Supplemental material

Netrin-DCC, Robo-Slit and HSPGs coordinate lateral positioning of dopaminergic diencephalospinal axons

Edda Kastenhuber, Ursula Kern, Joshua L. Bonkowsky, Chi-Bin Chien', Wolfgang Driever and Joern Schweitzer

Supplemental Text

Development of brain and axon tracts is grossly normal in *ast*

Early axonal scaffold development studies suggest that early-born neurons and their axons serve as guides for later neurons and their processes (for example see Pike et al., 1992; Pittman et al., 2008). Early DA axons projecting into the hindbrain grow in close proximity with the axons of the nucMLF (Fig. S1 A-C). The nucMLF neurons are among the first differentiating neurons in zebrafish sending out their axons into the hindbrain prior to 16 hpf (Hjorth and Key, 2002), and thus shortly before DA axonogenesis starts. Pathfinding errors observed in the Robo2 deficient mutants might therefore be the result of misguided pioneer axons of the nucMLF. To test this possibility, we inspected the morphology of the nucMLF neurons and their axons using acetylated tubulin antibody at 24 hpf. The nucMLF neurons of *ast* mutants (n=12) appeared normal compared to *ast/+* embryos (n=12) (Fig. S2A, B). Their longitudinally projecting axons displayed normal trajectories, indicating that the observed phenotypes of longitudinal THir axons in *ast* mutants are not a secondary consequence of misguided nucMLF axons.

robo2 is strongly expressed in the hindbrain during the phase of THir longitudinal tract formation (Fricke et al., 2001; Lee et al., 2001). Thus the observed phenotypes in *ast* mutants may be due to altered hindbrain structures, which may affect lateral positioning

of DA axons in a non-specific manner. We therefore inspected axonal outgrowth of neuronal populations, whose axons navigate in close proximity or through the same environment when compared to longitudinal DA tracts. The morphology of Mauthner and other reticulospinal neurons and their axons as demonstrated by 3A10 labeling at 72 hpf appeared to be normal in *ast* mutants (n=13; Fig. S2D) compared to *ast/+* embryos (n=12; Fig. S2C). Pathfinding of commissural hindbrain neurons in *ast* embryos (n=10; Fig. S2F) as revealed by zn5 labeling at 72 hpf was indistinguishable from *ast/+* embryos (n=9; Fig. S2E).

To control for normal DA neuronal specification in *ast* embryos we performed *th in situ* hybridization at 96 hpf which indicated that all DA groups in the ventral diencephalon are present in *ast* mutants (n=18; Fig. S2H) compared to *ast/+* embryos (n=16; Fig. S2G) demonstrating a normal development of DA neurons. In addition *ast* homozygotes develop to adulthood and are fertile indicating no gross developmental abnormalities (Campbell et al., 2007). These observations demonstrate that differentiation of DA neurons is unaffected in *ast* mutants. Furthermore, the observed pathfinding defects of DA neurons are not due to misguided pioneer axons or altered hindbrain structures in *ast* mutants.

Abnormal projections in *ast* mutants are of dopaminergic origin

In zebrafish the noradrenergic LC neurons start to express *th* at 24 hpf (Holzschuh et al., 2001). The LC neurons were shown to have ascending projections, which are associated with the MLF (Ma, 1994). In addition LC neurons project into the spinal cord (Ma, 1994). Since TH immunohistochemistry reveals both DA and NA neural circuitry we cannot exclude that NA axons from the LC contribute to the phenotypes we observe in *ast* mutants. To test for this, we used morpholino (MO)-antisense-oligo-mediated knockdown of *tfap2a* to prevent NA neuron differentiation. Tfap2a is a transcriptional activator required by all zebrafish hindbrain NA neurons to establish their noradrenergic identity (Holzschuh et al., 2003). Injection of 4.5 ng *tfap2a* MO into *ast* (n=16) or WT (n=12) embryos completely inhibited the formation of TH positive NA neurons in the LC (Fig.S3 C, G) and the medulla oblongata (data not shown) as revealed by TH immunohistochemistry. Elimination of NA neurons and their axonal projections in the *ast*

mutant background did not alter the observed THir projection phenotype in all inspected embryos. Pathfinding of THir longitudinal projections in *ast* embryos injected with either 4.5 ng control MO (n=14; Fig. S3A) or 4.5 ng *tfap2a* MO (n=16; Fig. S3C) did not show any obvious differences. Comparable amounts of TH positive fibers growing towards and occasional aberrantly crossing the midline were detectable in both *tfap2a* and control MO injected *ast* embryos. To control for any possible effect of *tfap2a* MO injection on DA axonal pathfinding, we examined development of DA longitudinal tracts in WT embryos injected with 4.5 ng *tfap2a* MO. Removal of NA neurons in WT (n=12; Fig. S3G) embryos does not affect longitudinal DA axon tract formation as compared to control MO injected WT embryos (n=10; Fig. S3E). To control for proper development of hindbrain structure s after injection of *tfap2a* MO, we inspected the morphology of reticulospinal Mauthner cells and their axons using 3A10 antibody (Hatta, 1992). This revealed normal development of Mauthner neurons and their axons (Fig. S3 B, D, F, H). Taken together these experiments indicate that the observed pathfinding defects in *ast* mutants are mainly of dopaminergic origin. In addition these experiments demonstrate that the majority of catecholaminergic circuitry innervating the spinal cord of zebrafish is dopaminergic.

Robo1 and Robo3 do not contribute to lateral positioning of longitudinal DA axons

Although our *in situ* hybridization analysis demonstrated only *robo2* expression in DA neurons in the ventral diencephalon, it is still possible that expression levels of other *robo* family members were below detection. Previous work has shown that Robo2 together with Robo1 are required for medial longitudinal fascicle formation (Farmer et al., 2008). Whether Robo3, which is required for proper midline crossing of commissural axons (Chen et al., 2008; Burgess et al., 2009), is also required for longitudinal tract formation has not been analyzed. We therefore decided to test for a potential requirement of Robo1 and Robo3, in addition to Robo2, during lateral positioning of longitudinal DA tracts *in vivo*. We first examined longitudinal tract formation of THir axons in WT embryos injected with 6,5 ng *robo1* MO (n=13) or in *twitch twice*/*robo3* (twt^{tw204}) mutants (n=7) at 48 hpf and 72 hpf respectively. Analysis of TH immunohistochemistry by confocal microscopy revealed normal lateral positioning of THir longitudinal tracts when

compared to twt^{tw204}/+ (n= 8) or WT embryos injected with 6,5 ng control MO (n=8) (data not shown). Next we analyzed longitudinal tract formation of THir axons in *ast*^{ti272}/*ast*^{ti272} mutant and *ast*^{ti272}/*ast*^{ti272}; *twt*^{tw204}/*twt*^{tw204} double mutant embryos injected with 6,5 ng *robo1* MO or control MO respectively. If repulsion by Robo1 and/or Robo3 function were additionally required to define lateral positioning of THir longitudinal tracts, we would expect a stronger phenotype when compared to *ast*^{ti272}/*ast*^{ti272} embryos only. Embryos at 72 hpf were labeled by TH immunohistochemistry and examined by confocal microscopy. This analysis demonstrated that a reduction of *robo1* gene function in *ast*^{ti272}/*ast*^{ti272} (Fig. S4B, n = 10) or *ast*^{ti272}/*ast*^{ti272}; *twt*^{tw204}/*twt*^{tw204} (Fig. S4D, n = 8) embryos did not yield a stronger phenotype when compared to *ast*^{ti272}/*ast*^{ti272} (Fig. S4A, n = 8) or *ast*^{ti272}/*ast*^{ti272}; *twt*^{tw204}/*twt*^{tw204} (Fig. S4C, n = 6) embryos injected with control MO. In *ast*^{ti272}/*ast*^{ti272}; *twt*^{tw204}/*twt*^{tw204} embryos injected with control MO (Fig. S4C) or *ast*^{ti272}/*ast*^{ti272} (Fig. S4B) and *ast*^{ti272}/*ast*^{ti272}; *twt*^{tw204}/*twt*^{tw204} (Fig S4D) embryos injected with *robo1* MO the gradual shifting of THir axons towards the midline or midline crossing is not enhanced when compared to *ast*^{ti272}/*ast*^{ti272} (Fig. S4A) embryos injected with control MO. These data, together with our expression analysis of *robo* family members in ventral diencephalic DA neurons, indicate that lateral positioning of longitudinal DA tracts is primarily specified by Robo2 function.

dcc or *netrin1* knockdown does not affect formation of DA neurons or overall axon guidance in the hindbrain

Although longitudinal DA tract formation in *ast* embryos is severely affected, some DA axons in the hindbrain still grow on their normal tracts towards the spinal cord (for example see Fig. 5B and S5B). Thus it is conceivable that the observed “rescue” in *ast* embryos could reflect the presence of these normal growing axons whereas misguided DA axons are not detectable due to a possible developmental delay by MO injections. For the following 4 reasons we do not think that this is the case. First, the overall morphology of *dcc*-MO1, *dcc*-MO2 and *ntn1a*/*ntn1b* MO injected embryos was grossly similar to WT and control morphants at 48 hpf (data not shown). Second, the formation of the MLF as revealed by acetylated-tubulin labeling was indistinguishable between *ast* embryos

injected with 4.5 ng *dcc*-MO1 (n=12), *ntn1a* and *ntn1b* MOs 4.5 ng each (n=12) and control morphants (n=12) at 24 hpf (Fig. S7A-C). Third, midline crossing of the axons of Mauthner neurons (MA) in the hindbrain as demonstrated by 3A10 labeling at 48 hpf showed no gross differences between *ast* embryos injected with either 4.5 ng *dcc*-MO1 (n=35, 100 % of the analyzed MA axons cross the midline), 4.5 ng each *ntn1a* and *ntn1b* MOs (n=27, 84 % of the analyzed MA axons cross the midline), and 9 ng control MO (n=25, 100 % of the analyzed MA axons cross the midline) (Fig. S7D-F), indicating normal development of the hindbrain. Fourth, to exclude that morpholino treatments affect formation of DA neurons in the ventral diencephalon and thereby may alter axonal pathfinding of THir longitudinal tracts in the hindbrain we detected *th* expression by *in situ* hybridization and counted DA neurons at 48 hpf. The number of DA neurons remained unchanged in *ast* embryos injected with 4.5 ng *dcc*-MO1 (n=15, average 42 ± 3,5 *th* positive cells) or 4.5 ng of each *ntn1a* and *ntn1b* MOs (n=15, average 44 ± 5,1 *th* positive cells) compared to 9 ng control MO (n=15, average 40 ± 4,2 *th* positive cells) or uninjected *ast* embryos (n=15, average 43 ± 2,7 *th* positive cells) (Fig. S7G-I and J). Thus, morpholino treatment does not affect DA neuron formation in the ventral diencephalon. Taken together these controls indicate that the restoration of longitudinal THir tracts in the hindbrain of *ast* is not a secondary consequence due to unspecific effects of MO injections but rather an effect of reduced attractive DCC/Netrin signaling.

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Supplemental Figure Legends

Figure S1. Longitudinal DA axons grow in close proximity to the MLF

Dorsal views of confocal z-projections of brain and hindbrain regions of 24 hpf embryos

(A-C) and 72 hpf embryos (D, E) are shown. Anterior to left. (A-C) In WT embryos TH positive axons (arrows in A) derived from neurons in the ventral diencephalon do not grow within the medial longitudinal fascicle (B) as demonstrated by the merged image in C (arrows in C indicates the position of TH positive axons which may be also positive for acetylated tubulin). The nucleus of the MLF (nucMLF) and the MLF are indicated in B. (D) Pathfinding of longitudinal DA axons (arrowheads) in the hindbrain of *ast*$^{te284/+}$ embryos is normal. (E) In *ast*^{te284} embryos lateral positioning of longitudinal DA axons is affected. Instead of retaining their lateral positions, axons grow towards the midline (arrows). Asterisks indicate LC neurons. Scale bar in A = 50 µm for A-C, in D = 50 µm for D and E.

Figure S2. Development of the brain and of hindbrain axon tracts is grossly normal in *ast*

A, B Single optical image planes showing the hindbrain of flat mounted embryos are shown; C-F Z-projections of confocal image stacks of the hindbrain are shown; G, H Z-projection of three pictures each at different focal planes of the posterior tuberculum and hypothalamus are shown. The developmental stages are indicated. Anterior to left. (A-H) Heterozygous *ast* embryos (A, C, E, G) were not different from *ast* mutants (B, D, F, H) in acetylated tubulin immunohistochemistry (A, B), 3A10 immunohistochemistry (C, D), zn5 immunohistochemistry (E, F) or *tyrosine hydoxylase* in situ hybridization (G, H). (A, B) The nucMLF (arrow) in the midbrain and the MLF axons (arrowheads) growing through the hindbrain are indicated. (C, D) The arrowhead indicates Mauthner neurons and the asterisk indicates Mauthner axons crossing the midline. White arrows point to reticulospinal axons. (E, F) Arrows indicate commissural tracts derived from hindbrain neurons. (G, H) Arrows indicate *th* positive cells. Numbers 1-7 indicate DA subgroups in the ventral diencephalon: group 1 of ventral thalamus, groups 2 and 4 of posterior tuberculum and groups 3, 5, 6, 7 of hypothalamus. LC; locus coeruleus. Scale bar in A = 100 µm for A and B, in C = 50 µm for C-F, in G = 50 µm for G and H

Figure S3. Abnormal projections in *ast* mutants are dopaminergic

Dorsal views of z-projections of the hindbrain of 60 hpf *ast* (A-D) or WT embryos (E-H) co-labeled with TH and 3A10 antibody are shown. Anterior to left. (A, C) Abnormal TH positive longitudinal projections in the hindbrain were similar in control MO (arrows in A) and *tfap2a* MO (arrows in C) injected *ast* embryos lacking noradrenergic neurons. (E, G) Injection of control (E) or *tfap2a* MO (G) does not lead to pathfinding errors of THir longitudinal axons in WT embryos (white arrowheads). (B, D, F, H) Midline crossing (asterisks) of reticulospinal Mauthner neurons (white arrows) in the same embryos as shown in A, C, E, G is unaffected by MO injections. Asterisks in A and E indicate noradrenergic LC neurons. Plus signs in C and G indicate the position were LC neurons would have been located. Scale bar in A = 50 µm for A-H.

Figure S4. Robo1 and Robo3 do not contribute to lateral positioning of longitudinal dopaminergic tracts in the hindbrain

Zebrafish embryos at 72 hpf were labeled with anti-TH. Confocal z-projections of dorsal views are shown, anterior at left. (A-D) Aberrant pathfinding of TH positive longitudinal projections were similar in *ast* or *ast*;*twt* embryos injected with either control or *robo1* MOs. Asteriks indicate noradrenergic LC neurons, white arrows point to axons shifted towards the midline and arrowheads indicate THir axons crossing the midline. Scale bar in A = 50 µm for A-D.

Figure S5. Ubiquitous *slit2* expression in heat shock treated *hsp70lslit/+* embryos exceeds levels of endogenous midline *slit2* expression and leads to abnormal pathfinding of Mauthner axons

Dorsal views of single image planes of 30 hpf (A-D) or z-projections of confocal images of 38 hpf (E and F) embryos at the hindbrain level are shown. Anterior to the left. (A-D): Detection of *slit2* expression levels by whole mount in situ hybridization. (A, C) In WT embryos *slit2* expression is detected at the midline (arrow in C). (B, D) In *hsp70l:slit2gfp/+* embryos heat shocked at 16- and 26 hpf to induce ubiquitous *slit2* expression levels are stronger than endogenous slit2 expression at the midline. In C and D higher magnifications of the midline region framed in A and B are shown. In the red framed areas in C and D brightness was identically adjusted using “levels” tool in

Photoshop to reveal structures in D. (E, F) Immunohistochemistry for 3A10 antibody indicates abnormal pathfinding of reticulospinal Mauthner neurons in *hsp70l:slit2gfp/+* embryos. Asterisks indicate Mauther neurons, which show normal projection behavior (white arrowheads in E and F). Plus sign indicates a Mauthner neuron abnormally projecting anteriorly (arrow in F). Scale bar in A = 50 µm for A and B, in C = 100 µm for C and D, in E = 50 µm for E and F.

Figure S6. Knockdown of *netrin1* restores lateral positioning of longitudinal DA tracts in *ast*

Dorsal views of confocal z-projections of the hindbrain labeled with TH antibody at 48 hpf are shown. Anterior to left. (A) Formation of DA longitudinal tracts (arrows) is unaffected in WT embryos injected with 9 ng control MO. (B) *ast* embryos injected with 9 ng control MO show TH positive axons projecting towards and crossing the midline (arrowheads). The arrow in B indicates some TH positive axons growing at their normal positions. (C, D) Injection of 4,5 ng *ntn1a* and *ntn1b* MOs each leads to a rescue of the *ast* phenotype. Instead of turning towards the midline after the level of the LC, THir longitudinal axons project in a WT-like fashion (arrows in C, D). Note that some axons still ectopically cross the midline (small arrow in D). (E) Injection of 4,5 ng *ntn1a* MO alone also causes a rescue of THir longitudinal tracts (arrows) with some axons still ectopically crossing the midline (small arrow). (F) Quantification of rescued *ast* embryos after *ntn1* MO injections. Asterisks indicate LC neurons. Scale bar in A = 50 µm for A-F.

Figure S7. *dcc* and *ntn1* morpholino knockdown experiments specifically affect DA axonogenesis but not DA neuron specification or formation of the general axonal scaffold.

Dorsal views of single optical sections of the midbrain-hindbrain region (A-C) or the ventral diencephalon (G-I) and z-projections of confocal image stacks of the hindbrain (D-F) of *ast* embryos at the indicated stages are shown. Anterior to left. (A-F) Control morphants (A and D) were not different from *dcc*-MO1 (B and E) and *ntn1a*/*ntn1b* MO (C and F) injected embryos with respect to general axonal scaffold (visualized by anti acetylated tubulin immunohistochemistry; A-C) and Mauthner neuron reticulospinal

projections (visualized by 3A10 immunohistochemistry; D-F). (A-C) The nucMLF in the midbrain and the MLF axons growing through the hindbrain are indicated. (D-F) The arrowheads indicate Mauthner neurons and the arrows points to Mauthner axons crossing the midline. (G-J) Quantification of *tyrosine hydoxylase* expressing cells demonstrated no developmental differences of DA neurons in the diencephalon between control MO (G), *dcc*-MO1 (H) and *ntn1a*/*ntn1b* MO (I) injected *ast* embryos (J). Arrows in G-I indicate DA neurons in the ventral diencephalon, arrowheads in G-I point to NA LC neurons. Scale bar in A = 50 µm for A-C, in D = 50 µm for D-F, in G =100 µm for G-I