Sim1a and Arnt2 contribute to hypothalamo-spinal axon guidance by regulating Robo2 activity via a Robo3-dependent mechanism

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SUMMARY

Precise spatiotemporal control of axon guidance factor expression is a prerequisite for formation of functional neuronal connections. Although Netrin/Dcc- and Robo/Slit-mediated attractive and repulsive guidance of commissural axons have been extensively studied, little is known about mechanisms controlling mediolateral positioning of longitudinal axons in vertebrates. Here, we use a genetic approach in zebrafish embryos to study pathfinding mechanisms of dopaminergic and neuroendocrine longitudinal axons projecting from the hypothalamus into hindbrain and spinal cord. The transcription factors Sim1a and Arnt2 contribute to differentiation of a defined population of dopaminergic and neuroendocrine neurons. We show that both factors also control aspects of axon guidance: Sim1a or Arnt2 depletion results in displacement of hypothalamo-spinal longitudinal axons towards the midline. This phenotype is suppressed in robo3a.1 mutation embryos. In the absence of Sim1a and Arnt2, expression of the robo3 splice isoform robo3a.1 is increased in the hypothalamus, indicating negative control of robo3a.1 transcription by these factors. We further provide evidence that increased Robo3a.1 levels interfere with Robo2-mediated repulsive axon guidance. Finally, we show that the N-terminal domain unique to Robo3a.1 mediates the block of Robo2 repulsive activity. Therefore, Sim1a and Arnt2 contribute to control of lateral positioning of longitudinal hypothalamic-spinal axons by negative regulation of robo3a.1 expression, which in turn attenuates the repulsive activity of Robo2.

KEY WORDS: Robo/Slit signalling, Robo receptor isoforms, Dcc/Netrin signalling, Dopaminergic neurons, Neuroendocrine systems, Hypocretinergic neurons, Hypothalamo-spinal projections, Orthopedia, Zebrafish

INTRODUCTION

Understanding how transcription factors genetically encode the specification of connectivity in the nervous system is a challenge in neurobiology. Knowledge of molecular mechanisms that link transcription factor function to regulation of axon guidance in vertebrates is limited to a few well-studied systems, including spinal motoneuron projections and retinotectal trajectories (for a review, see Butler and Tear, 2007; Polleux et al., 2007). However, for most central nervous system projection pathways, it is unknown how connectivity is transcriptionally determined.

Hypothalamic projections into the spinal cord (HTS) are an attractive model for understanding how CNS nuclei establish long-range projections. A well-characterised transcription-specific hypothalamic cell lineages. The homeodomain transcription factor Orthopedia (Otp) is co-expressed with the two PAS domain factors Sim1 and Arnt2, the latter two forming functional heterodimers. Otp/Sim1/Arnt2-dependent cell lineages in mammals (Acampora et al., 1999; Michaud et al., 2000; Michaud et al., 1998; Wang and Lufkin, 2000) and zebrafish (Blechman et al., 2007; Del Giacco et al., 2006; Eaton and Glasgow, 2006; Eaton and Glasgow, 2007; Löhr et al., 2009; Ryu et al., 2007) include neuroendocrine cells characterised by expression of the peptide hormones corticotropin-releasing hormone, thyrotropin-releasing hormone, arginine vasopressin, oxytocin and somatostatin. In addition to neuroendocrine lineages, Otp/Sim1/Arnt2 have been shown to control development of hypothalamic A11-type dopaminergic (DA) neurons in zebrafish and, for Otp, mammals (Borodovsky et al., 2009; Löhr et al., 2009; Ryu et al., 2007). Despite the diverse neurotransmitter/neuropetide phenotypes, Otp/Sim1/Arnt2-specified cell type share similar descending ipsilateral projections. Neuroendocrine cell lineages have been shown to innervate spinal target fields in mammals (Hancock, 1976; Swanson, 1977) and zebrafish (M. Hammerschmidt, personal communication). Furthermore, A11-type DA neurons project directly to the spinal cord in mammals (Björklund and Skagerberg, 1979) and zebrafish (Tay et al., 2011). The similarity in projection patterns of diverse Otp/Sim1/Arnt2-specified hypothalamic neurons prompted us to investigate whether this transcription factor network might also regulate axon guidance. Ipsilateral longitudinal axon guidance is regulated in part by the Robo/Slit and Netrin/Dcc receptor/ligand pairs (Farmer et al., 2008; Kastenhuber et al., 2009). Slit and Netrin guidance cues are expressed by ventral midline cells and bind to receptors of the Robo and Dcc families, respectively (Brose et al., 1999; Keino-Masu et al., 1996). In mouse, loss of the two Robo receptors Robo1 or Robo2, as well as loss of Slit1 and Slit2 ligands, disrupts proper establishment of longitudinal pioneer tracts in the midbrain and hindbrain (Farmer et al., 2008), and Robo/Slit signalling also affects ascending nigrostriatal tracts of mesencephalic dopaminergic neurons (Dugan et al., 2011). Similarly, longitudinal axons of A11-related DA

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neurons in zebrafish integrate midline repulsion via Robo2/Slit and midline attraction via Dcc/Netrin1 to specify lateral positioning (Kastenhuber et al., 2009). A role for Robo3 during ipsilateral longitudinal axon guidance has not been reported. Robo3, with at least four splice variants (Ypsilanti et al., 2010), displays the greatest structural heterogeneity within the Robo receptor family, and controls pre- and post-crossing behaviour of commissural axons (Chen et al., 2008; Sabatier et al., 2004).

Here, we provide evidence that Arnt2 and Sim1a are required to determine the lateral position of neuroendocrine and DA longitudinal axons en route to the spinal cord. Loss of Arnt2 or Sim1a function leads to displacement of HTS axons towards the midline. By performing expression analyses together with gain- and loss-of-function studies, we show that the abnormal projection pattern observed is caused by misexpression of the specific Robo3 isoform rob3a.1. We further show that rob3a.1 is sufficient to block Robo2-mediated midline repulsion of HTS longitudinal axons. Our data suggest that Sim1a and Arnt2 negatively regulate rob3a.1, which in turn attenuates repulsive activity of Robo2, and thereby controls the lateral positioning of HTS longitudinal axons.

MATERIALS AND METHODS

Fish maintenance and strains

Zebrafish were maintained at 28.5°C (West菲尔der, 1995). To inhibit pigmentation, embryos were incubated in 0.2 mM phenyliodoheax. For a complete list of used zebrafish lines, see supplementary material Table S1. Compound mutants were identified by PCR. Genotyping of arnt212636bR, asi2272z, asl226bR and tnl2206 alleles was previously described (Chalasani et al., 2007; Fricke et al., 2001; Golling et al., 2002; Kastenhuber et al., 2009).

Immunohistochemistry and in situ hybridization

Whole-mount immunohistochemistry (Holzscheuch et al., 2003), in situ hybridization and fluorescent in situ hybridization were performed as described previously (Filippi et al., 2007). To enhance signal intensity in fluorescent in situ hybridization, 5% dextrane sulphate was added in the hybridization buffer and 450 µg/ml iodophenol during peroxidase reaction. For confocal microscopy stained embryos were embedded in medium containing 1.2% low melting agarose in 20% glycerol. For details on antibodies and probes used, see supplementary material Table S2.

Plasmid construction and injections

For expression constructs, a multisite Gateway system was used (Kwan et al., 2007) (Kwan et al., 2007) were co-injected into one-cell stage sim1a Morpholino oligonucleotides (MOs) were obtained from Gene Tools (Philomath, OR). The sim1a (sim1a e2i2), arnt2 (arnt2 e2i2) and ddc (ddc-MO2) morpholinos have been described previously (Lühr et al., 2009; Suli et al., 2006). For controls, standard negative control morpholino from Gene Tools was used. Morpholinos were injected at one-cell stage: 0.25-1.0 ng (sim1a MO), 1-4.5 ng (arnt2 MO) and 4.5 ng (ddc MO) per embryo.

Photomorph caging experiments (SuperNova Life Science) was designed to bind and block the sim1a morpholino. Prior to injection, caging strand and sim1a morpholino were mixed at a molar ratio of 7:1 and hybridised at 70°C for 30 minutes. Subsequently, the mixture was cooled down to 4°C and stored overnight prior to injection. Special care was taken to keep the caged MO solution and injected embryos in the dark until time of uncaging. Uncaging of the morpholino was performed by exposure to UV light (Piolab, UV superbright light table, 312 nm, 100% intensity) for 30 minutes. Efficiency and function of the Photomorph caging strand was confirmed by PCR on cDNA derived from embryos injected with Photomorph caging strand and sim1a MO (with and without UV light treatment) using primers as described previously (Lühr et al., 2009).

Microscopy, quantification and statistical analyses

Confocal z-stacks were recorded using Zeiss LSM 510. LSM or NIH ImageJ software was used to create z-projections. Adobe Photoshop or Illustrator were used to assemble figures. Distances between axons were determined using Zeiss LSM510 Software. Midline crossing of MA axons was determined using a Leica MZ16f stereomicroscope. Quantification of fluorescence from in situ hybridization analyses: to determine the regions of interest for single neurons, we used the GFP channel and the region of interest manager embedded in ImageJ. Ten neurons in the anterior and six neurons in the posterior hypothalamus (see Fig. 4J) were randomly selected per embryo; on average, three slices per fish were used. To measure the average signal intensity within each selected neuron, as outlined by the regions of interest, we switched to the channel carrying the gene expression information maintaining the region of interest masks from the GFP channel. All samples were analysed at the LSM510 using identical acquisition parameters. Statistical analysis was performed using Student’s t-test. Error bars represent s.d.

RESULTS

A novel role for Sim1 and Arnt2 in establishment of HTS axon tracts

Absence of Otp, Sim1 or Arnt2 results in loss of cell type-specific terminal differentiation markers of defined hypothalamic neuroendocrine cell groups and of A11-related DA neurons in zebrafish (Lühr et al., 2009; Ryu et al., 2007). Although not fully differentiated, these cells are maintained at least until 4 dpf, as judged by persistent expression of otp and sim1 transcripts. To study the role of Sim1a and Arnt2 during longitudinal HTS tract formation, we used a transgenic zebrafish line visualizing otpb-expressing neurons in the hypothalamus and their longitudinal projections towards the spinal cord by GFP (supplementary material Fig. S1) (Fujimoto et al., 2011). To analyse a potential effect of Sim1a on HTS tract formation, we injected a sim1a morpholino into otpb:gfp transgenic embryos. otpb:gfp-positive longitudinal axons were significantly displaced towards the midline after depletion of sim1a when compared with controls (Fig. 1A-D; supplementary material Table S4). A similar medial displacement of otpb:gfp longitudinal HTS axons was observed upon injection of arnt2 MO (Fig. 1E,F; supplementary material Table S4) and in arnt212636bR mutant embryos (supplementary material Fig. S2A-E; Table S4). Moreover, co-injection of sub-threshold amounts of sim1a (0.25 ng) and arnt2 (1.0 ng) morpholinos, which do not display a phenotype when injected individually, did result in a medial displacement of otpb:gfp HTS axons (Fig. 1G,H,I; supplementary material Table S4). To determine the specificity of the observed phenotype, we used a transgenic reporter line that labels hypocretinergic (Hcrt) neurons (hert:tdTomato). Hcrt neurons reside in the lateral hypothalamus and also project to spinal target regions (Faraco et al., 2006; van den Pol, 1999), but do not depend on Sim1a and Arnt2 transcription factors for differentiation (data not shown). Analysis of otpb:gfp:hert:tdTomato transgenic embryos revealed that HTS axons of Hcrt neurons and otpb:gfp-positive neurons run at similar mediolateral positions, yet longitudinal axons of Hcrt neurons did not display any change in lateral positioning upon sim1a knockdown (supplementary material Fig. S3A-G, Table S4). These results demonstrate that sim1a and arnt2 are selectively and synergistically required for lateral positioning of otpb:gfp-positive HTS axons.

Temporally controlled knockdown links Sim1 function to axonal pathfinding of dopaminergic neurons

The medial displacement of otpb:gfp HTS axons upon depletion of arnt2 and sim1a could be caused by transdifferentiation of DA (and
Sim1a and Arnt2 in axon guidance

We decided to knock down sim1a at a time point when DA terminal differentiation marker tyrosine hydroxylase (TH) expression has already been initiated, but HTS development is still ongoing. The sim1a morpholino was kept inactive by hybridization with a complementary caging strand (Tomasini et al., 2009) until uncaging and activation of the sim1a morpholino was achieved by photocleavage (supplementary material Fig. S4). Injection of sim1a morpholino resulted in complete loss of TH expression of A11-related DA cells and their HTS projections (Fig. 2C,D). Conversely, caged sim1a morpholino-injected embryos displayed the full complement of hypothalamic DA neurons and developed HTS tracts similar to controls (Fig. 2A,B,E,F). Next, we injected a caged sim1a morpholino and photo-activated the morpholino at 22 hpf. This time point was chosen, because the earliest A11-related DA neurons differentiate between 18 and 24 hpf. Following this treatment, we detected individual TH-positive DA neurons in 84% of the embryos analysed. These embryos displayed a significant medial displacement of TH-positive HTS axons (Fig. 2G,H,I; supplementary material Table S5). This reveals that Sim1a is essential for lateral positioning of DA HTS axons, and thus has multiple functions during distinct steps of DA cell differentiation and maturation.

Robo2 is required for lateral positioning of HTS axon tracts in Sim1a- and Arnt2-dependent and -independent cell lineages

Proper formation of DA HTS tracts requires Robo2 function (Kastenhuber et al., 2009). As DA neurons are a fraction of otpb:gfp-positive cells, we analysed whether the role of Robo2 applies to otpb:gfp longitudinal axons in general. Owing to similarities in longitudinal projection tract formation, we also included Hcrt neurons in our experiments. robo2 transcripts are present in otpb:gfp-positive cells and in Hcrt neurons (data not shown).

To analyse mediolateral positioning of Hcrt axonal projections in comparison with otpb:gfp-positive projections, we used otpb:gfp:hct:tdtomato double transgenic zebrafish that were crossed into Robo2-deficient (asp1272) mutants. In ast homozygous mutant embryos, we observed a significant medial displacement of HTS axons labelled by both transgenic lines when compared with heterozygous siblings (Fig. 3A-G; supplementary material Table S6). Thus, otpb:gfp-positive cells, as well as Hcrt neurons, require Robo2 for lateral positioning of HTS projection tracts.

Expression of robo3a.1 is increased in otpb:gfp-positive neurons in the hypothalamus after sim1a or arnt2 depletion

We next analysed the molecular mechanisms underlying the medial displacement of otpb:gfp HTS tracts in sim1a morphants and arnt2hi2639 mutants. A previous study in mouse demonstrated that Robo3 transcripts were upregulated in mammillary body neurons of Sim1−/−/Sim2−/− double mutants (Marion et al., 2005). In zebrafish, two robo3 isoforms (robo3v1 and robo3v2) have been described, which only differ in a short stretch of amino acids at the N terminus (Challa et al., 2005). Based on its specific and spatially restricted expression in the diencephalon, we focused on robo3a.1 (Challa et al., 2005). To analyse expression of robo3a.1 in relation to otpb:gfp neurons, we performed fluorescent in situ hybridization to robo3a.1 in combination with anti-GFP immunohistochemistry. At 48 hpf, this analysis revealed a bilateral robo3a.1 expression domain in the hypothalamus, which extends medially towards the midline. Based on robo3a.1 and otpb:gfp expression profiles, the robo3a.1 domain could be separated into an anterior (aH) and a...
posterior (pH) part (Fig. 4G,J). In aH, robo3a.1 is expressed only in a subset of GFP-positive cells at very low levels, whereas in pH, robo3a.1 is co-expressed in most GFP-positive neurons (Fig. 4A-H). This expression profile coincides with the dynamic regulation of robo3a.1 transcription during DA cell development (supplementary material Fig. S5).

We next evaluated robo3a.1 expression in otpb:gfp transgenic arnt2nh2659 mutant embryos and in otpb:gfp embryos after sim1a MO knockdown (Fig. 4B-F). Our analysis reveals that, in response to arnt2 or sim1a loss of function, robo3a.1 expression levels are significantly increased in a subset of GFP neurons in the aH, whereas expression in pH was unchanged (Fig. 4K). By contrast, a change in expression levels of other Robo receptors or of Dcc was not detected (Fig. 4K and data not shown).

Taken together, these results provide evidence that robo3a.1 transcription is dynamically regulated during development of otpb:gfp and DA neurons, and that Sim1a and Arnt2 transcription factors are required for downregulation of robo3a.1 in aH as development proceeds.

**Robo3 is required to define lateral positioning of otpb:gfp-positive longitudinal axons**

We next tested a potential role of Robo3 during pathfinding of otpb:gfp-positive HTS longitudinal axons using Robo3 twitch twice (twttw204) mutants (Burgess et al., 2009). In twttw mutant otpb:gfp embryos, the distance between longitudinal HTS axons was significantly increased when compared with heterozygous siblings (Fig. 5A-E; supplementary material Table S7). In addition, we analysed pathfinding of Hcrt HTS axons, which do not express robo3a.1 (supplementary material Fig. S6A-C). Formation of hcret:tdtomato-positive longitudinal axons did not reveal a significant difference between twttw mutant and heterozygous sibling embryos (supplementary material Fig. S6D-H, Table S7). Therefore, Robo3 function specifically contributes to mediolateral positioning of otpb:gfp-positive longitudinal axons but not of Hcrt axons.
Medial displacement of otpb:gfp- and TH-positive longitudinal axons after sim1a or arnt2 depletion is suppressed in robo3 mutants

To examine whether an increase of robo3a.1 expression might be the cause for medial displacement of otpb:gfp-positive longitudinal axons, we assayed longitudinal tract formation after sim1a or arnt2 knockdown in Robo3-deficient embryos. In twt heterozygous otpb:gfp embryos injected with sim1a MO, we observed a medial displacement of longitudinal axons. By contrast, this displacement upon sim1a knockdown was strongly attenuated in twt mutant otpb:gfp embryos (Fig. 5F-J; supplementary material Table S8). Knockdown of arnt2 function resulted in similar findings (supplementary material Fig. S7A-E; Table S8). In order to demonstrate that negative regulation of robo3 by Sim1a is required for the positioning of longitudinal DA tracts, we again used stage-specific knockdown of sim1a by photoactivation of morpholinos (supplementary material Fig. S8A-D). This analysis demonstrated a significant increase in the average distance of TH-positive longitudinal axons for sim1a photomorphants of twt mutant genotype when compared with twt heterozygous embryos (supplementary material Fig. S8E, Table S9). These two sets of experiments indicate that negative control of robo3a.1 by Sim1a and Arnt2 is required for proper lateral positioning of otpb:gfp and DA longitudinal axons.

Overexpression of robo3a.1 induces medial displacement of Robo2-dependent longitudinal axons

We next analysed whether mis-expression of robo3a.1 was sufficient to induce medial displacement of Robo2-dependent longitudinal axons. In addition to robo3a.1, we also decided to analyse a potential role of robo3b.1 in HTS formation. We analysed Otpb and Hcrt neurons, which require Robo2 function for longitudinal tract formation. To temporally control expression of robo3a.1 or robo3b.1, we generated hsp70l:robo3a.1 and hsp70l:robo3b.1 transgenic lines, which enable comparable levels of ubiquitous robo3a.1 or robo3b.1 expression upon heat-shock treatment (data not shown). The heat-shock lines were crossed to the otpb:gfp and hcre:tdtomato transgenic lines. Transgenic embryos were heat-induced (39°C for 50 minutes) at 24 and 28 hpf to maintain robo3 expression during longitudinal HTS pathfinding. Heat-shock treatment did not affect formation of longitudinal axons in control or hsp70l:robo3b.1 embryos. By contrast, mis-expression of robo3a.1 resulted in medial displacement of longitudinal axons (Fig. 6). Quantification revealed a significant medial displacement phenotype after overexpression of robo3a.1 when compared with controls or with overexpression of robo3b.1 (Fig. 6G,N; supplementary material Tables S10, S11). In addition to the results...
described above, we observed different phenotypes upon mis-expression of \textit{robo3a.1} (enables midline crossing) and \textit{robo3b.1} (prevents midline crossing) in commissural Mauthner neuron axons (J.S., unpublished).

Our findings demonstrate that mis-expression of \textit{robo3a.1}, but not of \textit{robo3b.1}, is sufficient to induce medial displacement of longitudinal HTS axons. Thus, the Robo3a.1 isoform is important for proper mediolateral positioning of HTS tracts.

\textbf{Ectopic Robo3a.1 blocks Robo2-dependent repulsion}

To analyse how ectopic \textit{robo3a.1} expression may affect HTS tract formation, we first tested for a potential role of Robo3a.1 as a chemoattractive receptor. The medial displacement of DA longitudinal axons towards the midline in Robo2-deficient \textit{ast} embryos can be rescued upon knockdown of attractive Dcc/Netrin signalling (Kastenhuber et al., 2009). If Robo3a.1 serves as an attractive receptor, we would predict that a combined knockdown of \textit{sim1a} (thereby increasing Robo3a.1) and \textit{dcc} (thereby removing Dcc-mediated attraction) should still result in medial displacement phenotype of \textit{otpb:gfp} HTS axons. However, we found that medial displacement of longitudinal axons was significantly reduced following co-injection of \textit{sim1a} MO and \textit{dcc} MO when compared with controls (Fig. 7A-E; supplementary material Table S12). This indicates that attraction mediated by Dcc causes medial displacement of longitudinal axons after \textit{sim1a} knockdown, and argues against a role of Robo3a.1 as an attractive receptor.
We next analysed whether Robo3a.1 interferes with repulsion by Robo2 as suggested previously (Chen et al., 2008). If Robo3a.1 compromised Robo2 repulsion, then increased levels of robo3a.1 in the ast mutant background should not lead to a stronger medial displacement phenotype. Quantification of medial displacement of longitudinal axons after depletion of sim1a in ast/ast; twt/+ embryos (increased robo3a.1 expression), and in ast; twt double mutant embryos (no robo3a.1 expression) compared with ast/ast; twt/twt embryos (Fig. 8A-G; supplementary material Table S13) revealed similar phenotypic strength. To further support these findings, we made use of two different ast/robo2 alleles, the strong ast<sup>272z</sup> and the weaker ast<sup>284</sup> allele (Chalasani et al., 2007; Fricke et al., 2001). Consistently, the medial displacement phenotype of HTS axons is less pronounced in the weak ast mutants (Fig. 9A,J,I,R). We hypothesised that residual Robo2 signalling in weak ast embryos should be rendered less effective after robo3a.1 mis-expression. If so, the resulting reduced effectiveness of Robo2 signalling would be predicted to increase HTS medial displacement in weak, but not strong, ast mutants. The experiment showed that the medial displacement phenotype was not increased upon overexpression of robo3a.1 in the strong ast mutants. By contrast, a significant increase in medial displacement phenotype was observed after overexpression of robo3a.1 in the weak ast mutant background (Fig. 9; supplementary material Table S14). Taken together, these results are consistent with Robo3a.1 functioning to attenuate repulsion by Robo2, and that attraction by Dcc then directs longitudinal axons towards the midline.

**The extracellular N-terminal domain present in the Robo3a.1 isoform is important for interfering with Robo2-dependent repulsion**

To identify structural characteristics of Robo3 isoforms required for interference with Robo2-dependent repulsion, we used the Gal4/UAS system for Hcrt-specific expression of different robo3 isoforms together with tdTomato. A hcr:gal4 driver was co-injected with UAS:robo3-P2A-tdtomatoCAAX responders harbouring different Robo3 isoforms, and embryos showing transient expression of the tdTomato reporter were analysed. Whole-mount in situ hybridization for tdTomato revealed similar expression levels for the different robo3 isoforms (supplementary material Fig. S9). The medial displacement phenotype was quantified when tdTomato-positive longitudinal axons were in close contact with 3A10-positive MLF axons. (Fig. 10C,D). All midline crossing events caudal to the

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**Fig. 5. Robo3 is required for lateral positioning of otpb:gfp-positive longitudinal axons.** Dorsal views of confocal hindbrain z-projections (72 hpf) are shown. Anterior is towards the left. (A,B) In twt/+ embryos, otpb:gfp longitudinal axons in the midbrain (A, arrowheads) and hindbrain (A, arrows) grow by a defined distance towards the midline. Arrows in B indicate Mauthner neuron somata. (C,D) In twt/twt embryos, otpb:gfp longitudinal axons project more laterally in the midbrain (C, arrowheads) and hindbrain (C, arrows). (E) Quantification of the separation between otpb:gfp longitudinal axons in mid- and hindbrain (double-headed arrows, II and V in B) and between nucMLF somata (I, see asterisks in B), MLF axons (IV) or MA neurons (III) in twt/+ or twt/twt embryos. (F,G) In sim1a MO-injected twt/+ embryos, otpb:gfp longitudinal axons are medially displaced (f, arrows). (H,I) In sim1a MO-injected twt/twt embryos, otpb:gfp longitudinal axons project more laterally (H, arrows). (J) Quantification of the distance between MA neurons (II, marked in G) and vagal axons (IV in H), or of the distance between otpb:gfp longitudinal axons (I in H and III in G) in sim1a MO-injected twt/+ and twt/twt embryos. Arrowheads in B,D,G,I indicate pathfinding of Mauthner neurons in twt/+ and twt/twt embryos. *P<0.001. Scale bar: 50 µm.
level of the nucMLF were considered to be ectopic (Fig. 10C). Expression of tdTomato alone or of robo3b.1 in Hcrt neurons did not affect longitudinal tract formation. By contrast, mis-expression of robo3a.1 resulted in medial displacement of longitudinal axons and ectopic midline crossing events (Fig. 10A,F; supplementary material Table S15). To analyse requirement of Robo3A cytoplasmatic domains, we mis-expressed mouse Robo3A.1 and Robo3A.2 in Hcrt neurons. The two mouse Robo3A isoforms encode

![Image with figure and graph](image-url)
a similar N-terminus to zebrafish robo3a.1, but distinct C termini and contribute to different aspects of commissural axon guidance (Chen et al., 2008). Similar to our findings upon mis-expression of zebrafish robo3a.1, mis-expression of mouse Robo3A.1 or Robo3A.2 resulted in medial displacement of longitudinal axons and ectopic midline crossing (Fig. 10G-J,K; supplementary material Table S15).

Fig. 7. Dcc-mediated attraction causes medial displacement of otpb:gfp longitudinal axons after sim1a knockdown. Dorsal views of hindbrain confocal z-projections of 48 hpf otpb:gfp embryos. Anterior is towards the left. (A-D) GFP-positive longitudinal axons are medially displaced towards the midline (A, arrows) after co-injection of sim1a MO and control MO in otpb:gfp embryos. Medial displacement of longitudinal otpb:gfp axons (C, arrows) is reduced after combined injection of sim1a MO and dcc MO. (E) Quantification of the distance between otpb:gfp-positive longitudinal axons (I) or of the distance between Mauthner neurons (II) of indicated treatments. Arrowheads in B,D indicate normal midline crossing of MA neurons. *P<0.001; n.s., not significant. Scale bar: 50 µm.

Fig. 8. Medial displacement phenotype of otpb:gfp longitudinal HTS axons is not affected by depletion of sim1a in ast/ast; twt/+ and ast/ast; twt/twt embryos. Dorsal views of hindbrain confocal z-projections of 72 hpf otpb:gfp embryos. Anterior is towards the left. (A,B) otpb:gfp HTS axons (arrows) in a wild-type sibling. (C-H) Phenotypic strength of medial displacement of otpb:gfp-positive longitudinal axons is similar in ast/ast; twt/twt embryos injected with control MO (C, arrows), and in ast/ast; twt/+ (E, arrows) or ast/ast; twt/twt (G, arrows) sim1a morphant embryos. Arrowheads in B,D,F,H indicate pathfinding of Mauthner neurons. (I) Quantification of the distance between of otpb:gfp longitudinal axons and vagal axons, and of the distance between Mauthner neurons in ast/ast; twt/twt embryos injected with control MO, and in ast/ast; twt/+ or ast/ast; twt/twt embryos injected with sim1a MO. n.s., not significant. Scale bar: 50 µm.
Taken together, our results show that ectopic expression of zebrafish robos.1 and mouse Robo3A.1 or Robo3A.2 isoforms is sufficient to induce medial displacement of Robo2-dependent longitudinal axons. This suggests that blocking of Robo2 activity primarily occurs via the specific N-terminal domain present in the Robo3A isoforms.

**DISCUSSION**

Our data reveal a novel function for Arnt2 and Sim1a transcription factors in establishment of HTS longitudinal axon tracts. Arnt2 and Sim1 are key regulators for differentiation of at least five different neuroendocrine cell lineages and of A11-related DA neurons in the hypothalamus (Löhr et al., 2009; Michaud et al., 2000; Michaud et al., 1998). As these diverse neuronal types all develop descending projections to the spinal cord, we analysed whether Sim1a and Arnt2 would contribute to control of axonal pathfinding. We found that loss of Arnt2 or Sim1a function results in a medial shift of HTS trajectories while passing the hindbrain. Our data reveal that Sim1a and Arnt2 negatively regulate expression of robos.1, a specific robos isoform, which in turn inhibits Robo2 signalling activity.

In mouse embryos that lack Sim1 and/or Sim2 function, projection behaviour of hypothalamic neurons of the mammillary body is altered, resulting in aberrant midline crossing (Marion et al., 2005). These findings indicate that Sim transcription factor function may contribute to axon pathfinding for diverse neural populations. By contrast, Arnt2 function has been reported to be dispensable in this system. Our data indicate that sim1a and arnt2, similar to their role during neuronal differentiation (Michaud et al., 2000), act synergistically during HTS axon guidance. Robo3 transcripts have been reported to be upregulated in the mammillary body of Sim1−/−/Sim2−/− double mutants, suggesting that Robo3 might contribute to the reported axon guidance defects (Marion et al., 2005). Our analysis of HTS tract formation reveals similar mechanisms for Sim1a and Arnt2, and elucidates for both factors downstream molecular

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**Fig. 9.** Overexpression of robos.1 leads to increased medial displacement of longitudinal HTS axons in astt284 but not in astt272z embryos. Dorsal views of confocal z-projections of the brain and hindbrain of 72 hpf hcr:ttdtna embryos. Anterior is towards the left. (A-H) Medial displacement phenotype of hcr:wtdtna HTS axons is similar in astt272z and astt272z; hsp70:robos.1 embryos after heat-shock treatment. (J-Q) Medial displacement phenotype of hcr:ttdtna HTS axons is increased in astt284; hsp70:robos.1 embryos when compared with astt272z embryos. (I, R) Quantification of the number of hcr:ttdtna longitudinal axons in outer or inner sections in astt272z (I) or astt284 (R). Brackets in B, C, F, K, O indicate outer and inner sections used for quantification, defined by Mauthner neuron somata (outer section) and the area in between (inner section). Asterisks and plus signs indicate longitudinal hcr:ttdtna axons in outer and inner sections, respectively. Scale bars: in A, 50 µm for A, E, J, N; in B, 25 µm for B-D, F-H, K-M, O-Q.
mechanisms affecting axon guidance. However, mediolateral positioning of HTS axons is not completely restored upon depletion of sim1a or arnt2 in robo3 mutant embryos, suggesting additional HTS axon guidance control mechanisms by Sim1a and Arnt2. Downstream targets of Sim1 and Arnt2 include transcriptional regulators, as well as cell migration-related genes (Liu et al., 2003), which may contribute to HTS axon guidance independent of Robo3. In Drosophila, abrogation of sim leads to an axonal fasciculation phenotype (Freer et al., 2011), which differs from the mouse Sim1 or zebrafish sim1a loss-of-function defects. Thus, Sim1 may control different aspects of axonal pathfinding by regulating different axon guidance factors.

How does upregulated robo3a.1 misguide pathfinding of HTS longitudinal axons? Previous work suggested that Robo3a.1 may inactivate Robo1 and Robo2 receptors and thereby render pre-crossing commissural axons unresponsive to Slit repulsion (Chen et al., 2008). Several observations argue that a similar mechanism between Robo3A.1 and Robo2 may lead to the medial displacement phenotype of HTS longitudinal axons. The sim1a or arnt2 loss-of-function axon tract phenotypes share many features with Robo2 mutants. These similarities suggest that Robo3A.1 interferes with Robo2 activity. This notion is further strengthened by our findings that increased activity of Robo3a.1 (by either sim1a depletion or robo3a.1 overexpression) enhances the medial displacement phenotype of HTS axons in weak, but not strong, ast mutants. These genetic experiments reveal that Robo3 acts only through Robo2 in this developmental context, but has no Slit-dependent signalling activity independent of Robo2. This indicates that Robo3A.1 does not serve as an attractive receptor. Furthermore, our observations after combined knockdown of sim1a and arnt2
and dcc suggest that attraction mediated by Dcc is the driving force that directs otpb:gfp longitudinal axons towards the midline. This finding relates to our previous results demonstrating that knockdown of dcc in ast mutants restores mediolateral positioning of DA longitudinal axons (Kastenhuber et al., 2009). Our data indicate that Robo3a.1 inhibits Robo2-mediated repulsion and that attraction by Dcc then directs HTS longitudinal axons towards the midline.

**Fig. 11. Model for control of Robo2 activity during longitudinal HTS pathfinding.**
(A) During early stages, Robo3a.1 reduces repulsive activity of Robo2, while HTS axons navigate through areas of high slit expression levels in mid- and hindbrain. Double-headed arrow indicates potential Robo2/Robo3a.1 interaction domains. (B) Sim1a and Arnt2 negatively regulate robo3a.1 at later stages when HTS axons have passed regions of high slit expression. Lateral positioning in the spinal cord is then controlled by full activity of Robo2/Slit signalling. (C) Model of abnormal HTS axon trajectories in Arnt2 and Sim1a loss-of-function embryos: ‘attenuation’ of Robo/Slit signalling activity persists when axons travel through hindbrain because Robo3a.1 downregulation fails to occur after Arnt2/Sim1a depletion. As a result, HTS axons receive less Robo/Slit signalling activity and are medially displaced. For conserved Robo3 domains, see key. DC, diencephalon; MB/HB, midbrain-hindbrain; sc, spinal cord.
How does Robo3A.1 interfere with Robo2 activity? Our results on the mis-expression of different combinations of N- and C-terminal zebrafish and mouse Robo3a and Robo3b isoforms in Hcrt neurons suggest that the N-terminal domain present in Robo3A is important for inhibiting Robo2 activity during HTS axon guidance. A recent study demonstrated that different C-terminal domains present in mouse Robo3A.1 and Robo3A.2 contribute to distinct aspects of commissural axon guidance (Chen et al., 2008). Robo3A.1 allows midline crossing by potentially blocking activity of Robo1 and Robo2, whereas Robo3A.2 may prevent re-crossing by collaboration with Robo1 and Robo2. How can the observed differences be explained? Commisural and ipsilateral axon guidance mechanisms use similar sets of guidance cues, but respond with different projections (for a review, see Evans and Bashaw, 2010a). Thus, it is conceivable that DA and otpb:gfp-positive longitudinal growth cones, in contrast to commisural neurons, use a distinct pathway involving solely the N-terminal domain found in Robo3A isoforms to attenuate Robo2 activity. In Drosophila, different functions of Robo2 and Robo3 are specified by their ectodomains, and do not reflect differences in cytoplasmic signalling (Evans and Bashaw, 2010b; Spitzweck et al., 2010). Alternatively, as we failed to identify different C-terminal robo3 isoforms in zebrafish (data not shown), it is also possible that zebrafish solely use the N-terminal Robo3a.1 and Robo3b.1 isoforms to control different aspects of axon guidance. In addition to the results presented here, a role for Robo3a.1 and Robo3b.1 during axon guidance, has also been suggested by others (Challa et al., 2005; Devine and Key, 2008). Our findings indicate that inhibition of Robo2 activity via the N-terminal domain present in Robo3A isoforms occurs extracellularly during pathfinding of HTS axons. However, the precise molecular interactions remain elusive. The Robo3A specific domain could interfere with Robo2 by direct physical interaction, and thereby mask the Slit-binding domain of Robo2 mapped within the immunoglobulin-like domains 1 and 2 (Hohenester, 2008). Alternatively, repulsion by Robo2 may be inhibited by ectodomain-dependent heterodimerisation of Robo3A.1 and Robo2 in a Slit-independent fashion, similar to findings in Drosophila (Evans and Bashaw, 2010b).

What is the role of Robo3A.1 during longitudinal pathfinding? Lateral positioning of longitudinal axons is primarily governed by repulsion through midline derived Slit proteins (Farmer et al., 2008; Kastenhuber et al., 2009). In zebrafish, the four known Slit genes display strong expression at the midline of the midbrain and hindbrain, and lower expression within the spinal cord, suggesting different Slit levels (supplementary material Fig. S10) (Hutson et al., 2003; Yeo et al., 2001). Therefore, in order to maintain constant mediolateral positioning in regions with different Slit levels, the growth cones of DA and otpb:gfp-positive longitudinal axons may have adopted a mechanism to modulate Slit-dependent repulsion from the midline (Fig. 11). Robo3A.1 may contribute to this mechanism by reducing the activity of Robo2 and thus attenuating repulsion by Slit proteins. The Robo3A.1 dependent ‘attenuation’ mechanism may then enable the navigation of longitudinal otpb:gfp or DA growth cones to keep lateral positioning in the mid- and hindbrain despite high expression levels of slits (Fig. 11A, A’). Sim1a and Arnt2 contribute to this mechanism by controlling negative regulation of robo3a.1 in a stage-dependent manner synchronized with progress of axonogenesis. Complete downregulation of robo3a.1 then leads to full activation of Robo2, which allows for proper lateral positioning within the lower slit levels expressed in the spinal cord (Fig. 11B, 11B’). In arnt2 or sim1a loss-of-Function embryos, stage-dependent negative control of robo3a.1 does not occur. Therefore, Robo3a.1 is still present and inhibits Robo2 activity when longitudinal axons approach the spinal cord. When Robo2 activity is severely attenuated by Robo3a.1 overexpression, repulsion and attraction are imbalanced and longitudinal growth cones are directed towards the midline by Dcc/Netrin-mediated attraction (Fig. 11C, 11C’).

In summary, we have shown that Arnt2 and Sim1a transcription factors contribute to proper positioning of HTS longitudinal axon tracts. Sim1a and Arnt2 perform this function by negative regulation of robo3a.1 transcription at a specific stage of neuroendocrine and DA cell differentiation. Precise control of robo3a.1 expression is essential for proper longitudinal tract formation, as Robo3A.1 attenuates Robo2 activity to adjust lateral positioning within different Slit levels. Thus, our findings reveal a novel mechanism to ensure correct lateral positioning of longitudinal projections navigating through changing signalling environments.

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References


