to serial dilutions (3×10^{-8} to 3×10^{-6} M in egg water) of all-trans retinoic acid (RA, Sigma; stock solution 3×10^{-1}M in DMSO) for defined time periods in the dark. After treatment, embryos were washed under safety red light three times each for 10 minutes with egg water (Westerfield, 1995) to remove RA, and fixed at desired stages. For *th* expression analysis zebrafish embryos were exposed to RA for 9 hours between 24 hpf and 33 hpf, or for 24 hours between 24 hpf and 48 hpf. Hox gene expression analysis was performed on zebrafish embryos treated with RA for 9 hours between 24 hpf and 33 hpf.

Cycloheximide (CHX, Calbiochem; stock of 10 mg/ml in 100% ethanol) was applied at a concentration of 100 μg/ml in egg water. Embryos were exposed to CHX from 21 hpf to 32 hpf. For combined CHX and RA treatments, RA was added at 24 hpf to the embryos (final concentration of 3×10^{-7} M RA) and incubated for 8 hours. At 32 hpf, the embryos were washed as described above and fixed at desired stages. Controls for each experiment included sibling embryos incubated in the same concentration of Ethanol and/or DMSO as the experimental embryos.

Whole-mount in situ hybridization and immunohistochemistry

Whole-mount in situ hybridization (WISH) was performed according to Hauptmann and Gerster (Hauptmann and Gerster, 1994). Digoxigenin- or fluorescein-labeled antisense RNA probes were prepared using RNA labeling reagents (Boehringer).We prepared RNA probes for the following genes: *th* (Holzschuh et al., 2001); *phox2a* (Guo et al., 1999a); *hoxa2*, *hoxb2*, *hoxb3* and *hoxd3* (Prince et al., 1998); *isl1* (Inoue et al., 1994b); and *ret1* (Bisgrove et al., 1997). To generate a probe specific for *dopamine beta hydroxylase* (*dbh*)

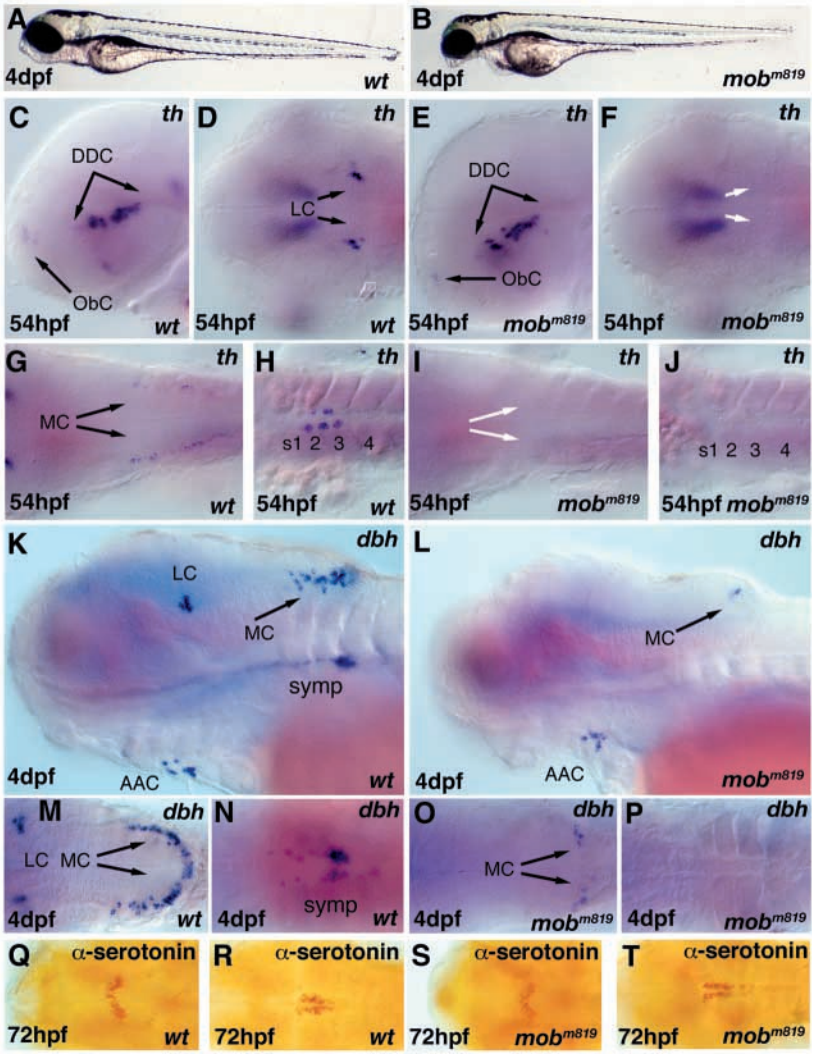

Fig. 1. The phenotype of *tfap2a* mutant embryos. (A) Live wild-type and (B) *tfap2a* mutant embryos at 4 dpf. Jaw defects, a slightly smaller head and reduced pigmentation in the tail characterize *mob* mutant embryos. (C-J) Detection of *th* expression in zebrafish embryos at 54 hpf by whole-mount in situ hybridization. (C,E) *th* expression is normal in the forebrain of *tfap2a* mutant embryos. (D,F,G,I) At 54 hpf, no *th* expression is detectable (white arrows) in the hindbrain of *tfap2a* mutant embryos. (H,J) *th* expression cannot be detected in *tfap2a* mutant embryos in the region where sympathetic ganglia form in wild-type. (K-P) Detection of *dbh* expression in zebrafish embryos at 4 dpf by whole-mount in situ hybridization. (K,M,N) At 4 dpf, *dbh* expression can be detected in the wild type in the locus coeruleus, the medulla oblongata/area postrema, sympathetic neurons as well as a group of arch associated neurons that may later contribute to the carotid body. (L,O,P) In *tfap2a*/*mob*^{m819}mutant embryos, the arch associated NA neurons develop normally, but cells of the locus coeruleus and sympathicus do not express *dbh*. A small number of cells starts to express *dbh* in the medulla oblongata/area postrema: both the number of cells and the expression level of *dbh* are severely reduced when compared with wild type. (Q-T) Immunohistochemistry with anti-serotonin antibodies reveals that the development of serotonergic neurons in the forebrain (Q,S) and hindbrain (R,T) is not affected in *tfap2a* mutant embryos. (A-T) anterior towards the left; (A-C,E,K,L) lateral views; (D,F-J,M-T) dorsal views. (A,C,D,G,H,K,M,N,Q,R) Wild-type; (B,E,F,I,J,L,O,P,S,T) *tfap2a*/*mob*^{m819} mutant embryo. AAC, arch associated cluster (carotid body); DDC, ventral diencephalic dopaminergic cluster; LC, locus coeruleus; MC, medulla oblongata noradrenergic cluster; ObC, olfactory bulb dopaminergic cluster; s, somite; symp, sympathetic neurons.

expression, we used a PCR-based approach to combine genomic fragments (Sanger Centre, Hinxton, UK) which contain Dbh-coding region, as judged from the sequence two genomic fragments (z35723-a1914e04.q1c and zfishC-a1746c09.p1c) in public databases and from comparison with mammalian Dbh sequences. To generate the probe containing 735 bp of the *dbh* coding sequence, we linearized with *Spe*I and transcribed with T7 polymerase. To make *tfap2a* probes, the EST fc31a07 was linearized with *Sal*I (MBI Fermentas) and RNA probe was transcribed with Sp6 RNA polymerase (Boehringer). Immunohistochemistry anti-serotonin antibodies (Chemicon), anti HuC/HuD antibodies (16A11, Molecular Probes), and anti-acetylated tubulin antibodies (Sigma) was performed as described (Solnica-Krezel and Driever, 1994), except that fixed embryos older than 3 dpf were permeabilized by 30 minutes incubation in proteinase K (Sigma; 10 μg/ml) and successive inhibition of proteinase K with phenyl-methyl-sulfonylfluoride (PMSF, Sigma). The cartilage of zebrafish embryos was stained with Alcian Blue as described elsewhere (Neuhauss et al., 1996).

Results

The *mont blanc* mutation disrupts the development of noradrenergic centers in the CNS and of sympathetic ganglia

Mutations of the *mont blanc* (*mob*) complementation group were initially isolated based on their prominent craniofacial phenotype (*mob*$^{m780/610}$) (Neuhauss et al., 1996). We isolated the new *mob*^{m819} allele in a haploid genetic screen for mutations affecting the catecholaminergic systems (CA) in zebrafish. All three alleles are characterized by absence of derivatives of the second pharyngeal arch (ceratohyal, basihyal cartilages) and strongly reduced gill arch derivates (basibranchial, ceratobranchials; Fig. 1A,B, Fig. 4I,J). As *mob*$^{m610/780}$, *low*^{ts213} and *mob*^{m819} map to the same region of LG24 (this paper) (A.B. and E.K., unpublished) (Knight et al., 2003), we carried out complementation tests with all three mutations. Both alleles from the previous screens failed to complement with *mob*^{m819} (data not shown).

The main CNS neuronal phenotype of *mob*^{m819} mutant embryos affects the noradrenergic (NA) centers in the hindbrain (Fig. 1C-P). The NA defects of *mob*$^{m610/780}$ and *mob*^{m819} are similar, indicating a similar allelic strength for these alleles (data not shown; A.B. and E.K., unpublished). Analysis of *th* expression in *mob*^{m819} mutant embryos revealed that in areas of the hindbrain where NA neurons normally develop, the locus coeruleus (LC) and the medulla, as well as in the PNS, fully differentiated *th*-expressing NA neurons could not be detected at 54 hpf (Fig. 1C-J). By contrast, *th* expression in the forebrain appears normal. We

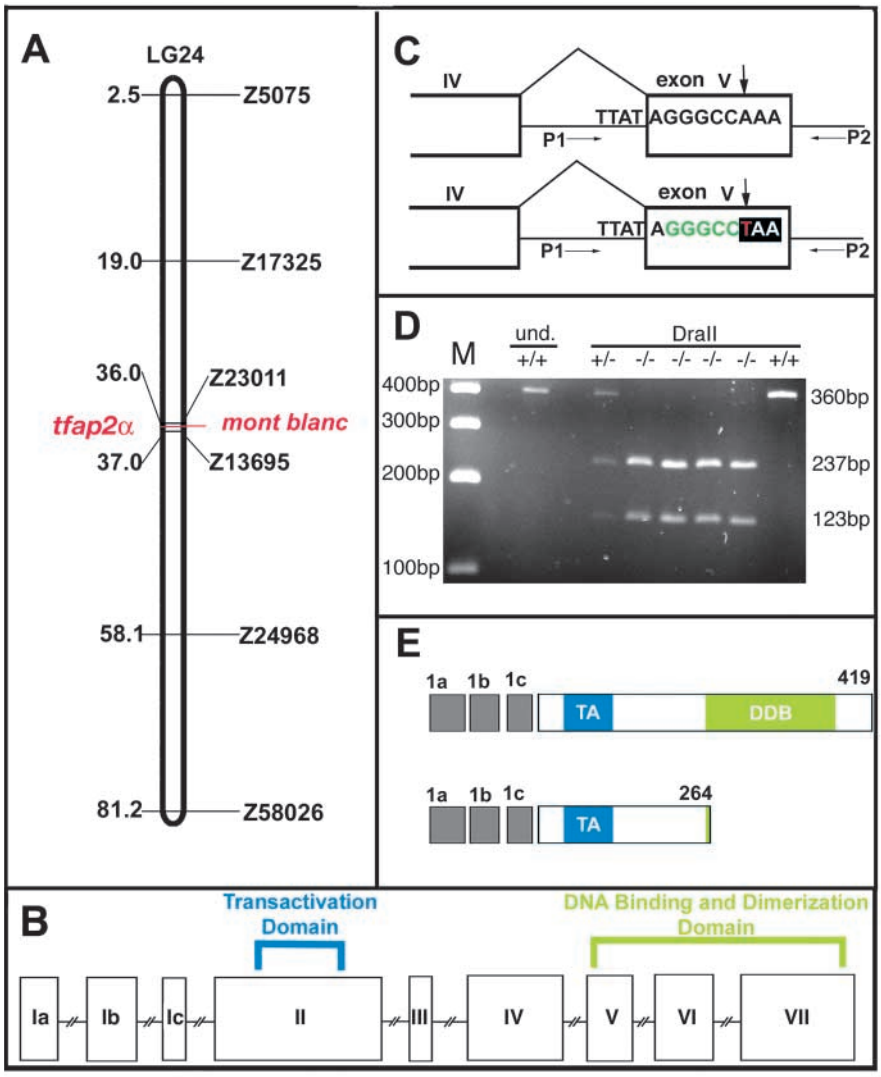

Fig. 2. Genomic organization of the zebrafish*tfap2aα* locus. (A) Genetic map of linkage group 24 (LG24) showing the position of *mont blanc/tfap2a* in relation to some SSLP markers. (B) The genomic organization of the zebrafish *tfap2a* gene; exons Ia, Ib and Ic represent alternative first exons for isoforms 1, 2 and 3. (C) Identification of the *mob*^{m819} mutation revealed an A to T transition (red) at the beginning of exon V, which introduces a stop codon (black box) and a *Dra*II restriction site (green with red T). (D) RFLP was used for linkage analysis. Intron primers P1 and P2 give rise to a 360 bp PCR product. Amplified mutant DNA can be cleaved by *Dra*II into 123 bp and 237 bp fragments. (E) The stop codon causes a truncation of Tfap2a at amino acid 264. The truncated protein lacks the dimerization and DNA binding domain (DDB, indicated in green). 1a, 1b and 1c are alternative first exons; M, 100 bp marker; TA, transactivation domain; und., undigested PCR product.

cloned zebrafish *dopamine beta hydroxylase* (*dbh*) to specifically label noradrenergic neurons. At 4 dpf, expression of *dbh* can be detected in the locus coeruleus, the medulla oblongata/area postrema, sympathetic neurons, as well as a group of arch associated neurons that may later contribute to the carotid body (Fig. 1K,M,N; a detailed analysis of *dbh* expression will be presented elsewhere). We analyzed expression of *dbh* in *mob*^{m819} mutant embryos at 4 dpf and found that *dbh* is not expressed in the locus coeruleus (Fig. 1L,O) and the enteric region (Fig. 1L,P). By contrast, *dbh* is still expressed in the carotid bodies of *mob*^{m819} mutant embryos (Fig. 1K). In the medulla oblongata/area postrema, a small number of cells express *dbh* at 4 dpf (Fig. 1K,O). The number of *dbh*-expressing cells in the medulla oblongata is strongly reduced when compared with wild type. Furthermore, the expression level of *dbh* in mutant medulla oblongata cells appears lower than in wild type (Fig. 1K,L,M.O; WISH procedure was performed identically on mutants and wild type in the same staining batch – which is confirmed by the fact that expression levels of *dbh* detected in the carotid bodies are similar in mutant and wild type). We conclude that *mob*^{m819} does affect *th* expression and noradrenergic differentiation in the hindbrain; however, in the medulla oblongata, other factors may later during development partially compensate for the absence of *mob*^{m819} activity. To test whether other monoaminergic neurons are affected in *mob*^{m819} mutants, we stained serotonergic neurons in the CNS with anti-serotonin antibodies. Serotonergic neurons appear normal in *mob*^{m819} mutant embryos at 72 hpf (Fig. 1Q-T). The development of reticulospinal neurons of the hindbrain also appeared normal as revealed by backfill labeling with rhodamine-dextran (data not shown). Furthermore, the overall pattern of neural development appeared normal at 1 dpf, as judged from anti-acetylated tubulin immunohistochemistry (data not shown).

mont blanc encodes the transcription factor Tfap2a

To identify the gene affected by *mont blanc* (*mob*) mutations, we mapped the mutation using simple sequence length polymorphism markers (SSLP). *mob* is located between the markers z23011 (2.04 cM from *mob*; 11 recombinants in 538 meiosis) and z13695 (2.75 cM from *mob*; 15 recombinants in 546 meiosis) on LG 24 (Fig. 2A). Two EST clones map to this region: fc31a07 (W. Talbot, http://zebrafish.stanford.edu) and fb83f04 (I. Dawid, http://dir.nichd.nih.gov/lmg/lmgdevb.htm), both predicted to be homologous to mammalian Tfap2a (S. Johnson, AI584805, AI722745; WUZGR; http//zfish.wustl.edu). *Tcfap2a* mutant mouse embryos (Schorle et al., 1996; Zhang et al., 1996) develop craniofacial and PNS phenotypes similar to those of *mob*^{m819} zebrafish mutants. Thus, *tfap2a* appeared to be a likely candidate gene for *mont blanc* mutations. As in mouse, the zebrafish *tfap2a* locus encodes three mRNA isoforms, each using different first exons. Zebrafish *tfap2a* isoforms *tfap2a1* and *tfap2a2* have been isolated (M. Fuerthauer, C. Thisse and B. Thisse, GenBank Accession Numbers AF457191, AF457192). We cloned and sequenced a full-length cDNA that encodes the zebrafish

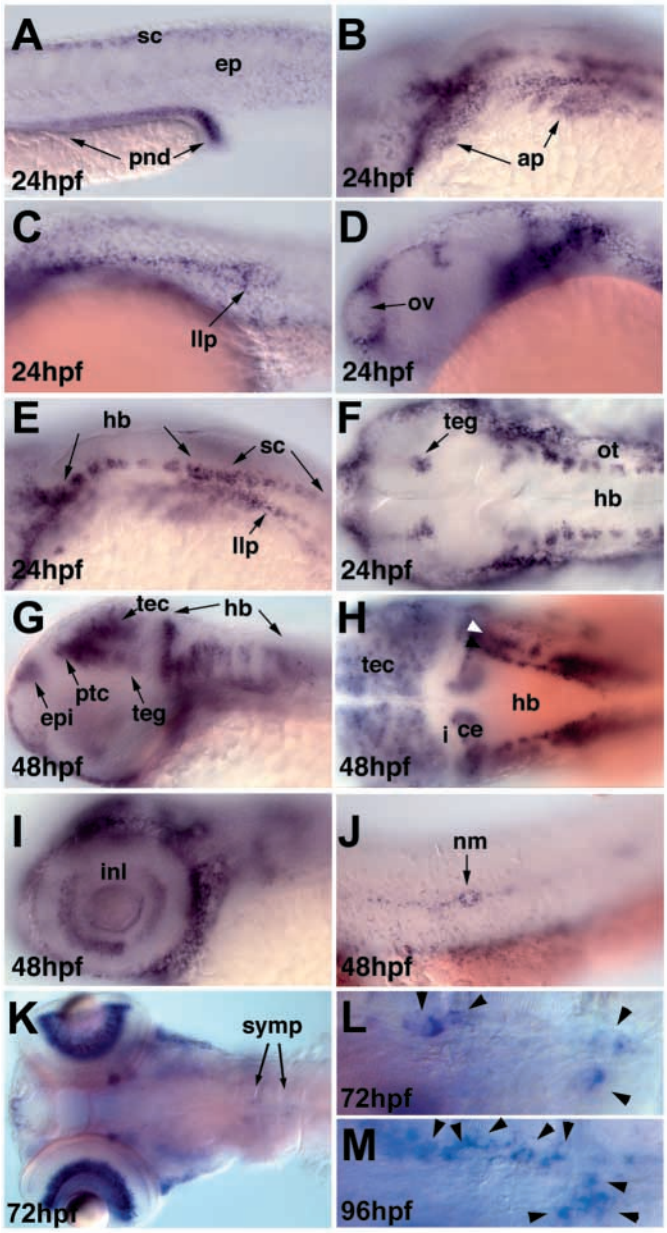

Fig. 3. Expression of *tfap2a* during development of zebrafish embryos. (A-F) *tfap2a* is expressed widely at 24 hpf in (A) spinal cord neurons, the pronephric duct and widely in the epidermis; in (B) neural crest cells in the arch primordia; in (C) migrating posterior lateral line primordium; in (D) epidermal cells surrounding the olfactory vesicle; in (E) segmentally arranged cell groups in the lateral hindbrain; and in (F) bilateral clusters of cells in the tegmentum. (G,H) Expression of *tfap2a* is increased in the central nervous system at 48 hpf. (G) *tfap2a* is expressed in the telencephalon, tectum, pretectum and tegmentum. (H) Two laterally stripes of hindbrain neurons express *tfap2a*. The white arrowhead indicates the lateral and the black arrowhead indicates the more medial stripe of the *tfap2a* expression domain. In the isthmus, expression of *tfap2a* is not detected, while strong expression is established in the adjacent optic tectum and cerebellum. (I) The cells in the inner nuclear layer of the retina express *tfap2a*. (J) The lateral line organs express *tfap2a*. (K,L) At 72 hpf, a few cells adjacent to the forming gut tube express *tfap2a.* which are probably neurons of the sympathetic ganglia. (L,M) Higher magnification of the area adjacent to the gut tube where sympathetic ganglia form (72 hpf and 96 hpf respectively; arrowheads indicate *tfap2a*-expressing cells). (A-M) Anterior towards the left; (A-D,G,I,J) lateral views; (F,H,K-M) dorsal views. ap, arch primordia; ce, cerebellum; ep, epidermal cells; epi, epiphysis; hb, hindbrain; i, isthmus; inl, inner nuclear layer of the retina; llp, lateral line primordia; nm, neuromast cells; ot, otic vesicle; ov, olfactory vesicle; pnd, pronephric duct; sc, spinal cord; symp, cells of sympathetic ganglia; teg, tegmentum; tec, tectum.

tfap2a3, a homolog of the mouse *Tcfap2a* isoform 3. The amino acid sequences of all three Tfap2a isoforms are highly conserved: isoforms 1 and 3 each share 85% amino acid identity between mouse and zebrafish, while isoform 2 shares 82% identity. The three zebrafish isoforms differ from each other only in the first 15 (isoform 1), 21 (isoform 2) or 9 (isoform 3) amino acids, which are encoded by three alternative first exons (Fig. 2). We assembled the genomic structure of *tfap2a* (Fig. 2B) through in silico chromosome walk on publicly available zebrafish genomic sequences (http://www.sanger.ac.uk/Projects/D_rerio). Sequencing of cDNAs from homozygous *mob*^{m819} embryos identified a point mutation in exon V, close to the splice-acceptor site (Fig. 2C). This A to T transition created a *Dra*II restriction site, which made it possible to confirm linkage to *mob*^{m819} by RFLP analysis (Fig. 2C,D). We did not find a single recombinant in 546 meiosis, indicating that *mob* and *tfap2a* map within an interval smaller than 0.18 cM. The mutation introduces a stop codon that truncates the protein at amino acid 264, located at the N-terminal end of the dimerization and DNA-binding domain (DDB; Fig. 2C,E). This domain is necessary for the dimerization of Tfap2 proteins, which in turn is required for DNA binding of Tfap2a protein to the target promoters and the formation of a functional transcriptional activation complex (Williams and Tjian, 1991a; Williams and Tjian, 1991b). Therefore, *mob*^{m819} is most likely a null allele. All three *tfap2a* isoforms are affected by the mutation.

tfap2a is expressed in the CNS, epidermis and neural crest

We examined the spatiotemporal expression patterns of *tfap2a* by whole-mount in situ hybridization. Using a full-length probe, we found an expression pattern throughout development that resembles the known expression pattern of *tfap2a1* (http://zfin.org). *tfap2a* expression is first detected at gastrula stages in non-neural ectoderm (data not shown), and expression continues in some epidermal cells (Fig. 3D; a potential requirement in *Xenopus* non-neural ectoderm has already been described (Luo et al., 2002). The neural crest primordia express *tfap2a* from the one-somite stage on (data not shown). During the segmentation period *tfap2a* expression begins in the pronephric duct and in migrating neural crest cells (Fig. 3A,B). From late segmentation stages, *tfap2a* is also expressed in the CNS in two bilateral cell groups in the tegmentum and in bilateral stripes by hindbrain and spinal chord neurons (Fig. 3E,F). During these stages, the migrating cells of the lateral line primordium also express *tfap2a* (Fig. 3C). By 48 hpf, expression of *tfap2a* can be detected in the inner nuclear layer of the retina, the epiphyseal region, the pretectum and tectum, the tegmentum, the cerebellum, and two lateral stripes on each

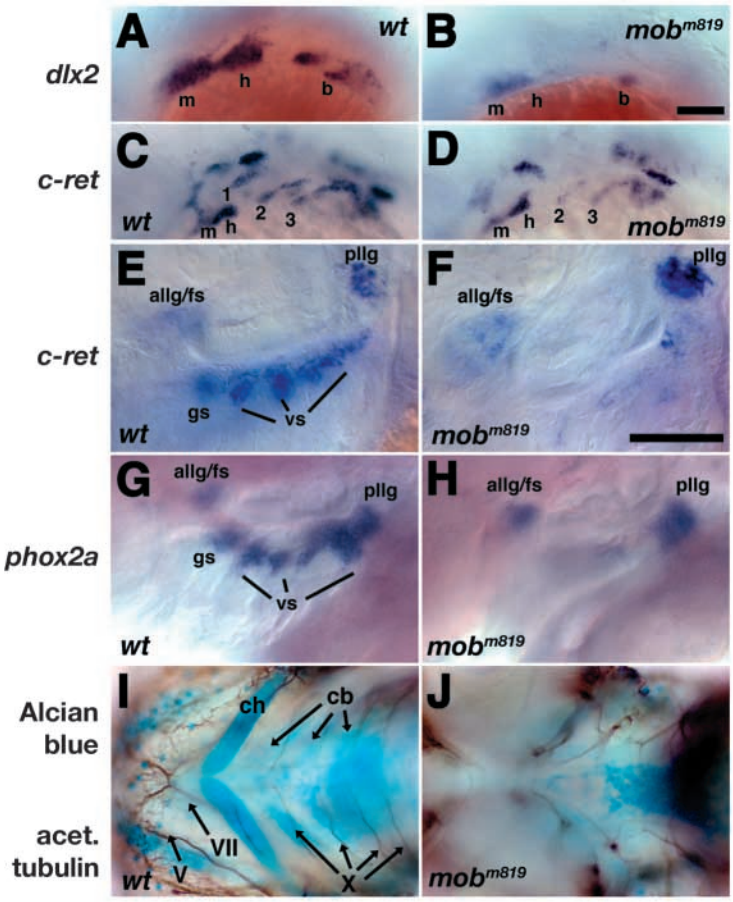

Fig. 4. Early development of the visceral sensory ganglia is affected in *tfap2a* mutant embryos. (A,B) In situ hybridization at 24 hpf reveals a strong reduction in *dlx2*-expressing neural crest cells in branchial arches of *tfap2a* mutant embryos. (C,D) By 24 hpf, *ret1* expression in the branchial arches is reduced in *tfap2a* mutant embryos. (E,F) At 4 dpf, *ret1* expression reveals a complete loss of epibranchial placode-derived sensory ganglia in *tfap2a* mutant embryos. (G,H) Expression of *phox2a*, which marks placodal-derived neurons, is also missing in *tfap2a* mutant embryos. (I,J) Alcian Blue staining of cartilage combined with anti-acetylated-tubulin immunohistochemistry in 4 dpf embryos. The axon bundles of the cranial nerves are missing in *tfap2a* mutant embryos. (A-H) Lateral views, anterior towards the left. (I,J) Ventral views, anterior towards the left. (A,C,E,G,I) wild-type; (B,D,F,H,J) *tfap2a* mutant embryos. allg, anterior lateral line ganglion; b, branchial stream, cb, ceratobranchials; ch; ceratohyal; fs, facial sensory ganglion; gs, glossopharyngeal sensory ganglion; h, hyoid stream; m, mandibular stream; pllg; posterior lateral line ganglion; vs, vagal sensory ganglia; V, axons of the facial cranial nerve; VII, axons of the glossopharyngeal cranial nerve; X axons of the vagus cranial nerve. Scale bars: 50 μm.

side of the hindbrain (Fig. 3G-I). The neuromasts of the lateral line organ continue to express *tfap2a* (Fig. 3J). From 3 dpf onwards, *tfap2a* can be detected in cells adjacent to the gut tube, which probably correspond to neurons of the sympathetic ganglia (Fig. 3K-M).

Defects of the cranial ganglia in *tfap2a* mutants

To analyze the cranial ganglia in *tfap2a* mutant zebrafish, we used expression of *dlx2*, *ret1* (previously *c-ret*) and *phox2a* as markers. *dlx2* is expressed in neural crest cells contributing to the branchial arches (Akimenko et al., 1994). In mouse embryos, the receptor tyrosine kinase *Ret* is expressed early together with *Phox2a* in derivatives of the epibranchial placode (Pachnis et al., 1993; Valarche et al., 1993; Robertson and Mason, 1995; Tiveron et al., 1996). The zebrafish homolog of *Ret*, *ret1*, is expressed in the branchial arches (Bisgrove et al., 1997; Marcos-Gutierrez et al., 1997), and we could also detect expression in cranial neural placodes (Fig. 4E). The closely related homeobox containing transcription factors Phox2a and Phox2b are expressed in epibranchial placodes and their derivatives, the VIIth, IXth and Xth cranial sensory ganglia (Tiveron et al., 1996; Pattyn et al., 1997; Fode et al., 1998; Begbie et al., 1999). Loss-of-function experiments in mouse have shown that Phox2a and Phox2b are required for the development of these ganglia, as well as for the development of NA neurons in the hindbrain (Morin et al., 1997; Pattyn et al., 1999).

Expression of *dlx2* and *ret1* is greatly reduced in *tfap2a* mutant embryos at 24 hpf (Fig. 4A-D). As in wild-type embryos, at this stage the neural crest and the placodal-derived cells migrate to their final positions. Later in development, when the sensory ganglia of the VIIth (facial), IXth (glossopharyngeal) and Xth (vagal) cranial nerves are forming, expression of *ret1* and *phox2a* is not detectable above the branchial clefts in mutant embryos (Fig. 4E-H). However, the anterior and posterior lateral line ganglia express *phox2a*. We further analyzed *tfap2a* mutant embryos by immunohistochemistry with anti-acetylated tubulin antibodies to visualize axon tracts. In *tfap2a* mutant embryos, the early axon scaffold tracts of the CNS appear normal at 24 hpf (data not shown). By 4 dpf, however, the pattern of peripheral axons in the jaw and pharyngeal regions of mutant embryos is disturbed, suggesting that either the fibers of the VII and IX cranial nerves are absent, or that they fail to reach this region because craniofacial structures are disturbed (Fig. 4I,J).

phox2a and *tfap2a* expression in the locus coeruleus and the medulla

The homeodomain transcription factor Phox2a is expressed by noradrenergic neurons in the locus coeruleus and is required for their development in mammals and zebrafish (Morin et al., 1997; Pattyn et al., 1997; Guo et al., 1999a). The *phox2a* gene is disrupted in zebrafish *soulless* (*sou*) mutant embryos, in which NA neurons of the LC do not form (Guo et al., 1999a). Tfap2a and tyrosine hydroxylase are co-localized in noradrenergic neurons in the embryonic mouse hindbrain (Kim et al., 2001). To test whether *tfap2a* and *phox2a* are co-expressed in zebrafish noradrenergic neurons of the hindbrain, we performed double in situ hybridizations with *tfap2a* and *phox2a* (Fig. 5A-C,E-J) or *th* probes (Fig. 5D). *phox2a* and *tfap2a* are indeed co-expressed in the zebrafish LC (Fig. 5C,G,I). When the first cells in the posterior hindbrain differentiate into NA neurons between 36 and 48 hpf, the expression domains of *phox2a* and *tfap2a* overlap in the ventral half of the hindbrain (Fig. 5E,F). *tfap2a* expression also expands to more dorsal areas where NA neurons differentiate.

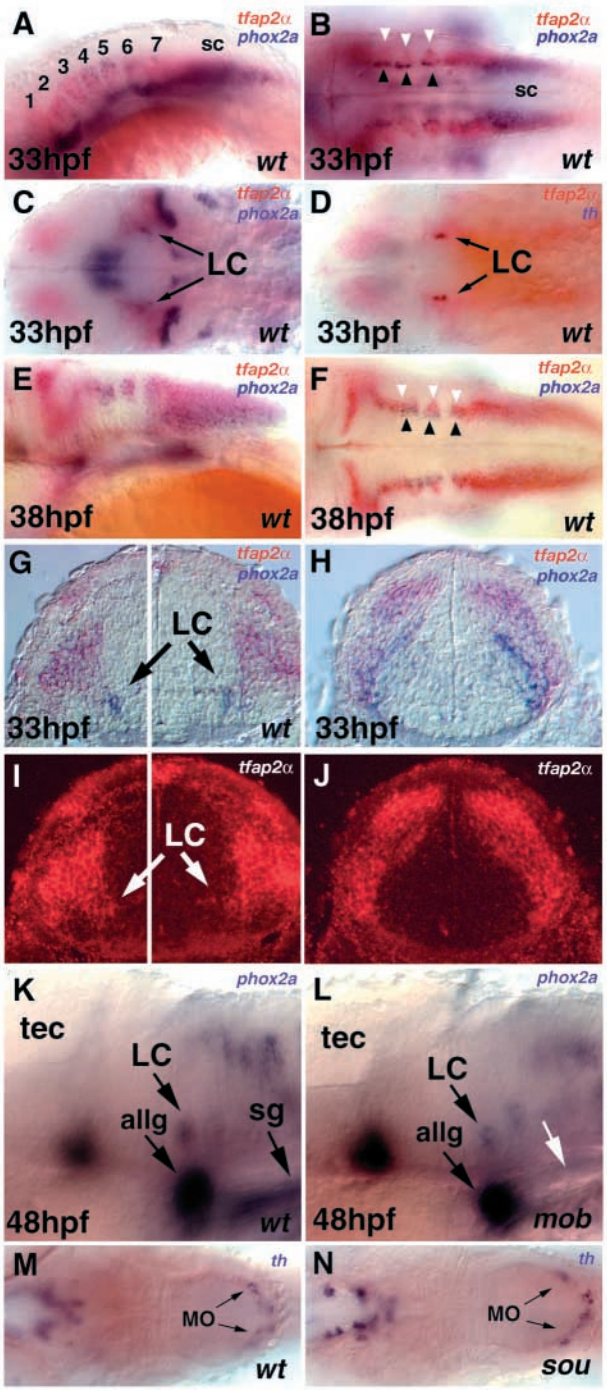

Fig. 5. *tfap2a* and *phox2a* expression and NA neuron specification in the LC. (A) Lateral view and (B) dorsal view of a wild-type embryo at 33 hpf showing expression of *tfap2a* (red) and *phox2a* (blue) in the hindbrain. *phox2a* and *tfap2a* are expressed in a segmental pattern reflecting rhombomeres 1-7 (r1-r7). In the rhombomeres, small groups of neurons in the ventral medial expression domain of *tfap2a* co-express *phox2a* (black arrowheads; white arrowheads indicate the lateral expression of *tfap2a*). In the spinal cord, both genes are expressed widely. (C) Dorsal view of a wild-type embryo at 33 hpf. In the locus coeruleus, *tfap2a* (red) and *phox2a* (blue) are co-expressed. (D) Dorsal view of a wild-type embryo at 33 hpf. Double staining for *th* (blue) and *tfap2a* (red) confirmed the co-localization of *th* and *phox2a* in the LC. (E) Lateral view of a wild-type embryo at 38 hpf. In the ventral part of r6-r7, most of the cells co-express *tfap2a* (red) and *phox2a* (blue), while in the dorsal part, only *tfap2a* is expressed. (F) Dorsal view of the same embryo. Co-expression of *tfap2a* and *phox2a* continues in the medial line of *tfap2a* expression (black arrowheads; white arrowheads indicate the lateral expression of *tfap2a*). (G-J) Transversal sections of embryos at 33 hpf showing expression of *tfap2a* and *phox2a* at the level of the LC (G,I; left and right halves show left and right LC from different sections) and rhombomere 6-7 and the medulla (H,J). In I and J, the same sections as in G and H are shown, but the Fast Red dye used to detect *tfap2a* expression is visualized by fluorescence microscopy. The *phox2a* expression domains in LC and medulla are part of the *tfap2a* expression domain, albeit *tfap2a* is expressed at relatively low levels in the region of *phox2a*-expressing cells. (K) Lateral view of a wild-type embryo at 48 hpf. *phox2a* is expressed in the LC and anterior lateral line ganglia (allg) and sensory ganglia (sg). (L) Lateral view of a *tfap2a* mutant embryo at 48 hpf. The LC develops in the mutant embryo, as revealed by the expression of *phox2a* in this region. The white arrow highlights the absence of *phox2a* expression at the position of the sensory ganglia. (M) Dorsal view of the hindbrain of a wild-type embryo at 72 hpf showing *th* expression in the posterior hindbrain. (N) Dorsal view of a *soulless/phox2a* mutant embryo, with *th* expression in the posterior hindbrain that is not distinguishable from wild-type. (A-F,K-N), anterior towards the left; (G-J), transverse sections. LC, locus coeruleus; MO, medulla oblongata; sc, spinal cord; sg, sensory ganglia; tec, tectum; 1-7, rhombomeres 1-7.

Consistent with the fact that *phox2a* is not expressed in the part of the medulla/area postrema where NA neurons develop, we find normal NA differentiation in the medulla of *soulless/phox2a* mutant embryos (Fig. 5M,N).

To test whether absence of the LC in *mob/tfap2a* mutants might be due to changes in *phox2a* expression, we examined the expression of *phox2a* in *mob/tfap2a* mutant embryos. *phox2a* is expressed in the LC of *mob/tfap2a* mutant embryos at all developmental stages analyzed (Fig. 5K,L; also data not shown). To find out if *tfap2a* could be a downstream target of *phox2a*, we assayed *tfap2a* expression in *soulless/phox2a* mutant embryos. We could not detect any alterations in the hindbrain expression pattern of *tfap2a* (data not shown). Our data indicate that *tfap2a* and *phox2a* act in parallel to control NA identity in the LC.

Sympathetic neurons fail to differentiate in *tfap2a* mutants

Dorsal root ganglia (DRG), enteric and sympathetic ganglia are neuronal derivatives of trunk neural crest. The absence of *th* and *dbh* expression from the intestinal tract of *mob/tfap2a* mutants could either reflect a differentiation defect resulting in absence of terminal differentiation markers, or could be caused by the mutation affecting neural crest precursor cells, and thus resulting in migration defects or reduced survival. To test whether precursors of trunk neural crest-derived peripheral neurons initially form, we analyzed Hu-antigen and *isl1* expression. The anti-Hu antibody recognizes a subset of ELAV-related proteins expressed very early in neuron differentiation (Marusich et al., 1994a). At 72 hpf the presence of Hu-immunoreactive cells reveals that precursors of sympathetic ganglia as well as enteric neurons of the anterior intestine are present at similar cell numbers in wild-type and *mob/tfap2a* mutant embryos (Fig. 6A,B). In contrast, we did not detect enteric neurons colonizing the posterior part of the gut tube in

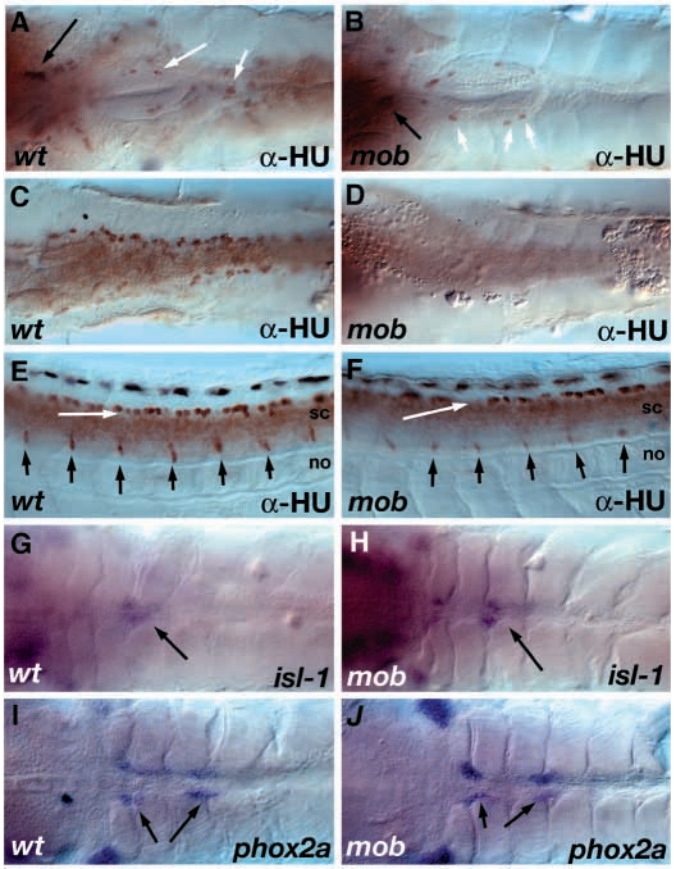

Fig. 6. Development of the peripheral nervous system in *tfap2a*. (A-F) Detection of PNS and CNS components by whole-mount immunohistochemistry with anti-Hu antibodies. (A,B) Dorsal view of the anterior intestine at 72 hpf in (A) wild-type and (B) *tfap2a* mutant embryos showing sympathetic (black arrows) and enteric neurons (white arrows). (C,D) Dorsal view of the posterior gut tube at 48 hpf. (D) In *tfap2a* mutant embryos no enteric neurons can be detected in the posterior gut tube. (E,F) Lateral view of the trunk region of (E) wild-type and (F) *tfap2a* mutant embryos 48 hpf: Rohan Beard sensory neurons (white arrows) and dorsal root ganglia (black arrows) can be detected in mutants, but the number of neurons is reduced in each group. (G,H) Whole-mount in situ hybridization with *isl1* riboprobes revealing presence of sympathetic neurons (black arrows) in (G) wild-type and (H) *tfap2a* mutant embryos. (I,J) Visualization of sympathetic neurons (black arrows) in (I) wild-type and (J) *tfap2a* mutant embryos by whole-mount in situ hybridization with *phox2a* riboprobes. (A-J) anterior towards the left. no, notocord; sc, spinal cord.

mob/tfap2a mutant embryos (Fig. 6C,D). DRG and Rohon-Beard cells (RB; dorsal primary sensory neurons of lateral neural plate origin in close lineage relation with neural crest) (Artinger et al., 1999) develop in *mob/tfap2a* mutant embryos, thought at 48 hpf they appear slightly reduced in cell number (Fig. 6E,F; for further discussion of DRGs in *mob* see A.B.-G. and E.W.K., unpublished). The LIM homedomain containing transcription factor Isl1 is expressed during early differentiation of dorsal root and sympathetic ganglia, as well as other neurons (Ericson et al., 1992; Inoue et al., 1994a). In 72 hpf old *mob/tfap2a* mutant embryos cells express *isl1* at the location where sympathetic neurons form in wild type (Fig. 6G,H). Furthermore, *phox2a* is expressed in *mob/tfap2a* mutant embryos in cells at the location where sympathetic neurons form in wild type (Fig. 6I,J). Taken together our data suggest that the lack of *th* and *dbh* expression for the noradrenergic transmitter phenotype is not caused by a failure of neural crest formation or migration, but reflects a block in terminal differentiation of sympathetic neurons.

Development of noradrenergic neurons in the hindbrain medulla and in the sympathetic nervous system depends on retinoic acid signaling

Tfap2a is expressed in many embryonic structures that are influenced by the level of all-*trans*-retinoic acid during development (Luscher et al., 1989; Philipp et al., 1994; Xie et al., 1998). The expression of *Tcfap2a* has been shown to be modulated by retinoic acid treatment in cell cultures (Williams et al., 1988; Luscher et al., 1989), suggesting a link between RA and the regulation of *Tcfap2a*. Therefore we investigated whether RA signaling plays a role in NA neuron differentiation in the hindbrain. To assay the effects of RA, we incubated groups of zebrafish embryos with different, increasing concentrations of RA. As RA primarily affects anterioposterior patterning and establishment of the Hox code in the neural tube during gastrulation and until mid-somitogenesis (Holder and Hill, 1991; Hill et al., 1995; Yan et al., 1998), embryo at these stages were not assayed. Exposure of zebrafish embryos to RA for 9 hours between 24 hpf and 33 hpf, or for 24 hours between 24 hpf and 48 hpf induced ectopic *th*-expressing cells in the hindbrain and the sympathetic nervous system, which we detected at 33 hpf, 48 hpf and 72 hpf (Fig. 7I,J,M,N; see Fig. 9E,G,I,K,M,O, data not shown). Induction of ectopic NA neurons was never found rostral to rhombomere 2, and we did not detect changes in the LC (Fig. 7E,F), or the DA neurons of the forebrain (Fig. 7A,B). At 33 hpf we counted 10.7±0.9 (mean±s.d.; n=3) *th*-expressing sympathetic cells in wild-type embryos, while we detected 23.7±3.4 (n=3) in embryos treated with RA between 24 hpf and 33 hpf. At 72 hpf we counted 44.1±4.5 (n=8) *th*-expressing sympathetic cells in wild-type embryos, and 116±16.2 (n=8) at 72 hpf in embryos treated with RA between 24 hpf and 33 hpf. To investigate effects of reduced RA levels during NA differentiation, we analyzed zebrafish *neckless* (*nls*) mutants, which are defective in retinaldehyde dehydrogenase type 2 (RALDH2), a key enzyme for RA synthesis. DA neurons in the forebrain and NA neurons in the LC of *nls* mutants are not affected by reduced RA signaling in *nls* mutants (Fig. 7C,G; also data not shown). By contrast, *nls* mutant embryos lack *th*-expressing neurons in the medulla/area postrema (Fig. 7K). Furthermore, the number of NA neurons in the sympathetic nervous system is strongly reduced in *nls* mutant embryos (Fig. 7O). At 72 hpf we detected only 14.6±6.0 (n=8) sympathetic *th*-expressing cells in *nls* mutants. To test whether *tfap2a* may mediate RA dependent induction of *th* expression, we analyzed *th* expression in RA-treated *tfap2a* mutant embryos. In *tfap2a* mutant embryos, RA does not lead to ectopic expression of *th*, and is not able to rescue the NA phenotype (Fig. 7H,L,P). Thus, *tfap2a* may be required downstream of RA to induce NA differentiation.

RA signaling is involved in hindbrain patterning and rhombomere specification (reviewed by Gavalas and Krumlauf, 2000; Gavalas, 2002). To rule out the possibility that our conditions of RA treatment interfered with the specification of rhombomere identities, we analyzed the rhombomere-

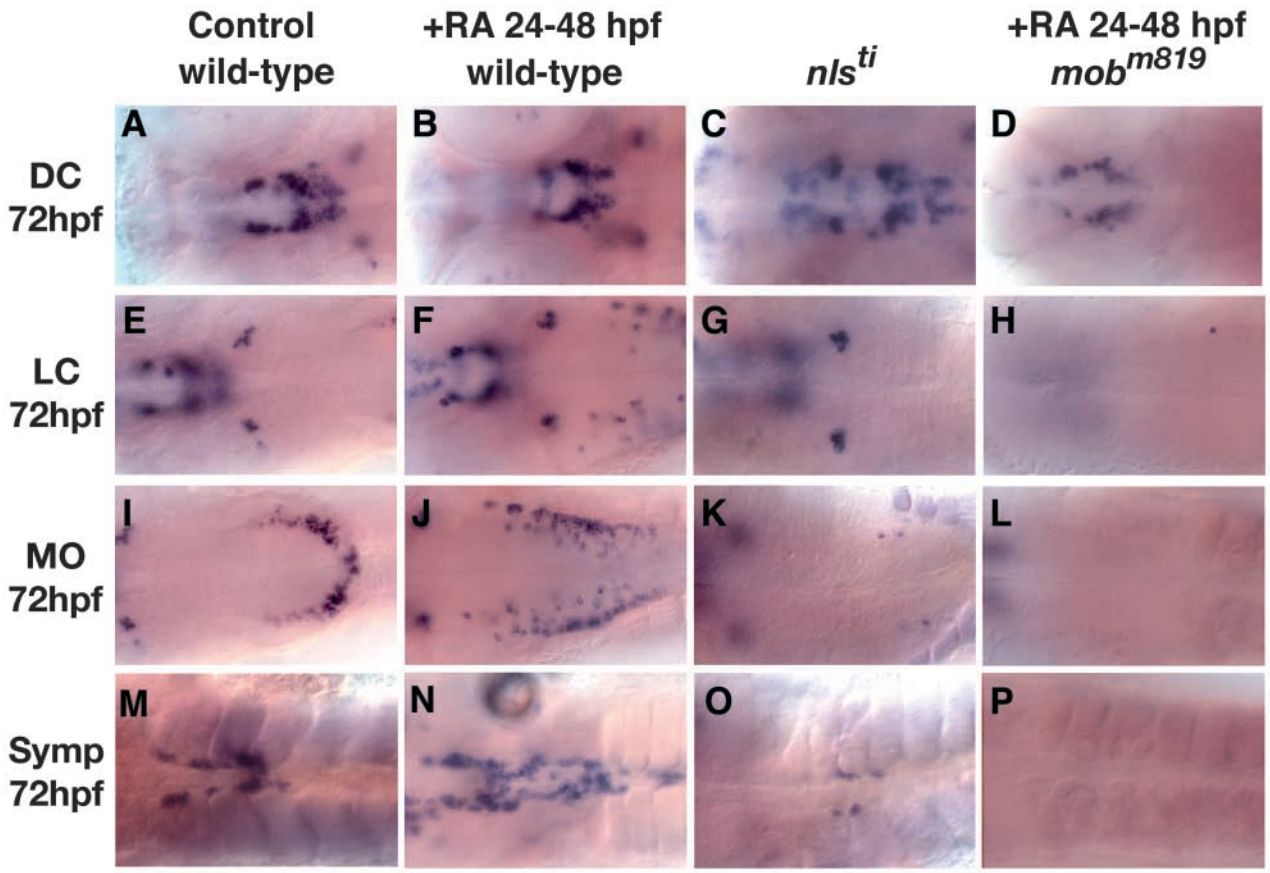

Fig. 7. Retinoic acid (RA) is required and sufficient to induce formation of *th*-expressing NA neurons in the posterior hindbrain. (A-P) Analysis of *th* expression in 72 hpf embryos by whole-mount in situ hybridization. In the diencephalon (DC) no differences of *th* expression are detected in (B) RA treated wild-type embryos, (C) *nls*/*raldh2* mutants and (D) *tfap2a* mutant embryos exposed to RA. (E-H) *th* expression is normal in the locus coeruleus (LC) of (F) RA-treated embryos, (G) *nls*/*raldh2* and (H) remains absent in *tfap2a* mutant embryos exposed to RA. (J) In the medulla oblongata and area postrema (MO), RA treatment induces *th* expression. (K) *nls/raldh2* mutant embryos express *th* in one or two cells of the MO only. (L) RA fails to induce *th* expression in the hindbrain of *tfap2a* mutant embryos. (M,N) A dramatic increase of enteric neurons expressing *th* in the gut region occurs after RA treatment. (O) Embryos mutant for *nls*/*raldh2* have a reduced number of *th*-expressing cells in the sympathetic ganglia (Symp). (P) RA is not able to induce *th* expression in the gut region of *tfap2a* mutant embryos. (A-P) Dorsal views, anterior towards the left.

specific expression domains of several Hox genes and *epha4* (Prince et al., 1998). Expression domains of *hoxa2* in rhombomere 2-5 (r2-r5), *hoxb2* in r3-r5, *hoxb3* in r4-r7 and *hoxd3* in r6-r7 are similar in wild-type controls and embryos treated with RA between 24-33 hpf (Fig. 8A-H). In addition, no alterations are seen in *epha4* expression in r1, r3, and r5 in RA-treated embryos at 33 hpf (Fig. 8I-L). These results demonstrate that RA treatment between 24 and 33 hpf does not change hindbrain patterning or posteriorize rhombomere identities. Thus, the ectopic *th* expression we observed is not due to an expansion of posterior rhombomere identities into more anterior territories.

Taken together, these results reveal an important role for RA in the development of NA neurons in the medulla/area postrema and in the sympathetic nervous system. In both NA neuronal populations, Tfap2a may be involved in mediating the effect of RA.

Induction of th expression by RA signaling may be mediated by Tfap2a

RA signaling is mediated by two classes of retinoid receptors, RARs and RXRs (Chambon, 1995). To determine whether RA may directly regulate the expression of *th* through its receptors, or whether it may act through the expression of intermediary proteins, we tested whether RA can induce ectopic *th* expression in the absence of new protein synthesis. To inhibit protein synthesis, we exposed zebrafish embryos to the protein synthesis inhibitor cycloheximide shortly before and during RA treatment. Embryos treated with cycloheximide alone had normal *th* expression in the CNS (Fig. 9B,F,J), but only a few *th*-expressing sympathetic neurons formed (Fig. 9N). Embryos treated with cycloheximide and RA did not express *th* ectopically in the hindbrain or peripheral nervous system (Fig. 9 compare C,G,K,O with D,H,L,P). Thus, protein synthesis is required to mediate the effect of RA on NA differentiation, indicating the involvement of intermediate targets of RA. To investigate whether *tfap2a* may mediate RA induced ectopic expression of *th* in the hindbrain, we analyzed *tfap2a* expression in RA-treated embryos. After RA exposure, *tfap2a* expression was upregulated in a restricted region of the hindbrain, in which *th* is also ectopically expressed in response to RA treatment (Fig. 9Q,R). We also examined the expression of *phox2a* and

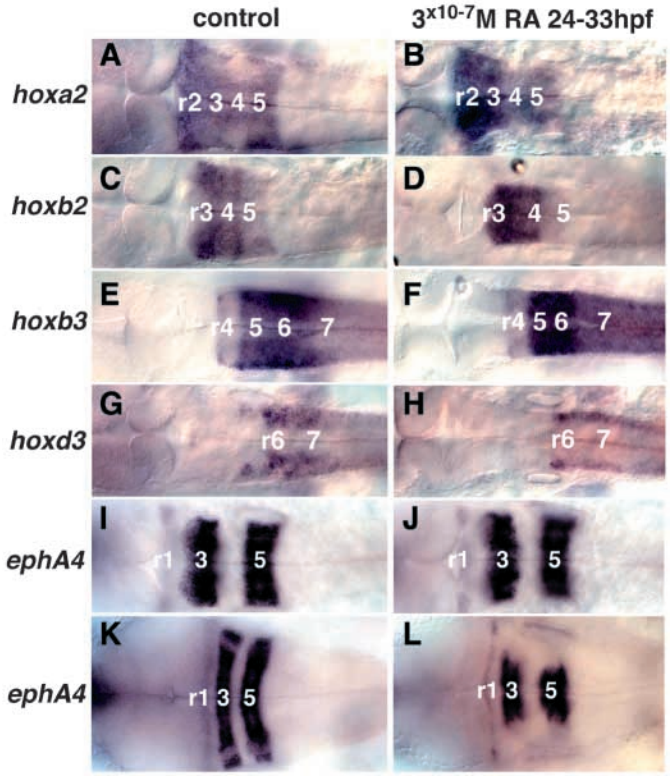

Fig. 8. Retinoic acid treatment between 24 and 33 hpf does not affect the anterioposterior pattern of the hindbrain. (A-H) Analysis of the *hoxa2, hoxb2, hoxb3* and *hoxd3* expression patterns did not reveal any changes in anterioposterior patterning of the hindbrain when embryos were exposed to RA between 24 and 33 hpf. (I,J) In situ hybridization for *epha4* expression did not reveal any anterioposterior shifts in the hindbrain rhombomere pattern of 33hpf embryos after RA treatment. (K,L) At 48 hpf, embryos treated with retinoic acid show a loss of the lateral expression domains of *epha4* in rhombomere 3 and 5. cont, control embryos exposed to DMSO; r, rhombomere; RA, retinoic acid. (A-L) Dorsal views of flat-mount preparations, anterior towards the left.

tfap2a in *nls* mutant embryos to elucidate if a reduction of RA signaling affects the expression pattern of these genes. At 34 hpf, *tfap2a* and *phox2a* expression was reduced in the hindbrain posterior to rhombomere 5 (Fig. 9S,T). These findings correlate with the lack of *th*-expressing cells in the posterior hindbrain and the normal development of the LC in *nls* mutants.

The failure of RA to induce ectopic *th* expression in the hindbrain when protein synthesis was blocked indicates that the response to RA treatment requires intermediary factors. The upregulation of *tfap2a* expression after RA treatment and the downregulation of *tfap2a* expression in RA-deficient *nls* mutant embryos suggest that Tfap2a may mediate RA induced *th* expression in the posterior hindbrain.

Discussion

The molecular mechanisms that lead to specification of noradrenergic neurons in the hindbrain, and to expression of the proper neurotransmitter phenotype in the LC and medulla, are poorly understood. Zebrafish embryos mutant for alleles of *mont blanc* are defective in NA neuron differentiation in the brainstem and in the sympathetic nervous system, and are also affected in visceral sensory ganglia and craniofacial development. We report that the pleiotropic *mob* phenotype is caused by a nonsense mutation in the *tfap2a* gene. Our analysis revealed a crucial role of *tfap2a* for expression of the neurotransmitter phenotype of NA neurons in the hindbrain. Expression of *phox2a*, a factor crucial for the specification of the locus coeruleus (LC), was not affected in *mob/tfap2a* mutant embryos, and *tfap2a* was not affected in *phox2a/sou* mutants. Thus, *phox2a* and *tfap2a* may represent parallel inputs into NA differentiation in the LC. Furthermore, our experiments provide the first in vivo evidence that *tfap2a*-dependent NA neuronal specification in the hindbrain is controlled by the retinoic acid signaling pathway.

The development of neural crest and epibranchial placode derivatives of the autonomic nervous system depend on *tfap2a* function

Neural crest cells arise from the dorsal neural tube, migrate throughout the body and differentiate into many different derivatives, including peripheral nervous system and craniofacial skeleton (reviewed by Le Douarin and Kalcheim, 1999). Loss-of-function experiments in mouse revealed that *tfap2a* contributes to a wide variety of developmental events, including skeletal formation in the thorax and limbs, aspects of organogenesis, formation of the peripheral nervous system, and neural tube closure (Schorle et al., 1996; Zhang et al., 1996). The analysis of *tfap2a* chimeric mice has shown that Tfap2a is indeed autonomously required for each of the pleiotropic aspects of the mutant phenotype investigated to date (Nottoli et al., 1998). In zebrafish, mutations in *mob/tfap2a* also cause extensive defects in the development of neural crest derivatives, including the craniofacial skeleton and pigment cells (Neuhauss et al., 1996; Schilling et al., 1996).

Primary sensory neurons of the trunk arise exclusively from the neural crest. By contrast, only some of the cranial sensory neurons are derived from neural crest. The majority of cranial sensory neurons have their origin in the dorsolateral trigeminal and vestibular placodes, and the distal epibranchial placodes (Ayer-Le Lievre and Le Douarin, 1982; D'Amico-Martel and Noden, 1983; Noden, 1993). The loss of the epibranchial derived sensory ganglia in *tfap2a* mutant embryos could be explained by defects in neural crest derived cranial structures. It was shown that neurogenic placodes form under the influence of the surrounding cranial tissues, including neural crest and pharyngeal endoderm (Vogel and Davies, 1993; Begbie et al., 1999). The reduction of *phox2a* and *ret1* expression in *tfap2a* mutants indicates that both components of the sensory ganglia, namely neural crest and neurogenic placodal cells, are directly affected by lack of *tfap2a*. We have not detected an increase in apoptosis in any of the cranial ganglia in *mob/tfap2a* mutants (data not shown). This is in contrast to *Tcfap2a* knockout mice, which develop increased apoptosis in the trigeminal ganglia (Zhang et al., 1996).

tfap2a is expressed in neural crest cells and required for their normal development (Luo et al., 2003), and thus it is likely that neural crest defects in *tfap2a* mutants mediate the sensory ganglia phenotype. By contrast, the phenotype in the sympathetic nervous system may reflect a direct involvement of *tfap2a* in specification of the NA neurotransmitter phenotype. Hu and *isl1* expression occurs early in the

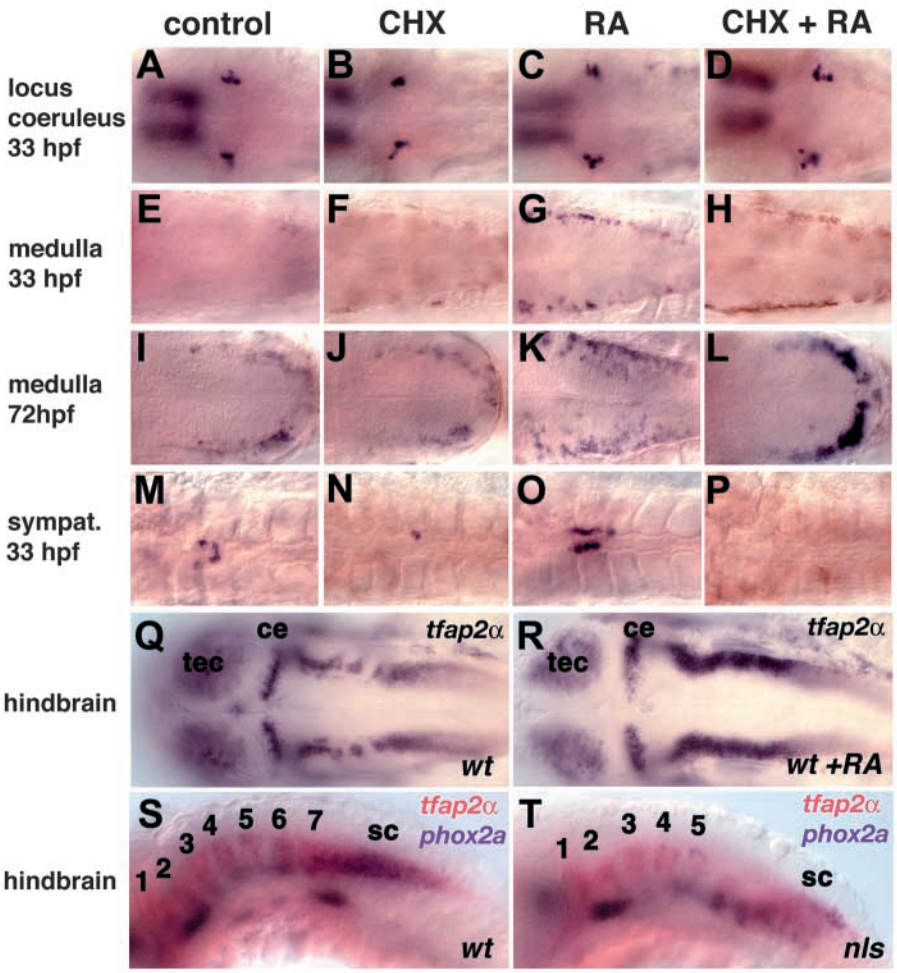

Fig. 9. Retinoic acid-mediated induction of *th*-expressing cells requires protein synthesis and coincides with increased *tfap2a* expression. (A-D) Cycloheximide (CHX; B), retinoic acid (RA; C) or a combination of both treatments (D) does not alter the expression of *th* in the locus coeruleus (LC) compared with untreated wild-type embryos (A). (E-H) *th* expression at 33 hpf in the hindbrain of wild-type embryos (H) and embryos exposed to (F) cycloheximide, (G) retinoic acid and (H) a combination of cycloheximide and RA. CHX blocks the RA induced expression of *th* in medulla at 33 hpf. (H) Brown cells are pigment cells and not NA neurons, which would stain blue from whole-mount in situ hybridization procedure. (I-L) Expression of *th* in the medulla oblongata of wild-type embryos at 72 hpf. (J) Cycloheximide-treated embryos, (K) retinoic acid-treated embryos, (L) embryos treated with both cycloheximide and RA. (M-P) Expression of *th* in the region in which sympathetic neurons form in wild-type embryos (M) at 33 hpf. Embryos exposed to cycloheximide (N), retinoic acid (O) and a combination of both (P). CHX blocks the RA-induced expression of *th* in sympathetic ganglia. (Q,R) Expression of *tfap2a* in wild-type embryos (Q) and embryos treated with RA between 24 and 33 hpf (R). RA induces the expression of *tfap2a* in the hindbrain. (S,T) Expression of *tfap2a* (red) and *phox2a* (blue) in the hindbrain of wild-type embryos (S) and *nls* mutant embryos (T). The expression of *tfap2a* (red) and *phox2a* (blue) is reduced in the posterior hindbrain (r6,7) of *neckless/raldh2*-deficient mutants. (A-R) dorsal views, anterior towards the left; (S,T) lateral views, anterior towards the left. ce, cerebellum; hb, hindbrain; sc, spinal cord; tec, tectum; 1-7, rhombomeres r1-7; sympat, region in which sympathetic neurons form.

maturation process and is observed in S-phase neural precursor cells of the sympathetic ganglia (Vogel et al., 1993; Marusich et al., 1994b; Avivi and Goldstein, 1999). The expression of Hu and *isl1* in cells in the sympathetic ganglia of *tfap2a* mutants indicates that neural crest cells migrate to their proper location, proliferate and start to differentiate, but fail to adopt their neurotransmitter phenotype. Thus, it is likely that expression of *tfap2a* in sympathetic precursors is required for correct further development and differentiation towards the noradrenergic phenotype.

tfap2a is required for noradrenergic identity of brainstem neurons

Two main groups of noradrenergic (NA) neurons, a caudal rhombencephalic group (A1-A3, in medulla and area postrema) and a rostral rhombencephalic group (A4-A7, in the locus coeruleus), can be recognized in the hindbrain of higher vertebrates (Smeets and Reiner, 1994; Smeets and Gonzalez, 2000) as well as in zebrafish (Ma, 1994a; Ma, 1994b; Ma, 1997; Holzschuh et al., 2001). The control of noradrenergic development in the mouse hindbrain has mainly been studied in the locus coeruleus (reviewed by Goridis and Rohrer, 2002), where important roles have been demonstrated for Mash1 (Hirsch et al., 1998), Phox2a (Morin et al., 1997), Phox2b (Pattyn et al., 1999) and Tlx3 (Qian et al., 2001). Although Mash1, Phox2a and Phox2b appear to act in a linear cascade (Hirsch et al., 1998; Pattyn et al., 2000a), Tlx3 is thought to act independently of Phox2b during LC development (Qian et al., 2001). *Tcfap2a* is expressed in NA and AD neurons in the mouse and has been implicated to contribute to activation of the TH and DBH promoters in cell culture systems (Kim et al., 1998; Kim et al., 2001); however, a function of *Tcfap2a* for NA differentiation in vivo was unknown. We demonstrate a novel requirement of Tfap2a for NA neuron differentiation in the LC.

The development of the NA groups in the posterior hindbrain has not been studied extensively. It was shown that Tlx3 and Phox2b are required for the development of medullary NA neurons (Pattyn et al., 2000a; Qian et al., 2001). We show that expression of *th* is missing in the entire hindbrain of *tfap2a* mutant embryos at 2 dpf when noradrenergic differentiation occurs in wild type. Thus, our studies reveal *tfap2a* as an additional transcription factor required for early NA neuronal differentiation throughout the hindbrain. However, low level expression of *dbh* can be detected in a small group of cells in the medulla/area postrema from 4 dpf onwards, which indicates that some NA neuronal precursors can bypass a requirement for *tfap2a* during late development.

We can only speculate that other transcription factors with later onset of expression, possibly additional members of the Tfap2 family, may substitute for *tfap2a*.

A loss of NA identity of the LC has also been reported for zebrafish *soulless/phox2a* mutant embryos, in which LC progenitor cells have been shown to exist (Guo et al., 1999a). We find that the medullary and area postrema NA neurons develop normally in *soulless/phox2a* mutants, in contrast to *tfap2a* mutant embryos. The genetic requirement for both *tfap2a* and *phox2a* to control *th* expression in the LC could be explained either by both of them providing independent inputs for NA specification, or by *tfap2a* acting downstream of *phox2a* in specification of the NA neurotransmitter phenotype. The latter idea would be consistent with our findings that the loss of functional Tfap2a does not cause a loss of *phox2a* expression in the LC, while *th* expression does not occurs in *tfap2a* mutant embryos. Thus, *tfap2a* could be a downstream target of *soulless/phox2a*. However, several observations argue against this hypothesis being true for the whole hindbrain. First, *tfap2a* expression is not detected in many *phox2a*-expressing cells in the hindbrain and vice versa. Second, the expression pattern of *tfap2a* is not altered in *soulless/phox2a* mutant embryos. Third, *phox2a* and *tfap2a* are not co-expressed in cells that give rise to NA neurons of the medulla/area postrema. Therefore, *tfap2a* and *phox2a* may provide parallel inputs into NA differentiation of LC neurons, while upstream factors other than *phox2a*, e.g. *phox2b*, may control *tfap2a* expression in the medulla/area postrema.

The role of retinoic acid in NA neuron specification

The capacity of RA to induce neuronal differentiation in cell culture has been well documented for several cell lines (reviewed by Guan et al., 2001). Furthermore, the expression of Tfap2a is enhanced during RA induced neuronal differentiation in a human teratocarcinoma cell line (Williams et al., 1988; Luscher et al., 1989). In cell culture, RA may induce the expression of the noradrenaline transporter, a protein specifically expressed in NA neurons (Matsuoka et al., 1997). RA may also increase the number of TH-positive cells in quail neural crest culture (Rockwood and Maxwell, 1996). These findings suggest that the RA signaling pathway may play a role in NA differentiation. To investigate a potential role of RA during zebrafish NA development in vivo, we analyzed embryos that are genetically deficient in one of the RA synthesis pathway enzymes, retinaldehyde dehydrogenase 2 (Raldh2), as well as embryos that have been exposed to high levels of RA. Incubation of embryos in the presence of high RA concentrations induced ectopic *th* expression in the anterior hindbrain caudal to rhombomere 1, as well as in the sympathetic nervous system. The capacity of RA to induce ectopic *th*-expressing cells was clearly restricted to rhombomere 2 to 7, and never occurred in rhombomere 1 or in the proximity of the LC. The nuclear transducers of RA signaling are the RAR- and RXR-type retinoid receptors, which bind to retinoic acid response elements (RARE) in target gene promoters as RXR homodimers, or as RXR heterodimers either with RAR, or with other nuclear receptors (Gudas, 1994; Chambon, 1995). Several zebrafish retinoic acid receptor genes have been cloned (Joore et al., 1994; White et al., 1994; Jones et al., 1995). At 24 hpf, *rara* is expressed in the hindbrain with a sharp posterior border at rhombomere 6 to 7. *rarg* is also expressed in the ventral part of the hindbrain and in two lateral patches of head mesenchyme (Joore et al., 1994). Following RA treatment, the expression of *rarg* is induced in the entire brain, whereas *rara* is ubiquitously induced in the embryo (Joore et al., 1994). As *rara* and *rarg* are both expressed in the posterior hindbrain and induced ectopically by RA, both are potential mediators for RA induced *th* expression in the hindbrain. The fact that both receptors are not expressed in rhombomere 1 may explain why RA is unable to induce *th* expression anterior to rhombomere 2. When exposing zebrafish embryos to the protein synthesis inhibitor cycloheximide, we prevented the induction of ectopic *th* expression by RA. Therefore, translation of an intermediary target of RA signaling is required to induce *th* expression, rather than *th* being directly activated by retinoid receptors.

We showed that, after treatment of embryos with excess RA, *tfap2a* expression is upregulated in the posterior hindbrain. Furthermore, in *tfap2a* mutant embryos, exogenously applied RA is not able to ectopically induce *th* expression or to rescue the deficiency in NA-expressing cells. These findings implicate that RA signaling may induce *th* expression via activation of *tfap2a* expression. Indeed, a requirement for RA was confirmed when we analyzed zebrafish *neckless /raldh2* mutant embryos, which are deprived of RA. Raldh2 is involved in the biosynthesis of RA from vitamin A (reviewed by Duester, 2000). Loss of function of Raldh2 in mouse mimicked the most severe phenotypes associated with vitamin A deficiency (VAD), implicating Raldh2 as the main source of RA in the vertebrate embryo (Niederreither et al., 1999; Niederreither et al., 2000). Zebrafish *neckless/raldh2* mutant embryos resemble many aspects of VAD (Begemann et al., 2001). In *nls* mutants, we find that the NA neurons are missing in the posterior hindbrain, and that the expression of *tfap2a* is downregulated.

It is well established that RA signaling is involved in AP patterning of the hindbrain by regulating the expression of Hox genes (Holder and Hill, 1991; Hill et al., 1995) (reviewed by Gavalas and Krumlauf, 2000; Gavalas, 2002). In our experiments, however, embryos were exposed to RA only from 24 hpf onwards, when rhombomere identity has already been determined in the zebrafish. Indeed, we can demonstrate that the AP patterning of the hindbrain is not changed in our experiments: Anterioposterior expression borders of *hoxa2*, *hoxb2*, *hoxb3*, *hoxd3* and *epha4* were unaffected in our RA-treated embryos. Similarly, treatment of chick embryos with an RA antagonist at late or post-somitogenesis stages has not resulted in any changes of rhombomere identity (Dupe and Lumsden, 2001).

In summary, our data provide evidence that two separate mechanisms control noradrenergic development in the zebrafish hindbrain. In the locus coeruleus, signals from the midbrain-hindbrain-boundary organizer initiate a cascade of transcription factors, including Phox2a and Phox2b (Guo et al., 1999a), which ultimately requires Tfap2a for NA precursor cells to express the NA neurotransmitter phenotype. In the posterior hindbrain, the medulla and area postrema, retinoic acid is an important signal, both necessary and sufficient to induce NA differentiation, and precursor neurons share a requirement for Tfap2a in order to be able to express the NA neurotransmitter phenotype. Thus, although the inductive signals may be different, hindbrain NA neurons of the LC and the posterior groups share a requirement for Phox2b, Tlx3 and

Tfap2a to establish their noradrenergic identity. Furthermore, our data reveal that noradrenergic precursors of the peripheral nervous system rely on *tfap2a* to adopt their neurotransmitter phenotype.

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