Netrin-3, a Mouse Homolog of Human NTN2L, Is Highly Expressed in Sensory Ganglia and Shows Differential Binding to Netrin Receptors

Hao Wang,¹ Neal G. Copeland,² Debra J. Gilbert,² Nancy A. Jenkins,² and Marc Tessier-Lavigne¹

¹Departments of Anatomy, and Biochemistry and Biophysics, Howard Hughes Medical Institute, University of California, San Francisco, California 94143-0452, and ²Mammalian Genetics Laboratory, Advanced Bioscience Laboratories Basic Research Program, Frederick Cancer Research and Development Center at the National Cancer Institute, Frederick, Maryland 21702

The netrins comprise a small phylogenetically conserved family of guidance cues important for guiding particular axonal growth cones to their targets. Two netrin genes, *netrin-1* and *netrin-2*, have been described in chicken, but in mouse so far a single netrin gene, an ortholog of chick *netrin-1*, has been reported. We report the identification of a second mouse netrin gene, which we name *netrin-3*. *Netrin-3* does not appear to be the ortholog of chick *netrin-2* but is the ortholog of a recently identified human netrin gene termed *NTN2L* ("netrin-2-like"), as evidenced by a high degree of sequence conservation and by chromosomal localization. *Netrin-3* is expressed in sensory ganglia, mesenchymal cells, and muscles during the time of peripheral nerve development but is largely excluded from the CNS at early stages of its development. The murine netrin-3

protein binds to netrin receptors of the DCC (deleted in colorectal cancer) family [DCC and neogenin] and the UNC5 family (UNC5H1, UNC5H2 and UNC5H3). Unlike chick netrin-1, however, murine netrin-3 binds to DCC with lower affinity than to the other four receptors. Consistent with this finding, although murine netrin-3 can mimic the outgrowth-promoting activity of netrin-1 on commissural axons, it has lower specific activity than netrin-1. Thus, like netrin-1, netrin-3 may also function in axon guidance during development but may function predominantly in the development of the peripheral nervous system and may act primarily through netrin receptors other than DCC.

Key words: *netrin-3*; axon guidance; sensory neurons; mouse chromosome 17; peripheral nervous system; DCC

An early step in the development of the nervous system is the guidance of axonal growth cones to their correct targets. Significant progress has been made in recent years in identifying molecules that guide axons in a variety of organisms, including nematodes, insects, and vertebrates. These molecules include members of the cadherin and immunoglobulin superfamilies, ephrins, semaphorins, and netrins, all of which appear to have phylogenetically conserved functions (Tessier-Lavigne and Goodman, 1996).

The netrins play important roles in guiding various classes of axons in all of these species. In particular, genetic and embryological studies have implicated netrins in attracting axons to the midline of the nervous systems of worms, flies, and vertebrates and in repelling other axons away from the midline, as well as in guiding other axons that do not navigate close to the midline (for review, see Tessier-Lavigne and Goodman, 1996; Culotti and Merz, 1998). The attractive effects of netrins appear to be mediated by receptors that are members of the DCC (deleted in colorectal cancer) subfamily of the immunoglobulin superfamily,

which include the UNC-40 protein of *Caenorhabditis elegans*, the Frazzled protein of *Drosophila*, and the DCC and neogenin proteins of vertebrates (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996; Fazeli et al., 1997). In the case of repulsion, genetic data implicate the UNC-5 protein of *C. elegans* as a strong candidate receptor involved in mediating repulsive actions of the netrin UNC-6 (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Hamelin et al., 1993). In vertebrates, the UNC-5 homologs UNC5H1, UNC5H2, and UNC5H3 are all netrin-binding proteins, consistent with the possibility that they too function as repulsive netrin receptors (Ackerman et al., 1997; Leonardo et al., 1997).

Within each species, netrins appear to be a small family. One netrin, the UNC-6 gene product, has been described in the nematode *C. elegans*, and two in *Drosophila* (Netrin-A and Netrin-B) (Ishii et al., 1992; Harris et al., 1996; Mitchell et al., 1996). In vertebrates, biochemical purification led to the identification of chick netrin-1 and netrin-2 (Serafini et al., 1994). Orthologs of chick *netrin-1* have been identified in other vertebrate species, including mouse (Serafini et al., 1996), *Xenopus* (de la Torre et al., 1997), zebrafish (Lauderdale et al., 1997; Strahle et al., 1997), and human (Meyerhardt et al., 1999). A netrin gene named *NTN2L* ("netrin-2-like") (van Raay et al., 1997) has been reported in human as well; it does not appear to be the strict ortholog of either chick *netrin-1* or chick *netrin-2*.

Here, we describe the cloning, expression, binding properties, and *in vitro* activities of a novel murine netrin, netrin-3, which is the ortholog of human *NTN2L*. Two properties of netrin-3 distinguish it from previously reported vertebrate netrins: its exclu-

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Correspondence should be addressed to Marc Tessier-Lavigne, Department of Anatomy, University of California, 513 Parnassus Avenue, Room S-1479, San Francisco, CA 94143-0452.

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Figure 1. Alignment of the predicted amino acid sequences of murine netrin-3, human NTN2L, chick netrin-2, and chick netrin-1 proteins. Amino acid residues identical among other netrins and murine netrin-3 are shaded in black. Similar residues are shaded in gray. The different domains of mouse netrin-3 (domains VI, V-1, V-2, V-3, and C) are indicated as defined by homology with other netrins (Serafini et al., 1994; Harris et al., 1996; Mitchell et al., 1996; de la Torre et al., 1997). The nucleotide sequence of mouse netrin-3 has been submitted to GenBank.

sion from the CNS at early stages of development and its selectivity in binding to various netrin receptors. These results suggest that netrin-3 may be more specialized for development of the peripheral nervous system and for functioning through a subset of known netrin receptors.

MATERIALS AND METHODS

Isolation of a murine netrin-3 cDNA. Two million phage plaques from a neonatal mouse brain cDNA library (Stratagene, La Jolla, CA) were screened with a 32 P-labeled murine *netrin-1* cDNA. Filters (Hybond-N; Amersham, Arlington Heights, IL) were hybridized at 42°C in 0.4 M NaPO₄, pH 7.2, 1% BSA, 1 mM EDTA, 7% SDS, 15% formamide, 250 µg/ml salmon sperm DNA, and 2×10^6 cpm/ml of the probe for 16 hr. The filters were washed repeatedly at 55°C in $0.2 \times$ SSC and 0.1% SDS and autoradiographed. Three positive clones were isolated and excised

into the phagemid vector pBluscript as directed by the manufacturer (Stratagene).

To isolate the 5' end of the cDNA, two rounds of 5' rapid amplification of cDNA ends (RACE) (Boehringer Mannheim, Indianapolis, IN) were conducted according to the manufacturer's instructions. Two micrograms of poly(A⁺) RNA from mouse embryonic day 12.5 (E12.5) embryos were reverse transcribed using a 3' primer complementary to the sequence 5'GGCAGAGCCCGTCTCCAA3' contained in the 5' end of the available cDNA sequence. A poly(A⁺) tail was then added using terminal transferase. The cDNA was used as template to amplify the 5' end, and a 0.5 kb PCR product was cloned into the pCRII vector (Invitrogen, San Diego, CA). Because this fragment still did not contain the entire open reading frame, a second round of 5' RACE was conducted using a primer complementary to 5'CCGACGCGGGCTACTCTGCAGAC3', a sequence in the 5' end of the newly cloned fragment. This second round of RACE resulted in the cloning of a 0.3 kb sequence, which contains the

5' end of the gene, including the putative ATG and 150 bp of 5'UTR. The continuity of these PCR products with the existing cDNA clone was confirmed by sequence comparison with the genomic DNA.

Isolation of genomic DNA. One pair of primers (5'ATCTTGGCACT-GCAGACCCGGCAGC3' and 5'GTCCGAGGAATCGCAGACGC-GATT3') was used to screen a mouse P1 genomic library by PCR (Genome Systems, Inc., St. Louis, MO). Two positive clones containing an 80 kb insert were mapped, and the genomic DNA spanning the cDNA region was subcloned into pBluscript SK(-).

Interspecific mouse backcross. Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*) F1 females and C57BL/6J males as described previously (Copeland and Jenkins, 1991). A total of 205 N2 mice were used to map the *Ntn3* locus. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described previously (Jenkins et al., 1982). All blots were prepared with Hybond-N⁺ nylon membrane (Amersham). The probe, an ~1.4 kb *Xba*I fragment of mouse genomic DNA from the 5' UTR, was labeled with [α -³²P] dCTP using a nick translation labeling kit (Boehringer Mannheim); washing was done to a final stringency of 1.0× SSCP and 0.1% SDS at 65°C. A fragment of 4.8 kb was detected in *Sca*I digested C57BL/6J DNA, and a fragment of 3.1 kb was detected in a *Sca*I digested *M. spretus*-specific fragment followed in backcross mice.

A description of the probes and restriction fragment length polymorphisms (RFLPs) for the loci linked to *Ntn3*, including *Masl*, *E4f1*, and *Pim1*, has been reported previously (Rooney et al., 1998). Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Expression of mouse netrin-3 protein and netrin-3(VI.V)-Fc in 293 EBNA cells. The genomic sequences encoding the signal peptide, domain VI and domain V, do not contain introns as revealed by sequence comparison to cDNA clones and 5' RACE products. Therefore, this region of the genomic DNA was fused with the cDNA and cloned into the mammalian expression vector pCEP4 (Invitrogen). In addition, a sequence encoding the myc epitope was also cloned in frame 5' to the stop codon to generate a plasmid we named pMN3^{myc}. For netrin-3 (VI.V)-Fc, human Fc DNA was fused in frame to generate netrin-3 (VI.V)-Fc. Transfection of pMN3^{myc} and netrin-3 (VI.V)-Fc into 293 EBNA cells was performed using LipofectAMINE (Life Technologies, Gaithersburg, MD) as directed by the manufacturer. Transfected cells were selected with G418 (250 μ g/ml) and hygromycin B (40 μ g/ml). After 7 d, the resistant cell colonies were pooled, expanded, and frozen as a stable source of transfected cells.

Purification of murine netrin-3 from transfected cells. Recombinant murine netrin-3 protein was purified from conditioned media by heparin affinity chromatography to 75–85% homogeneity, as assessed by silver staining. Netrin-3 (VI.V)-Fc protein was purified from conditioned media by protein-A affinity chromatography (Harlow and Lane, 1988) to greater than 90% homogeneity.

Northern blot analysis. Total RNA from various stages of embryos and brains was extracted (Chomczynski and Sacchi, 1987). Poly(A⁺) RNA was isolated by oligo-dT cellulose (Sambrook et al., 1989). For Northern analysis, 3 μ g of poly(A⁺) RNA was used. The blotted membrane was hybridized with ³²P-labeled cDNA in 0.32 M NaPO₄, pH7.2, 7% SDS, 1 mM EDTA, 1% BSA, and 250 μ g/ml salmon sperm DNA at 65°C for 16 hr. The membrane was washed repeatedly in 0.1× SSC and 1% SDS at 65°C and exposed to x-ray film at -80°C in the presence of an intensifying screen for 3 d.

In situ hybridization. Embryos from different stages were fixed in 4% paraformaldehyde in PBS, cryoprotected in 30% sucrose in PBS, embedded in O.C.T. compound, and cryostat sectioned at 16 μ m thickness. Antisense and sense RNA probes for *in situ* hybridization were transcribed from linearized plasmid using T7 or T3 RNA polymerase in the presence of [³⁵S]UTP. For murine *netrin-3*, a 1287 base probe spanning 1037 bases in the coding region and 250 bases in the 3'UTR was used. Another 368 base probe corresponding to 118 bases in the coding region and 250 bases in the 3'UTR gave similar patterns as the 1037 base probe (data not shown). We routinely used the 1037 base probe for murine *netrin-3* *in situ* hybridization. The murine *netrin-1* and chick *netrin-1* probes were the same as described previously (Kennedy et al., 1994; Serafini et al., 1996). For chick *netrin-2*, a 1243 base probe spanning 205 bases in the coding region and 1038 bases in the 3'UTR was used. For each cDNA, a sense probe was also synthesized and showed no signal under the same conditions as for the antisense probe. Sections were

Figure 2. *Ntn3* maps in the proximal region of mouse chromosome 17. *Ntn3* was placed on chromosome 17 by interspecific backcross analysis. The segregation patterns of *Ntn3* and flanking genes in 130 backcross animals that were typed for all loci are shown at the top. For individual pairs of loci, >130 animals were typed (see Materials and Methods). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*) F1 parent. The black boxes represent the presence of a C57BL/6J allele, and the white boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosomes is listed at the bottom of each column. A partial chromosome 17 linkage map showing the location of *Ntn3* in relation to linked genes is shown at the bottom. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the position of the loci in human chromosomes, where known, are shown to the right. References for the human map position of loci cited in this study can be obtained from Genome Data Base, a computerized database of human linkage information maintained by The William H. Welch Medical Library of the Johns Hopkins University (Baltimore, MD).

processed, hybridized, and washed at high stringency as described previously (Wang et al., 1994). Slides were coated with photographic emulsion (K5; Polysciences, Warrington, PA), developed after 3–7 d, stained with hematoxylin, dehydrated and cleared with xylene, and mounted in Permount (Fisher Scientific, Houston, TX).

Explant culture. E13 rat dorsal explant cultures with the cell aggregates were as described previously (Kennedy et al., 1994; Serafini et al., 1994), with micrographs of explants taken 16 hr after the onset of incubation of the cultures. Chemorepulsive activity was assayed as described previously (Colamarino and Tessier-Lavigne, 1995), with explants fixed 40 hr after the onset of incubation of the cultures and trochlear motor axons stained with the F84.1 antibody.

Binding experiments. Transient transfections of expression constructs

encoding rat DCC, rat neogenin, rat UNC5H1, rat UNC5H2, mouse UNC5H3, and rat L1 were performed using LipofectAMINE (Life Technologies). Forty-eight hours after transfection, the cells were incubated with 2 μ g/ml purified murine netrin-3 protein in PBS supplemented with 1% goat serum, 2 μ g/ml heparin, and 0.05% sodium azide at 37°C for 1 hr. After washing three times with PBS, the cells were fixed with 4% paraformaldehyde at room temperature for 10 min. The cells were then stained using monoclonal antibody 9E10, followed by a Cy 3-conjugated Goat anti-mouse IgG secondary antibody.

Equilibrium binding on 293T cells transiently expressing these receptors was quantified essentially as described previously (Keino-Masu et al., 1996; Leonardo et al., 1997). Briefly, the transfected cells were plated on a poly-D-lysine-coated 48-well plate. After washing with PBS containing 1% BSA and 0.05% sodium azide, followed by incubation with the ligand in binding buffer (PBS plus 1% BSA plus 0.05% sodium azide plus 2 μ g/ml heparin) for 3 hr on ice, the cells were washed twice with binding buffer. The cells were then fixed with 50% methanol, 100% methanol, and 4% paraformaldehyde sequentially. Fixed cells were washed three times before incubation with [125 I]goat anti human IgG (1 μ Ci/ml in 10% heat-inactivated goat serum) for 30 min at room temperature. The activity was counted after washing with PBS three times, followed by dissolving the cells in 1 M NaOH and 1% Triton X-100. Experiments were performed in triplicate for each concentration.

RESULTS

Cloning of the murine *netrin-3* gene

To search for other murine netrin homologs, we screened a neonatal mouse brain cDNA library with a mouse *netrin-1* probe. Three positive clones were isolated and sequenced. The documented netrins have a characteristic structure, comprising three domains called VI, V, and C. Domains VI and V are so named because of their homology to domains VI (a globular domain) and V (comprising epidermal growth factor-like repeats) of the laminin γ chains. The C-terminal domain C is highly basic. The sequence of each of the clones we obtained in our screen contained an open reading frame homologous to domains V and C of murine netrin-1. All three clones lacked sequences corresponding to domain VI and the signal peptide. The 5' end of the existing clones was obtained by two rounds of 5' RACE, yielding regions homologous to domain VI and the signal peptide. We later confirmed the continuity of these fragments with the cDNA clones by Northern analysis and by comparison to genomic sequence (data not shown).

The predicted amino acid sequence of the murine netrin-3 protein shows homology to all other known netrins (Fig. 1), including a high degree of homology at the amino acid level to the human NTN2L gene product (87.2% identity) and a lower level of homology to chick netrin-2 (71.6%) and chick netrin-1 (52.4%). Therefore, murine *netrin-3* is likely the strict ortholog of human NTN2L. However, the homology between mouse netrin-3 and chick netrin-2 is much lower, indicating that *netrin-3* is likely not the strict ortholog of chick *netrin-2*, as previously noted also for NTN2L (van Raay et al., 1997).

Chromosomal localization of the murine *netrin-3* gene

The chromosomal location of murine *netrin-3* (*Ntn3*) was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J $\times$ *M. spretus*) F1 $\times$ C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 2400 loci that are well distributed among all the autosomes, as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNA was digested with several enzymes and analyzed by Southern blot hybridization for informative RFLPs using a murine *Ntn3* probe. The 3.1 kb *ScaI* *M. spretus* RFLP (see Materials and Methods) was used to follow the segregation of the murine *netrin-3* locus in backcross mice. The

mapping results indicated that murine *netrin-3* is located in the proximal region of mouse chromosome 17 linked to *Masl*, *E4fl*, and *Piml*. Although 130 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 2), up to 192 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are as follows: Centromere-*Masl*-2/192-*Ntn3*/160-*E4fl*-1/132-*Piml*. The recombination frequencies [expressed as genetic distances in centimorgans (cM) plus the SE] are as follows: *Masl*-1.0 $\pm$ 0.7-[*Ntn3*, *E4fl*]-0.8 $\pm$ 0.8-*Piml*. No recombinants were detected between *Ntn3* and *E4fl* in 160 animals typed in common, suggesting that the two loci are within 1.9 cM of each other (upper 95% confidence limit).

Furthermore, we have compared the interspecific map of chromosome 17 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (provided from the Mouse Genome Database, maintained at The Jackson Laboratory, Bar Harbor, ME). *Ntn3* mapped in a region of the composite map that contains the t-complex, a $\sim$ 12 cM interval that contains numerous mutations, including those affecting tail length, male sterility, transmission ratio distortion, and prenatal lethality (Green, 1989). Whether mutations in *Ntn3* result in any of the t-complex phenotypes remains to be determined.

The proximal region of mouse chromosome 17 shares regions of homology with human chromosome 6 and 16 (Fig. 2). The placement of *Ntn3* in this interval in mouse is consistent with the human localization of NTN2L, 16p13.3 (van Raay et al., 1997).

Figure 3. Northern analysis of murine *netrin-3* expression in mRNA extracted from E9.5, E10.5, E11.5, and E12.5 whole embryos and E14.5, E16.5, and E18.5 neonatal and adult brain. The position of RNA molecular weight markers on the gel is indicated on the right. Elongation factor 1a (*EF1a*) was used as a control for loading of mRNA.

Figure 4. Comparison of expression of murine *netrin-1* (A, E, I), murine *netrin-3* (B, F, J), chick *netrin-1* (C, G, K), and chick *netrin-2* (D, H, L) in the spinal cord by *in situ* hybridization. Comparable developmental stages in mouse and chick are illustrated: A–D, E9.5 in mouse (A, B) and stage 18 in chick (C, D); E–H, E10.5 in mouse (E, F) and stage 22 in chick (G, H); I–L, E11.5 in mouse (I, J) and stage 26 in chick (K, L). (Note that the signal along the edge of the ventricular zone and in the central canal in I and J represents an edge effect and not a real signal). sc, Spinal cord; d, dorsal root ganglion; fp, floor plate; vz, ventricular zone; dm, dermomyotome; s, somite. Scale bar, 0.25 mm.

Sites of expression of murine *netrin-3*

Northern analysis shows that several transcripts are made from the murine *netrin-3* gene (Fig. 3). The major transcripts are 6.5, 4.0, and 3.0 kb, respectively. In mRNA derived from whole embryos during early stages of nervous system development, the expression of murine *netrin-3* is highest at approximately E12.5. Expression in brain tissue was also detected from E14.5 (the earliest stage examined) into adulthood (Fig. 3). In adult brain, the 3.0 kb transcript predominates.

To determine the regional distribution of murine *netrin-3* transcripts at early embryonic stages and whether, like *netrin-1*, it could play a role in axon guidance, we performed *in situ* hybridization analysis of murine *netrin-3* expression on sections of E9.5–E14.5 mouse embryos. In contrast to the two chicken *netrin* genes and murine *netrin-1*, murine *netrin-3* is not detected at early stages of spinal cord development (E9.5 and E10.5 in the mouse) (Fig. 4A–H). Instead, the highest signal is detected in the dorsal root ganglia (Fig. 4B,F). At E11.5, murine *netrin-3* mRNA is also detected at low levels in the motor column in the ventral spinal cord (Fig. 4J). At all these developmental stages, no signal was detected in the floor plate or in the ventricular zone, two sites of expression of the chick *netrin-1*, chick *netrin-2*, and mouse *netrin-1* genes. The murine *netrin-3* signal in the ventral spinal cord is maintained at a low level through E14.5 (Figs. 5B,C, 6D).

At E9.5, the expression of *netrin-3* visualized on transverse sections at the forelimb level was highest in condensing dorsal root ganglia (Fig. 4A). This expression in the dorsal root ganglia persists through E12.5 (Fig. 5A–C) but then reduces in intensity by E14.5 (Fig. 5D). We also observed expression in mesenchymal tissues. At E9.5 and E10.5, low diffuse signals were seen in the mesenchyme (Fig. 5A). At E11.5 and E12.5, this signal is mostly

localized in the condensing mesenchyme (Fig. 5B,C). At E14.5, the expression of *netrin-3* was found in the differentiating myotubes (Fig. 5D–F). The expression of murine *netrin-1* was also examined at E14.5 and compared with that of murine *netrin-3*. Interestingly, at this later stage of development, with the exception of the spinal cord, murine *netrin-3* and murine *netrin-1* have very similar expression patterns, especially in the muscles and in the bronchi of the lung (Fig. 5G–I), indicating that the two genes may have similar or redundant functions in these tissues at this stage of development.

In the head region, expression at E9.5 is highest in the differentiating trigeminal (V) ganglion, the geniculate (VII) ganglion, the vestibular (VIII) ganglion, the glossopharyngeal (IX) ganglion, and the nodose and jugular (X) ganglia (Fig. 6A and data not shown). This expression persists through E12.5 (Fig. 6) and is reduced by E14.5 (data not shown). At E11.5 and E12.5, the murine *netrin-3* gene is also expressed in the developing thalamus (Fig. 6C,D). In addition, a lower level of expression was also observed in mesenchymal tissues at all these stages. At later stages (after E16.5), expression of *netrin-3* is still observed in many regions of the brain but at moderate or low levels and very diffusely (data not shown).

Murine *netrin-3* binds to known netrin receptors

To help elucidate the possible function of murine *netrin-3*, we examined whether the *netrin-3* protein binds to known netrin receptors. Murine *netrin-3* protein was expressed with a C-terminal c-myc epitope tag in 293 EBNA cells (see Materials and Methods). The expression of the protein was confirmed by Western analysis using monoclonal antibody 9E10, which recognizes the myc epitope (data not shown). Netrin-3 protein was

Figure 5. Expression of murine *netrin-3* and murine *netrin-1* at different stages at the forelimb level. *A–C*, Murine *netrin-3* at E10.5 (*A*), E11.5 (*B*), and E12.5 (*C*). *D–F*, Expression of murine *netrin-3* at E14.5 in transverse sections (*D*), with higher magnification showing expression in the lung (*E*) and in the muscles (*F*). *G–I*, Expression of murine *netrin-1* at E14.5 in sections adjacent to those in *D–F*, respectively. Note that the expression patterns of *netrin-1* and *netrin-3* are similar in muscles and the lung. *d*, Dorsal root ganglion; *lb*, limb bud; *o*, oesophagus; *cm*, condensing mesenchyme; *m*, muscle; *l*, lung; *sc*, spinal cord. Scale bar: *A–D*, *G*, 1.4 mm; *E*, *F*, *H*, *I*, 0.33 mm.

purified and used in binding assays (see Materials and Methods). Netrin-3 was found to bind all the receptors to which netrin-1 binds, including DCC, neogenin, UNC5H1, UNC5H2, and UNC5H3 (Fig. 7), suggesting that netrin-3 may function through these receptors.

The affinity of the receptors for murine netrin-3 was estimated in equilibrium binding experiments using netrin-3(VI.V)-Fc, a fusion of the N-terminal two-thirds of netrin-3 to the constant portion of human IgG. A similar fusion of chick netrin-1 to Fc, the netrin-1(VI.V)-Fc protein, has been used to estimate the affinity of the receptors for netrin-1. This netrin-1 derivative is bioactive but, unlike full-length netrin-1, it does not aggregate at high concentrations, thereby improving the reliability of binding estimates (Keino-Masu et al., 1996; Leonardo et al., 1997). Specific binding of netrin-3(VI.V)-Fc to each of the receptors showed saturation, and binding curves were fitted to the Hill equation, yielding K_d values of 11.5, 3.0, 6.2, 3.3, and 4.5 nM for DCC, neogenin, UNC5H1, UNC5H2, and UNC5H3, respectively (Fig. 8). These values are comparable with those observed for the binding of netrin-1(VI.V)-Fc to its receptors (Keino-Masu et al., 1996; Leonardo et al., 1997), with the exception of the affinity for DCC, which is four times lower for netrin-3(VI.V)-Fc than for

netrin-1(VI.V)-Fc. We were not able to perform binding experiments with full-length netrin-3 because it also aggregates at high concentrations, particularly those necessary to evaluate binding to DCC. However, it seems reasonable to make use of the estimates obtained using netrin-3(VI.V)-Fc because the (VI.V)-Fc construct has been shown to be a reliable indicator of binding interactions in the case of netrin-1 (Keino-Masu et al., 1996).

Murine netrin-3 has outgrowth-promoting activity for spinal commissural axons and chemorepulsive activity for trochlear motor axons

Given that netrin-3 and netrin-1 bind to the same receptors, we examined further whether murine netrin-3 can mimic the previously documented activities of chick netrin-1 *in vitro*. We therefore first tested whether cells expressing murine netrin-3 or purified murine netrin-3 protein can also elicit commissural axon outgrowth from E13 dorsal explants, an activity that in the case of netrin-1 requires the function of the DCC receptor (Keino-Masu et al., 1996). Aggregates of transfected cells secreting recombinant murine netrin-3 always elicited directional axon outgrowth ($n > 20$) (Fig. 9*B*), whereas purified murine netrin-3 protein always elicited radial axon outgrowth from spinal cord explants

Figure 6. Expression of murine *netrin-3* in the head region of the mouse embryo at E9.5 (*A*), E10.5 (*B*), E11.5 (*C*), and E12.5 (*D*). *V*, Trigeminal ganglion; *VII*, geniculate ganglion; *VIII*, vestibular ganglion; *IX*, glossopharyngeal ganglion; *f*, forebrain; *h*, hindbrain; *m*, mesenchyme. *Arrows*, Thalamus; *arrowheads*, positive signal in the ventral aspect of the neural tube in the hindbrain. Scale bar, 1.4 mm.

Figure 7. Murine *netrin-3* protein binds to the netrin receptors DCC (*A*), neogenin (*B*), UNC5H1 (*D*), UNC5H2 (*E*), and UNC5H3 (*F*) but not a control protein of the immunoglobulin gene superfamily, L1 (*C*). Scale bar, 40 μ m.

($n > 15$) (Fig. 9C). However, the concentration of murine *netrin-3* required to elicit optimal commissural axon outgrowth from E13 explants is 120 μ g/ml (data not shown), approximately fourfold higher than that of *netrin-1* (Kennedy et al., 1994), consistent with the lower apparent affinity of *netrin-3*(VI.V)-Fc for DCC.

We next examined whether *netrin-3* also possesses chemorepulsive activity toward trochlear motor axons, an activity previously demonstrated for *netrin-1* (Colamarino and Tessier-Lavigne, 1995). We therefore cocultured aggregates of transfected cells expressing *netrin-3* with explants of ventral neural tube taken from the hindbrain–midbrain junction (HMJ) from E11 rats, which

Figure 8. Equilibrium binding of the netrin-3(VI.V)-Fc fusion protein to UNC5H1, UNC5H2, UNC5H3, neogenin, and DCC. Binding of netrin-3(VI.V)-Fc was determined by measuring the radioactivity associated with cells after subsequent incubation with radiolabeled anti-human IgG antibody. Specific binding curves were fitted using the Hill equation. K_d values for the interaction of netrin-3(VI.V)-Fc with UNC5H1, UNC5H2, UNC5H3, neogenin, and DCC are 6.2, 3.3, 4.5, 3.0, and 11.5 nM, respectively.

contain the cell bodies of trochlear motor neurons, as well as floor plate cells. In control cultures, trochlear motoneurons extend axons along a dorsally directed trajectory away from the floor plate within the explant. When they reach the cut edge of the explant, they project out into the collagen matrix and continue along their dorsal trajectory, unimpeded by control cells ($n = 9$ of 10 explants with a large axon bundle directed to the cells) (Fig. 9D) (Colamarino and Tessier-Lavigne, 1995). In contrast, when cultured with cells expressing netrin-3, the axons grew dorsally within the explant but, as they emerged into the collagen, they redirected their growth away from the exogenous source of netrin-3 ($n = 0$ of 10 explants with large bundles; two of these had short and splayed bundles, whereas the other eight had redirected their axons completely, as in Fig. 9E). These effects were similar to those observed with cells secreting netrin-1 (Colamarino and Tessier-Lavigne, 1995). Thus, netrin-3 also possesses chemorepulsive activity. Unfortunately, the qualitative nature of the assay did not make it possible to assess the specific activity of netrin-3 in repulsion.

DISCUSSION

We have described the cloning, expression, binding properties, and *in vitro* outgrowth-promoting activity of murine netrin-3. We found that this netrin gene is expressed in sensory ganglia, some CNS regions, mesenchymal tissues, and differentiating muscles. In addition, murine netrin-3 binds to all the receptor molecules to which netrin-1 is known to bind and mimics the outgrowth-promoting activity of netrin-1 on spinal cord commissural neurons and its chemorepulsive activity on trochlear motor axons. However, unlike netrin-1, netrin-3 binds to these receptors dif-

ferentially, showing lower affinity for DCC and, presumably as a consequence, lower specific activity than netrin-1 in the commissural axon outgrowth assay (its specific activity in the chemorepulsive assay has not been assessed).

The high level of homology between murine *netrin-3* and human *NTN2L* indicates that murine *netrin-3* is likely to be the ortholog of human *NTN2L*. This conclusion is further supported by the fact that murine *netrin-3* maps to a region of mouse chromosome 17 that is syntenic to the region of human chromosome 16p13.3 to which *NTN2L* maps. In contrast, the lower homology between murine *netrin-3* and chick *netrin-2* suggests that murine *netrin-3* may not be an ortholog of chick *netrin-2*. In agreement with this, we found that the expression pattern of murine *netrin-3*, especially during early stages of development, does not correspond to that of chick *netrin-2*. One important difference is that chick *netrin-2* is expressed in the ventral two-thirds of the spinal cord at approximately the time when commissural axons extend to the midline and has been suggested to form with *netrin-1* (which is expressed in the floor plate), a gradient of netrin protein in the chick spinal cord (Kennedy et al., 1994). In contrast, in the mouse, *netrin-3* is not expressed in the ventral spinal cord at early stages. Instead, murine *netrin-1* alone is expressed both in the floor plate and the ventral two-thirds of the spinal cord (Serafini et al., 1996), suggesting that it may subserve the functions of both chick *netrin-1* and chick *netrin-2*. Outside the spinal cord, the distribution pattern of murine *netrin-3* is similar to that of chick *netrin-2* in some respects but different in others. For example, both chick *netrin-2* and murine *netrin-3* are expressed in dorsal root ganglia; however, the onset of expression of chick *netrin-2* occurs at a later developmental stage than that of mouse *netrin-3*, which appears to be expressed as soon as neuronal differentiation commences in the ganglia. Similarly, murine *netrin-3* is expressed as soon as neuronal differentiation starts in condensing cranial sensory ganglia, whereas no expression of chick *netrin-2* was detected in cranial ganglia at comparable developmental stages (stages 16 to 26; data not shown). Other differences include the expression pattern in the dermomyotome and mesenchymal cells.

In addition to differences in sites of expression, a further apparent difference is that murine netrin-3 protein has a lower affinity for DCC than does netrin-1. Presumably as a consequence, murine netrin-3 has a lower specific activity in promoting commissural axon outgrowth in cell culture. It is difficult to make a precise comparison between affinities of chicken netrin-2 and mouse netrin-3 because the K_d for interaction of chick netrin-2 with DCC has not been measured. However, during the purification of the netrins from chick brain no striking difference in specific activity of the two chick proteins was observed (Serafini et al., 1994), suggesting that chick netrin-1 and netrin-2 likely have similar affinities for DCC and that chick netrin-2 may also bind DCC with higher affinity than does mouse netrin-3. Together, the differences in sequence, in expression pattern, and possibly in binding characteristics suggest that murine *netrin-3* is neither the ortholog nor the functional homolog of chick *netrin-2*. It will be of interest to determine whether there is a mouse ortholog of chick *netrin-2* and, conversely, whether there is a chick ortholog of mouse *netrin-3*.

Particularly striking sites of expression of murine *netrin-3* are in sensory ganglia and, to a lesser extent, in the developing motor columns. Although we do not know whether it is expressed by neurons or by glia in the sensory ganglia and motor column, the onset of expression in the sensory ganglia correlates with the

Figure 9. Outgrowth-promoting and chemorepulsive actions of netrin-3 *in vitro*. *A–C*, E13 dorsal spinal cord explants were cocultured with 293 EBNA cells mock-transfected with the vector pCEP4 (*A*), with transfected 293 EBNA cells expressing netrin-3 (*B*) or with purified netrin-3 protein (120 μ g/ml) (*C*). Netrin-3 promoted the outgrowth of spinal commissural axons from these explants. *D, E*, Explants of the ventral half of the HMJ of E11 rat embryos were cultured with 293 EBNA cells mock-transfected with the vector pCEP4 (*D*) or with transfected 293 EBNA cells expressing netrin-3 (*E*). Trochlear motor axons (*arrows*) originating from cell bodies in the trochlear nucleus (*IV*) extended dorsally away from the floor plate (*FP*) and into the collagen matrix. They continued to grow dorsally in the presence of control cells (*D*) but were repelled by netrin-3-secreting cells (*E*). In *D* and *E*, dots show the outline of aggregates of transfected cells. Scale bar, 33 μ m.

onset of neuronal differentiation in these ganglia. If it is expressed by the neurons, it is possible that the neurons secrete netrin-3 and that secreted netrin-3 is displayed on axonal surfaces. It is known that the receptors neogenin, UNC5H1, and UNC5H2 are also expressed in the dorsal root ganglia and that DCC, neogenin, and UNC5H1 are expressed in the motor column (Keino-Masu et al., 1996; Leonardo et al., 1997). Netrin-3 protein produced by sensory neurons and motoneurons might therefore in principle bind to peripheral sensory and motor axons via these receptors. Indeed, in *Drosophila*, netrins are expressed in neurons throughout the CNS, and netrin protein can be detected immunohistochemically associated with axon tracts (Harris et al., 1996; Mitchell et al., 1996). Interestingly, in *Drosophila* mutants that carry a deletion of both *netrin* genes, occasional breaks in the longitudinal axon tracts are observed, suggesting that proper fasciculation may require netrin expression (Mitchell et al., 1996). Similarly, expression of UNC-6 in neurons in *C. elegans* is consistent with the hypothesis that netrin may produce some of its effects locally by being presented on axonal surfaces (Wadsworth et al., 1996). Thus, if netrin-3 is expressed on sensory axons in the mouse, it may regulate the fasciculation of axons or provide a guidance cue for later developing axons that encounter the axons of netrin-3-expressing neurons.

In addition to its expression in sensory ganglia, murine *netrin-3* is also expressed in a striking pattern in the differentiating muscles. Murine *netrin-3* mRNA is first detected in myoblasts at low

levels at approximately E10.5, and by E14.5, it is clearly expressed in the muscle cells. Interestingly, murine *netrin-1* shows similar expression in muscles at E14.5, although a much higher level expression of murine *netrin-1* is detected in the migrating myoblasts at E10.5 (Fig. 4). Although we do not know whether both genes are coexpressed in the same muscle cells at E14.5, the fact that they are both expressed in muscles suggests that mammalian netrin-1 and netrin-3 may both play a role in guiding peripheral axons to their correct muscle targets. A precedent for this is provided by the expression of netrin genes by subsets of muscle cells in *Drosophila*, and the demonstration that loss of netrin function, as well as ectopic expression of netrins in inappropriate muscles, can cause targeting defects for subsets of motor axons (Mitchell et al., 1996; Winberg et al., 1998). Whether netrins are required for peripheral motor axon guidance in mammals will require the examination of axon trajectories in mice lacking the function of *netrin-1* and *netrin-3*.

In vitro murine netrin-3 protein appears to bind all the receptor proteins to which netrin-1 binds and can mimic both the outgrowth-promoting activity of netrin-1 on commissural axons and the chemorepulsive activity of netrin-1 on trochlear motor axons. The affinity with which netrin-3(VI.V)-Fc binds to neogenin, UNC5H1, UNC5H2, and UNC5H3 is comparable with that of netrin-1(VI.V)-Fc (Keino-Masu et al., 1996; Leonardo et al., 1997). However, the affinity of netrin-3(VI.V)-Fc for DCC is lower than for all other netrin receptors we tested and is approx-

imately fourfold lower than that of netrin-1(VI.V)-Fc. It will therefore be of interest to determine whether netrin-3 *in vivo* is specialized for interactions with netrin receptors other than DCC and, in particular, for chemorepulsion. Examination of the loss-of-function phenotype of netrin-3 mutant mice should help address this issue and determine its role in axon guidance and in the development of non-neural tissues.

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