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Dev Dyn. Author manuscript; available in PMC 2008 December 30.

Published in final edited form as:
Dev Dyn. 2005 November ; 234(3): 726–731. doi:10.1002/dvdy.20474.

Identification of New Netrin Family Members in Zebrafish: Developmental Expression of *netrin2* and *netrin4*

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Abstract

Netrin1 is a diffusible factor that attracts commissural axons to the floor plate of the spinal cord. Recent evidence indicates that Netrin1 is widely expressed and functions in the development of multiple organ systems. In mammals, there are three genes encoding Netrins, whereas in zebrafish, only the Netrin1 orthologs *netrin1a* and *netrin1b* have been identified. Here, we have cloned two new zebrafish Netrins, *netrin2* and *netrin4*, and present a comparative sequence and expression analysis. Despite significant sequence similarity with *netrin1a/netrin1b*, *netrin2* displays a unique expression pattern. *Netrin2* transcript is first detected in the notochord and in developing somites at early somitogenesis. By late somitogenesis, *netrin2* is expressed in fourth rhombomere and is subsequently expressed in the hindbrain and otic vesicles. In contrast, *netrin4* is detected only at very low levels during early development. The nonoverlapping expression patterns of these four Netrins suggest that they may play unique roles in zebrafish development.

Keywords

axon guidance; notochord; otic vesicle; rhombomere; pectoral fins; somites; hindbrain; tectum

INTRODUCTION

The formation of neuronal connections requires both attractive and repulsive guidance cues that direct growth cones to their appropriate targets. The Netrins are diffusible guidance molecules that can either attract or repel growth cones, depending on the constellation of receptors on the target axon. They are evolutionarily conserved vertebrate homologs of the *Caenorhabditis elegans* UNC-6 protein that is involved in axon guidance and cell migration (Livesey, 1999). Whereas *unc-6* mutations disrupt the circumferential projection of growth cones along the dorsoventral axis of the *C. elegans* body wall (Hedgecock et al., 1990; Ishii et al., 1992), mutations in murine and *Drosophila* netrins cause defects in commissural axon guidance to the ventral midline of the central nervous system (CNS; Serafini et al., 1994; Harris et al., 1996; Mitchell et al., 1996). In addition to their roles in nervous system development, the Netrins appear to play roles in the development of other organ systems. Netrin1 regulates

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The Supplementary Material referred to in this article can be found at http://www.interscence.wiley.com/jpages/1058-8388/suppmat

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ear development in the mouse, as revealed by the examination of Netrin1 gene trap mice (Salminen et al., 2000). Recent evidence indicates that Netrin1 also regulates mammary gland morphogenesis as well as promoting angiogenesis both in vitro and in vivo (Srinivasan et al., 2003; Lu et al., 2004; Park et al., 2004). Netrin1 has also been implicated in the regulation of cell survival and tumorigenesis (Arakawa, 2004; Mazelin et al., 2004).

Netrin family proteins are characterized by two extracellular laminin-like domains (VI and V) and a highly basic region in the C-terminus of the protein. Laminin-like domains VI and V have been implicated in receptor interaction (Keino-Masu et al., 1996; Kruger et al., 2004), while recent evidence suggests that the C-terminal basic region also mediates interaction with integrin receptors to modulate pancreatic epithelial cell development (Yebra et al., 2003).

Two classes of receptors have been identified that recognize Netrins. The DCC (Deleted in Colorectal Cancer) family includes DCC and Neogenin in vertebrates, UNC-40 in *C. elegans*, and Frazzled in *Drosophila melanogaster*. The second family, the Unc5 receptors, includes vertebrate UNC5A/B/C/D (or UNC5H1/2/3/4), which are homologs of the *C. elegans* UNC-5 receptor. Ablation of the DCC receptor results in compromised axonal attraction (Fazeli et al., 1997), whereas loss of Neogenin is associated with impaired mammary gland ductal formation (Srinivasan et al., 2003). Studies in *C. elegans* showed that the UNC-5 receptor mediates repulsive guidance activities of Netrin (Hedgecock et al., 1990; Hamelin et al., 1993). Mutation of murine Unc5b leads to vessel branching defects during vascular development (Lu et al., 2004). In vertebrates, complex formation between DCC and Unc5b converts Netrin1-induced growth cone attraction to repulsion (Hong et al., 1999).

Three secreted members of the Netrin family have been identified to date in human and mouse: Netrin1, Netrin2-like (called Netrin3 in mouse), and Netrin4 (beta-Netrin) (Serafini et al., 1996; Wang et al., 1999; Koch et al., 2000; Yin et al., 2000). Mammalian Netrin2-like is so named because it is most similar to, but somewhat divergent from, chick Netrin2. Because all Netrin family members promote axonal outgrowth in in vitro assays, it is likely that there are functional redundancies among the Netrins. Apart from Netrin1, the specific roles of the other Netrins remain unknown and await combinatorial gene inactivation studies. In zebrafish, there are two Netrin1 orthologs, *netrin1a* (Lauderdale et al., 1997) and *netrin1b* (originally named *net1*; Strähle et al., 1997). These two genes are primarily expressed near the ventral midline of the CNS, throughout the length of the rostro-caudal axis. Here, we report the cloning and characterization of two new zebrafish Netrin family members, *netrin2* and *netrin4*. We compared the transcript distributions of all four zebrafish Netrins in developing embryos. Although *netrin2* and *netrin4* possess sequence and structural similarities to *netrin1a* and *netrin1b*, their expression patterns are markedly different, suggesting unique roles for these genes during development.

RESULTS AND DISCUSSION

Identification of *netrin2* and *netrin4*

To investigate whether additional Netrin homologs exist in zebrafish, database mining was performed with zebrafish Netrin1a and Netrin1b amino acid query sequences. We identified an expressed sequence tag (EST) clone (accession no. AL916140) that shows significant similarity to mammalian Netrin2-like. A genomic database search (www.sanger.ac.uk/projects/D_rerio) further revealed zebrafish clones homologous to mammalian Netrin4. Complete cDNA sequences for each of these genes were obtained by RACE polymerase chain reaction and nested polymerase chain reaction (PCR). The extended cDNA sequences were shown to contain full-length open reading frames, based on protein sequence alignments, the presence of upstream in-frame stop codons and an N-terminal signal peptide. ClustalW alignments and phylogenetic analysis demonstrated that the predicted

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protein sequences of *netrin2* and *netrin4* represent Netrin family members with the highest degree of similarity to chick Netrin2 and mouse Netrin4, respectively (Fig. 1A, B). Zebrafish *netrin2* and *netrin4* encode the typical domains of Netrin family members: an N-terminal laminin-like domain VI followed by a laminin-like domain V, and an integrin-interacting basic region (domain C) at the C-terminus (Fig. 1C). Zebrafish genomic BLAST searches indicate that *netrin2* and *netrin4* map to linkage group 14 and 7, respectively. Together, sequence similarities and the presence of Netrin-specific domains indicate that these proteins represent new zebrafish orthologs of Netrin2 and Netrin4.

Developmental Expression Patterns

To gain insight into the expression profiles of these genes during zebrafish development, reverse transcriptase-PCR (RT-PCR) analyses were performed with total RNA from 24, 36, 48, and 72 hpf embryos. *Netrin1a*, *netrin1b*, and *netrin2* transcripts were detected in all four stages at relatively constant levels. *Netrin4* transcript was not detectable under the same conditions (Fig. 1D). *Netrin4* PCR product was detected only after extended amplification (data not shown). The relatively weak expression of zebrafish *netrin4* during early development is reminiscent of mouse Netrin4, further supporting the hypothesis that *netrin4* is a true Netrin4 ortholog (data not shown; Koch et al., 2000). Two zebrafish Netrin receptors, *dcc* and *neogenin1* (Hjorth et al., 2001;Shen et al., 2002; Fricke and Chien, manuscript submitted for publication) are expressed coincident with *netrin1a*, *1b*, and *2* during this developmental window (Fig. 1D).

We further investigated the expression pattern of all four zebrafish Netrin family members during embryonic development using RNA in situ hybridization (Fig. 2). *Netrin1a* and *netrin1b* transcripts are primarily detected in the developing CNS as previously reported (Lauderdale et al., 1997;Strähle et al., 1997). At 24 hours postfertilization (hpf), *netrin1a* is expressed in the brain, spinal cord, and somites. By 36 hpf, *netrin1a* is expressed in the optic nerve, and broadly in the ventral hindbrain and spinal cord. At 24 hpf, *netrin1b* is detected in the brain, the floor plate of the spinal cord, and the hypochord; at 30 hpf, it is expressed primarily in the floor plate of the hindbrain and spinal cord.

At 24 hpf, *netrin2* is expressed with remarkable specificity in hindbrain rhombomere 4, with weaker expression more posteriorly in the hindbrain as well as in ventral somites in the tail (Fig. 2I–L). Expression is also seen in a small cluster of cells in the developing otic vesicle (Fig. 2I). The hindbrain expression domain was identified as rhombomere 4 (Fig. 2K) using double staining with *krox20*, which is expressed in rhombomeres 3 and 5 (Oxtoby and Jowett, 1993). At 36 hpf, *netrin2* expression has extended anteriorly and posteriorly along the roof of the hindbrain and is present at the dorsal margin of the otic vesicle (Fig. 2M–O). Strong expression is evident in the ventromedial aspect of the otic epithelium by 36 hpf as well (Fig. 2N, O). *Netrin2* is also detectable in the notochord (Not) and spinal cord (SC) after long periods of colorimetric development (Fig. 2P). By 48 hpf and 72 hpf, *netrin2* expression is observed in expanded domains within the brain, otic vesicles, pharyngeal arches, and pectoral fin buds. Expression has extended anteriorly past the midbrain/hindbrain boundary around the medial and caudal margins of the tectum, and has extended posteriorly to the cerebellum and along the roof of the hindbrain (Fig. 2Q–V). Unlike the ventral expression of *netrin1a* and *1b*, *netrin2* expression in the hindbrain and midbrain is largely dorsal. *Netrin4* was not detected at any of these developmental stages, consistent with the RT-PCR results (data not shown).

To examine the onset of the zebrafish *netrin2* mRNA during early stages embryogenesis, embryos from cleavage through somitogenesis stages were collected and hybridized with *netrin2* riboprobe (Supplementary Figure S1, which can be viewed at http://www.interscence.wiley.com/jpages/1058-8388/suppmat). *Netrin2* mRNA was strongly detected in the notochord in the early somitogenesis staged embryos. *Netrin2* is also detected

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in developing somites (adaxial cells) in mid-somitogenesis embryos. Trunk expression is significantly reduced by 22 hpf. No signal was detected in cleavage, blastula, gastrula embryos, or in tail bud stage. Therefore, netrin2 expression commences by early somitogenesis stages. These data suggest that *netrin2* mRNA may play roles in early development even before axonal tracts and nerves are established.

How do the expression patterns of the zebrafish Netrins compare with those of their homologs in mouse and chick? Phylogenetic analysis shows that zebrafish *netrin2* is most similar to chick Netrin2 and somewhat less similar to mouse Netrin 3/human Netrin2-like (Fig. 1B). Chick *netrin2* is expressed in the ventral two thirds of the spinal cord and dermomyotome (Kennedy et al., 1994). Zebrafish *netrin2* is also detected in the spinal cord, albeit weakly, and ventrally in the caudal somites (Fig. 2L, P). In contrast, mouse Netrin3 is restricted to the sensory ganglia, mesenchymal cells, and muscles but is not expressed in the CNS during early mouse development (Wang et al., 1999). Furthermore, zebrafish *netrin2* is strongly expressed in the notochord and developing somites in early segmentation-staged embryos similar to the chick netrins (Supplemental Fig. 1). Thus, zebrafish *netrin2* is most closely related to chick Netrin2 both in sequence and in expression profile. Whether they share similar functions during development remains to be determined. Interestingly, mouse Netrin1 is expressed in the ear during development (Salminen et al., 2000), as is zebrafish *netrin2*, suggesting that they may have similar functional roles. The relatedness of mouse and zebrafish Netrin4 is supported by the finding that neither gene is expressed appreciably during early embryonic development.

Summary

Here, we have reported the identification of two new members of the Netrin family of secreted proteins, zebrafish *netrin2* and *netrin4*. Both *netrin2* and *netrin4* show significant differences in expression relative to the previously described zebrafish Netrins, *netrin1a* and 1b, which are far more robustly expressed in the spinal cord and trunk of developing embryos. These expression data suggest that each family member may play unique functional roles. In the future, it will be interesting to elucidate the divergent functions of these Netrin proteins during zebrafish development.

EXPERIMENTAL PROCEDURES

Cloning of Zebrafish *netrin2* and *netrin4*

A BLAST search of the zebrafish EST database with *netrin1a* and *1b* amino acid sequences yielded one sequence, AL916140. *Fugu rubripes netrin4* sequence was identified based on similarities with mouse Netrin4 (www.ensembl.org/Fugu_rubripes/). Genomic database searches (Sanger Institute) with the *Fugu netrin4* EST yielded a putative zebrafish *netrin4*. Total RNA was isolated from 24 hpf wild-type zebrafish embryos using TRIZOL (GIBCO-BRL) as previously reported (Lee et al., 2001). The RNA was reverse-transcribed with a RETRO-script kit (Ambion). RT-PCR was performed using primers spanning sequence from the clone AL916140 and from the genomic sequences for *netrin4*. These PCR fragments were extended by 5′ and 3′ rapid amplification of cDNA ends (RACE) using the BD SMART RACE cDNA amplification kit (BD Biosciences) according to the manufacturer's instructions. Amplified products were verified by sequence analysis. The sequences of the zebrafish *netrin2* and *netrin4* genes have been submitted to GenBank under the following accession numbers: zebrafish *netrin2*, AY928518; and zebrafish *netrin4*, AY928519.

RT-PCR Analysis

RNA was extracted from whole embryos staged at 24, 36, 48, and 72 hpf. Two micrograms of total RNA was reverse-transcribed using random hexamers provided with the SuperScript III kit (Invitrogen). The primer pairs for zebrafish β-actin were 5′-cccaaggccaacagggaaaa-3′

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(forward) and 5′-ggtgcccatctcctgctcaa-3′(reverse); *netrin1a*, 5′-tgacagatgcaaacctttcac-3′ (forward) and 5′-ggattcacagtctgatggctcttc-3′ (reverse); *netrin1b*, 5′-gcctgcgattgtcatcctgttgg-3′ (forward) and 5′-ccctctcttgtcgcgctgctgg-3′ (reverse); *netrin2*, 5′-tatagggacatgggcaaagc-3′ (forward) and 5′-ctcttttcagcgggtctcc-3′ (reverse); *netrin4*, 5′-ttttaaaaggaggctgcctgtg-3′ (forward) and 5′-gcatttgtctggatgactgagg-3′ (reverse) and 5′-acactgccaacactgtcagagc-3′ (forward) and 5′-gcctttatcatgagcacccaac-3′ (reverse); *dcc*, 5′-cagtttccaggtttcagggatg-3′ (forward) and 5′-ctggttcttgggaatggagttg-3′(reverse); *neogenin1*, 5′-gggcaactccaaagatctcaa-3′ (forward) and 5′-tgaaaggtcagtggtgcaaga-3′ (reverse). The following conditions were used for amplification of *netrin1a* and *netrin1b*: denaturation at 94°C for 30 sec, annealing at 67°C for 30 sec, and extension at 72°C for 60 sec, 25 cycles. The conditions used to amplify *netrin2*, *dcc*, and *neogenin1* were denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 60 sec, 25 cycles. The conditions used to amplify *netrin4* were denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec, and extension at 72°C for 60 sec, 25 cycles or 35 cycles.

Whole-Mount RNA In Situ Hybridization

Staging of embryos and whole-mount in situ hybridizations were performed as described previously (Lee et al., 2001). Briefly, antisense digoxigenin-labeled riboprobes were synthesized from the linearized plasmids encoding *netrin2* and *netrin4* cDNA. After in situ hybridization, images were taken using a Leica digital camera. Two-color in situ hybridization was performed as described previously with fluorescein- and digoxigenin-labeled probes (Jowett, 2001).

Acknowledgements

We thank Dr. Tatjana Piotrowski, Dr. Richard Dorsky, Arminda Suli, and Michael Jurynec for advice and technical support. The *krox20* plasmid was kindly provided by Dr. David Grunwald's laboratory. D.Y.L. was funded by the NIH and the American Cancer Society; K.W.P. and D.Y.L. were funded by the American Heart Association; and C.B.C. was funded by the NIH/NEI.

Grant sponsor: the NIH; Grant sponsor: American Heart Association; Grant sponsor: American Cancer Society; Grant sponsor: NIH/NEI.

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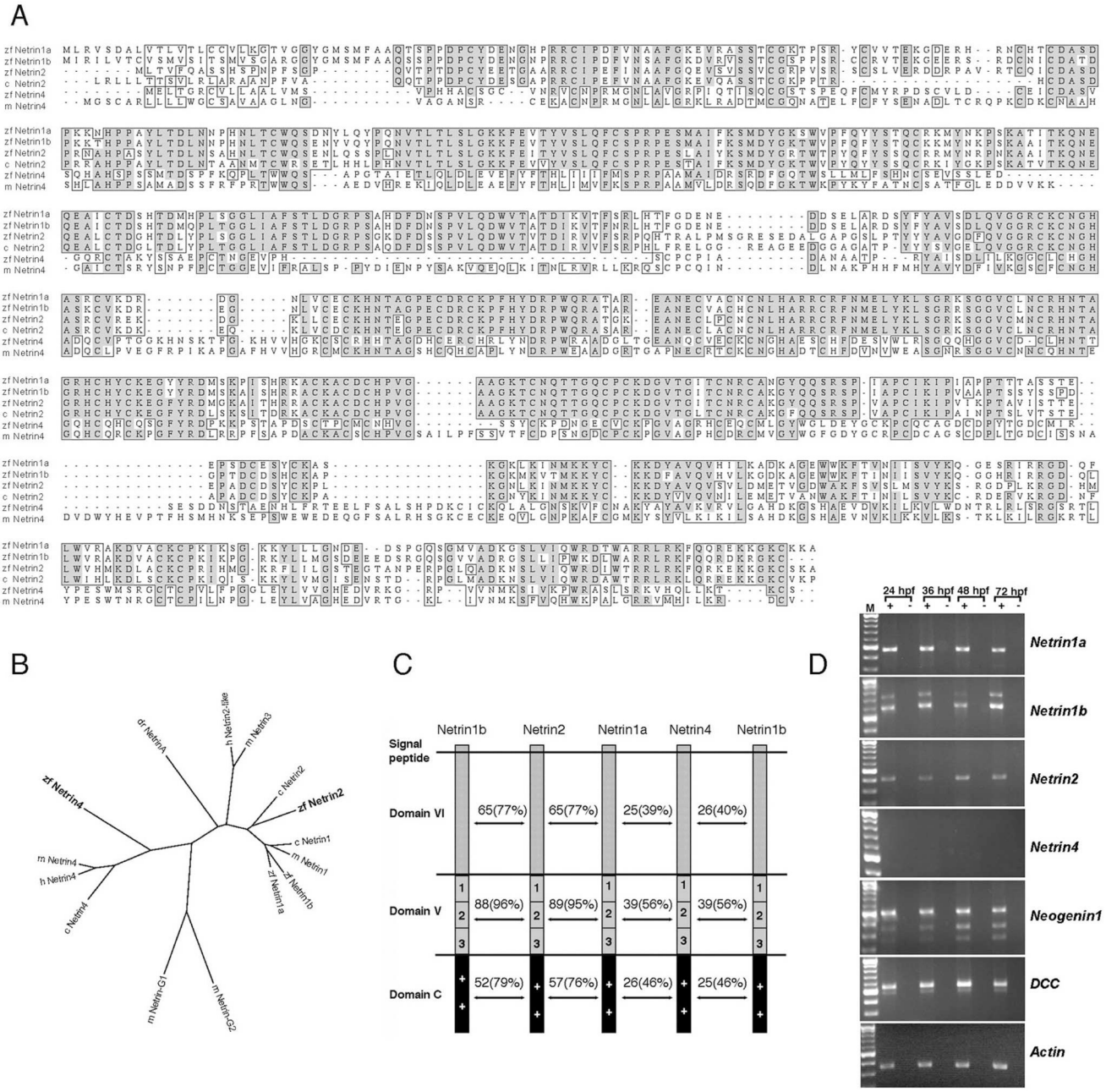

Fig. 1.

ClustalW amino acid alignment of Netrin sequences and reverse transcriptase-polymerase chain reaction (RT-PCR) expression analysis of Netrin and Netrin receptor genes. **A:** Alignment of chick Netrin2 and mouse Netrin4 with zebrafish Netrin2 and Netrin4. Dark and pale gray boxes represent, respectively, identical and similar amino acids. zf, zebrafish; m, mouse; c, chick. **B:** Phylogenetic analysis of predicted zebrafish Netrin proteins based on ClustalW alignment using the Biology Workbench site (http://workbench.sdsc.edu). zf, zebrafish; h, human; m, mouse; c, chick; dr, *Drosophila*. Zebrafish Netrin2 is most closely related to chick Netrin2, whereas zebrafish Netrin4 is closely related to human and mouse Netrin4. The GPI-linked proteins Netrin-G1 and -G2 are included for comparison. **C:** Zebrafish Netrin2 and Netrin4 contain all the domains (domains VI, V, and C) described in other Netrin

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family members. Numbers indicate identity, and parentheses represent similarities between two given proteins. Netrin2 is highly similar to Netrin1a and 1b, while Netrin4 is the most divergent member of the Netrin family. **D:** RT-PCR analyses were performed to detect *netrin* transcripts in total RNA collected from 24, 36, 48, and 72 hpf embryos. Actin amplification served as a loading control, and mock amplifications (−, no RT) confirmed that bands represent amplification of RNA. *Netrin2* is expressed from 24 hpf through 72 hpf, whereas *netrin4* is not detected at any of these stages (25 cycles). *Netrin1a*, *netrin1b*, *dcc*, and *neogenin1* are expressed at all stages.

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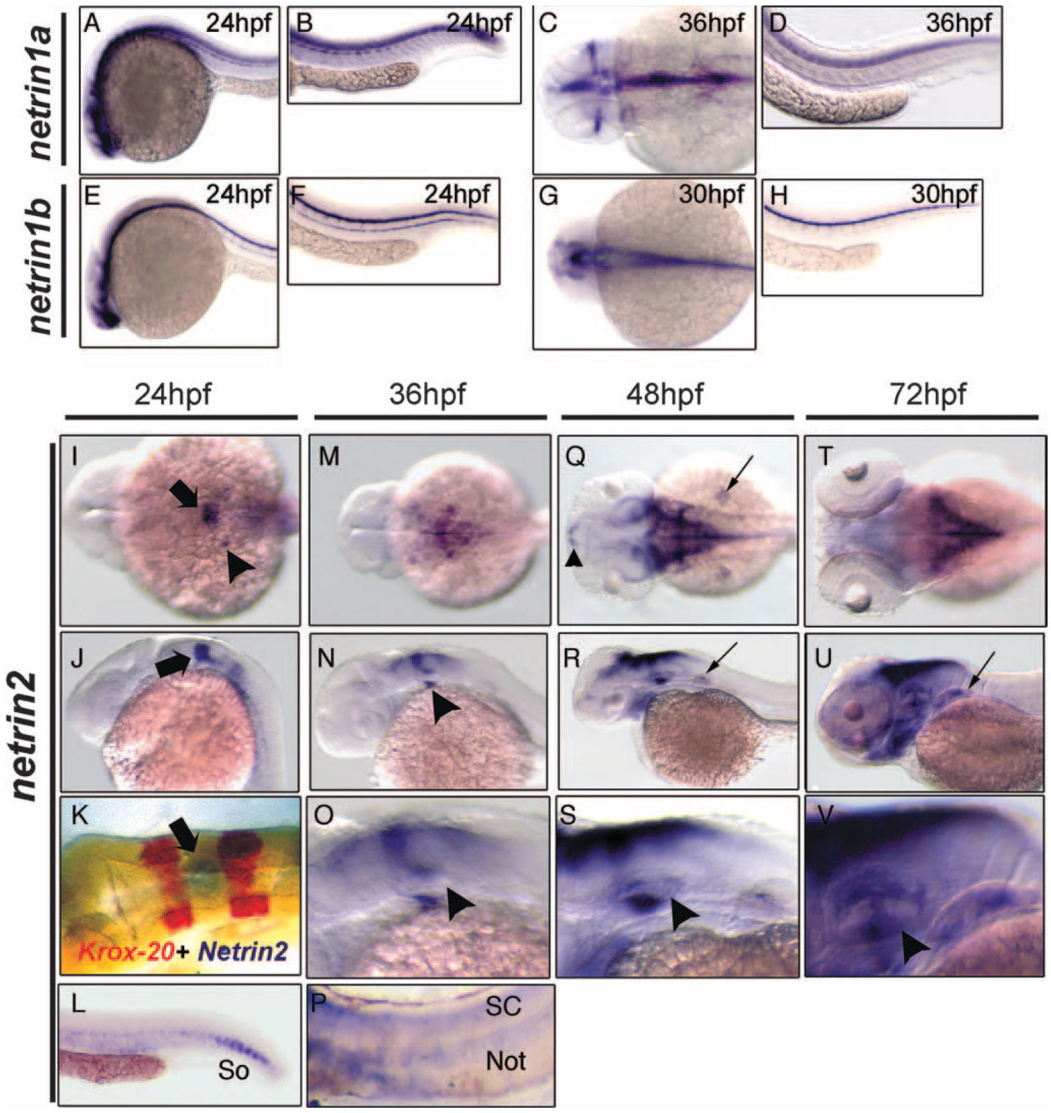

Fig. 2.

Netrin mRNA expression in zebrafish embryos. *Netrin1a*, *netrin1b*, and *netrin2* expression domains were analyzed by RNA in situ hybridization during the first 3 days of embryonic development. A–H: Lateral (A, B, D–F, H) and dorsal (C, G) views of *netrin1a* (A–D) and *netrin1b* (E–H). **A, B:** *netrin1a* is expressed in the brain, spinal cord, and somites at 24 hpf. **C, D:** By 36 hpf, somite expression has diminished and expression is visible in the optic nerve. **E, F:** *netrin1b* is detected in the ventral brain, the floor plate of the spinal cord, and the hypochord at 24 hpf. **G, H:** Hypochord expression diminishes by 30 hpf. I–V: Dorsal (I, K, M, Q, T) and lateral (J, L, N–P, R, S, U, V) views of *netrin2* expression at stages indicated. **I, J:** *netrin2* is expressed in the fourth rhombomere (arrow) and in the otic vesicle (arrowhead) at 24 hpf. **K:** *krox20* two-color in situ hybridizations confirm rhombomere 4 localization of *netrin2* (arrow). **L:** *netrin2* is expressed in the ventral somites (So) at 24 hours postfertilization (hpf). **M, N:** At 36 hpf, *netrin2* expression extends to the dorsal and ventral margins of the otic vesicle (arrowhead) and in the hindbrain region. **O:** Enlarged view of the hindbrain and otic vesicle (arrowhead). **P:** *netrin2* is also detected in notochord (Not) and spinal cord (SC) after prolonged colorimetric development. **Q, R:** At 48 hpf, *netrin2* is expressed in bilateral clusters

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in the telencephalon (small arrowhead), around the medial and caudal margins of the tectum, in the cerebellum, and along the dorsal hindbrain, and in the ventromedial margin of the otic vesicles. It is also expressed in the pharyngeal arches and pectoral fins (small arrows). In contrast to *netrin1a* and *1b*, expression in the brain is primarily constrained to the dorsal aspect. **S:** Higher magnification views of the hindbrain and otic vesicle; arrowhead denotes the strong expression in the ventral margin of the otic epithelium. **T, U:** By 72 hpf, expression in the pharyngeal arches is robust, and expression in the dorsal aspect of the otic epithelium and pectoral fins (small arrow) has strengthened. **V:** Higher magnification view of the otic vesicle; the arrowhead denotes the strong expression in the developing inner ear.