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An agency of Industry Canada

CA 2250263 C 2002/08/20

(11)(21) 2 250 263

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 1997/04/19
(87) Date publication PCT/PCT Publication Date: 1997/10/30
(45) Date de délivrance/Issue Date: 2002/08/20
(85) Entrée phase nationale/National Entry: 1998/09/28
(86) N° demande PCT/PCT Application No.: US 1997/006452
(87) N° publication PCT/PCT Publication No.: 1997/040064
(30) Priorité/Priority: 1996/04/19 (08/635,137) US

(51) Cl.Int.^{6}/Int.Cl.^{6} C12N 15/18, A61K 31/70, A61K 38/18, A61K 48/00, C07K 14/48, C12Q 1/68, C07H 21/00, G01N 33/53

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(54) Titre : NETRINE-I DE L'HOMME
(54) Title: HUMAN NETRIN-1

(57) Abrégé/Abstract:
Specific netrin proteins, nucleic acids which encode netrin proteins and hybridization reagents, probes and primers capable of hybridizing with netrin genes and methods for screening chemical libraries for lead compounds for pharmacological agents useful in the diagnosis or treatment of disease associated undesirable cell growth are provided. An exemplary screen involves forming a mixture comprising a recombinant netrin protein, a natural intracellular netrin protein binding target and a candidate pharmacological agent; incubating the mixture under conditions whereby, but for the presence of said candidate pharmacological agent, said netrin protein selectively binds said binding target; and detecting the presence or absence of specific binding of said netrin protein to said binding target.

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PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 1/00, 14/00, 17/00, A61K 38/00

A1

(11) International Publication Number: **WO 97/40064**

(43) International Publication Date: 30 October 1997 (30.10.97)

(21) International Application Number: PCT/US97/06452

(22) International Filing Date: 19 April 1997 (19.04.97)

(30) Priority Data:
08/635,137 19 April 1996 (19.04.96) US

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(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: HUMAN NETRIN-1

(57) Abstract

Specific netrin proteins, nucleic acids which encode netrin proteins and hybridization reagents, probes and primers capable of hybridizing with netrin genes and methods for screening chemical libraries for lead compounds for pharmacological agents useful in the diagnosis or treatment of disease associated undesirable cell growth are provided. An exemplary screen involves forming a mixture comprising a recombinant netrin protein, a natural intracellular netrin protein binding target and a candidate pharmacological agent; incubating the mixture under conditions whereby, but for the presence of said candidate pharmacological agent, said netrin protein selectively binds said binding target; and detecting the presence or absence of specific binding of said netrin protein to said binding target.

Human Netrin-1

INTRODUCTION

Field of the Invention

The field of this invention is human netrin proteins and genes.

Background

In the developing nervous system, axons project considerable distances along stereotyped pathways to reach their targets. Axon growth and guidance depends partly on the recognition of cell-surface and extracellular matrix cues along these pathways. The identification of such nerve cell growth and guidance cues is the holy grail of neurobiology. These are the compounds that tell neurons when to grow, where to grow, and when to stop growing. The medical applications of such compounds are enormous and include modulating neuronal growth regenerative capacity, treating neurodegenerative disease, and mapping (e.g. diagnosing) genetic neurological defects.

Over decades of concentrated research, various hypotheses involving chemo-attractants and repellents, labeled pathways, cell adhesion molecules, etc. have been invoked to explain guidance. Molecules such as N-CAM and N-cadherin have been reported to provide favorable substrates for axon growth and certain sensory axons may be responsive to NGF and NGF-like factors. Recent reports suggest the existence of diffusible chemotropic molecule(s) which influence the pattern and orientation of commissural axon growth.

Relevant Literature

Ishii et al. (1992) Neuron 9, 873-881 disclose a gene, unc-6, derived from C. elegans, which has sequence similarity to the disclosed netrins. Serafini et al (1994) Cell 78, 409-424 and Kennedy et al (1994) Cell 78, 425-435 at page 5, column 1 describe related vertebrate netrins. The work was also reported in *The New York Times*, Section B7, Tuesday, August 16, 1994 and more recently (May 19, 1995) described in Science 268, 971-973 (see also references cited therein).

SUMMARY OF THE INVENTION

The invention provides methods and compositions relating to a human netrin protein and gene. Netrins are a class of proteins which are naturally involved in neural axon guidance. The subject compositions include nucleic acids which encode the specified netrin protein and hybridization probes and primers capable of hybridizing with the specified netrin

gene. The netrin proteins finds particular use in modulating neural axon outgrowth. The disclosed compositions also find use variously in screening chemical libraries for regulators of axon outgrowth and orientation, in genetic mapping, as probes for netrin genes, as diagnostic reagents for genetic neurological disease and in the production of specific cellular and animal systems for the development of neurological disease therapy.

This invention provides an isolated netrin comprising the amino acid sequence of SEQ ID NO:2.

This invention also provides an isolated polynucleotide encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:2.

This invention also provides an isolated polynucleotide comprising SEQ ID NO:1 or a fragment thereof at least 100 nucleotides in length.

This invention also provides an isolated polynucleotide comprising a sequence fully complementary to SEQ ID NO:1 or a fragment thereof at least 100 nucleotides in length.

This invention also provides a method of determining the presence of a human netrin gene in a sample comprising a nucleic acid, said method comprising contacting said sample with a polynucleotide as described above, and detecting the presence of specific hybridization of said polynucleotide to said nucleic acid.

This invention also provides a method of determining the presence of a human netrin having the amino acid sequence of SEQ ID NO:2 in a sample, said method comprising contacting said sample with an antibody which specifically binds said netrin and detecting the presence of specific binding of said agent to said netrin.

This invention also provides a method of screening for candidate drugs, said method comprising the steps of: contacting a prospective agent with a mixture comprising a netrin according to claim 1 and a netrin binding target under conditions wherein, but for the presence of said prospective agent, said netrin and said netrin binding target have a first association; detecting a second association between said netrin and said netrin binding target; comparing said first and second association; wherein a difference between said first and second association identifies said prospective agent as a lead pharmaceutical compound which modulates the interaction of said netrin and said netrin binding target.

This invention also provides use, in the manufacture of an agent, of a netrin of this invention for modulating the growth, differentiation or morphology of a neuron.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides methods and compositions relating to a human netrin-1 protein and gene; including methods and compositions for identifying, purifying, characterizing, and producing the subject proteins and for identifying, characterizing, cloning, expressing, inhibiting the expression of and amplifying the subject nucleic acids. The subject proteins may be incomplete translates of the disclosed netrin cDNA sequence or deletion mutants of the corresponding conceptual translates, which translates or deletion mutants have the human netrin-1 binding activity and specificity described herein. The netrins are isolated, partially pure or pure and are typically recombinantly produced. An "isolated" protein for example, is unaccompanied by at least some of the material with which it is associated in its natural state; generally constituting at least about 0.5%, preferably at least about 2%, and more preferably at least about 10% by weight of the total protein in a given sample; and a pure protein constitutes at least about 50%, preferably at least about 90%, and more preferably at least about 99% by weight of the total protein in a given sample. A wide variety of molecular and biochemical methods are available for generating and expressing the subject compositions, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, et al. Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) or that are otherwise known in the art.

The disclosed netrin compositions may be used to generate human netrin-1 specific antibodies, to modulate axon outgrowth or guidance in situ or in vivo, etc. For in vivo applications, the compositions are added to a retained physiological fluid such as blood or synovial fluid. For CNS administration, a variety of techniques are available for promoting transfer of the therapeutic across the blood brain barrier including disruption by surgery or injection, drugs which transiently open adhesion contact between CNS vasculature endothelial cells, and compounds which facilitate translocation through such cells. Netrins

may also be amenable to direct injection or infusion, topical, intratracheal/nasal administration e.g. through aerosol, intraocularly, or within/on implants e.g. fibers e.g. collagen, osmotic pumps, grafts comprising appropriately transformed cells, etc. A particular method of administration involves coating, embedding or derivatizing fibers, such as collagen fibers, protein polymers, etc. with therapeutic proteins. Other useful approaches are described in Otto et al. (1989) J Neuroscience Research 22, 83-91 and Otto and Unsicker (1990) J Neuroscience 10, 1912-1921. Generally, the amount administered will be empirically determined, typically in the range of about 10 to 1000 μg/kg of the recipient and the concentration will generally be in the range of about 50 to 500 μg/ml in the dose administered. Other additives may be included, such as stabilizers, bactericides, etc. will be present in conventional amounts.

The invention provides netrin-specific binding agents including isolated binding targets such as membrane-bound netrin receptors and netrin-specific antibodies and binding agents identified in screens of natural and synthetic chemical libraries, and methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. Generally, netrin-specificity of the binding agent is shown by binding equilibrium constants. Such agents are capable of selectively binding the specified netrin, i.e. with an equilibrium constant at least about $10^7\ M^{-1}$, preferably at least about $10^8\ M^{-1}$, more preferably at least about $10^9\ M^{-1}$. A wide variety of cell-based and cell-free assays may be used to demonstrate netrin-specific binding; preferred are rapid in vitro, cell-free assays such as mediating or inhibiting netrin-cell/protein binding, immunoassays, etc.

The invention also provides nucleic acids encoding the subject proteins, which nucleic acids may be part of netrin-expression vectors and may be incorporated into recombinant cells for expression and screening, transgenic animals for functional studies (e.g. the efficacy of candidate drugs for neural disease or injury), etc. and nucleic acid hybridization probes and replication/amplification primers having a disclosed netrin cDNA specific sequence. The hybridization probes contain a sequence common or complementary to the corresponding netrin gene sufficient to make the probe capable of specifically hybridizing to the corresponding netrin gene, and only to the corresponding netrin gene, in the presence of other netrin genes. Hence, the subject probes and primers are uniquely specific to the disclosed cDNA. Hybridization probes having in excess of 100 continuous bases of netrin gene sequence are generally capable of hybridizing to the corresponding

netrin cDNA and remaining bound at a reduced final wash stringency of 0.2 X SSC (0.9 M saline/0.09 M sodium citrate) and 0.1% SDS buffer at a temperature of 65°C.

The subject nucleic acids are isolated, meaning they comprise a sequence joined to a nucleotide other than that which it is joined to on a natural chromosome, and usually constitute at least about 0.5% , preferably at least about 2%, and more preferably at least about 5% by weight of total nucleic acid present in a given fraction. A pure nucleic acid constitutes at least about 50%, preferably at least about 90%, and more preferably at least about 95% by weight of total nucleic acid present in a given fraction. The subject nucleic acids find a wide variety of applications including use as translatable transcripts, hybridization probes, PCR primers, therapeutic nucleic acids, etc.; use in detecting the presence of netrin genes and gene transcripts, e.g. allele-specific oligonucleotide (ASO) probes use to identify wild-type and mutant netrin alleles in clinical and laboratory samples, in detecting or amplifying nucleic acids encoding other netrins, and in gene therapy applications, e.g. antisense oligonucleotides capable of inhibiting the intracellular expression of a targeted netrin transcript.

The invention provides efficient methods of identifying pharmacological agents or lead compounds for agents capable of mimicking or modulating netrin function (e.g. bioactive netrin deletion mutants and netrin peptides). A wide variety of screens may be used; for example, cell-based assays may be used for monitoring netrin function and in vitro binding assays may be used to identify netrin-specific binding agents. Kennedy et al. (1994) Cell 78, 425-435 describe a particularly convenient COS cell-based netrin expression assay. Preferred methods are amenable to automated, cost-effective high throughput screening of natural and synthetic chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and human trials; for example, the reagents may be derivatized and rescreened in in vitro and in vivo assays to optimize activity and minimize toxicity for pharmaceutical development.

EXAMPLES

Human Netrin 1 (SEQ ID NO:2) and cDNA (SEQ ID NO:1).

We isolated chicken netrin 1 and 2 cDNAs as described in Serafini et al. (1974) *Cell* 78:409-424. Based on the chick netrin 1 and 2 cDNA sequences, we designed degenerate oligonucleotide primers and used these primers to amplify a cDNA encoding mouse netrin 1

from a murine cDNA library. We then isolated a human netrin cDNA using degenerate oligonucleotide primers constructed using amino acid sequences conserved in chick and mouse netrin sequences as a guide.

The starting material for PCR was 100 ng of human genomic DNA. Two rounds of PCR amplification were used. In the first round, the following program and conditions were used: 1. 94°C 30 sec.; 2. 50°C 45 sec; 3. 72°C, 1 minute; 4. go to 1, 30 times; 5. 4°C; 6. end. In the second round of PCR amplification, nested PCR and the following conditions and program were used: 1. 94°C 1 minute; 2. 42°C 1 minute; 3. increments of 1°C every 5 seconds to 72°C; 4. 72°C for 3 minutes; 5. go to 1, 2 times; 6. 94°C for 1 minute; 7. 46°C for 1 minute; 8. increments of 1°C every 6 seconds until 72°C; 9. 72°C 2 minutes; 10. go to 6, 7 times; 11. 94°C for 1 minute; 12. 55°C for 1 minute; 13. 72°C 2 minute; 14. go to 11, 24 times; 15. 94°C for 1 minute; 16. 55°C for 1 minute; 17. 72°C for 10 minutes; 18. 4°C; 19. end. PCR products were subcloned and individual clones containing inserts corresponding to human netrin sequence isolated using a Grunstein and Hogness screen (Sambrook, 1989). ^{32}P was incorporated into a probe using PCR with a portion of the mouse netrin-1 cDNA clone as a template. The final wash of the filters was at a reduced stringency of 1X SSC and 0.1%SDS at 65°C (Sambrook et al., 1989). This screen isolated an approximately 140 base pair human netrin cDNA clone. This cDNA fragment was used to isolate a longer human netrin cDNA from a Human fetal brain cDNA library (Stratagene cat#936206). The ~140 base pair human netrin cDNA was used as a template and ^{32}P incorporated into a human netrin cDNA probe using PCR. $1X10^6$ clones were screened at high stringency (Sambrook et al., 1989) identifying a single approximately 7 kb netrin cDNA (HBC-1, deposited with ATCC June 7, 1995, as plasmid HN-1, deposit number 97204).

Sequence analysis determined that an EcoRI subclone of the 7 kb HBC-1 clone corresponded to a splice variant of human netrin-1. The first 1086 base pairs of sequence show high homology to mouse netrin-1 and the remaining 626 base pairs are highly divergent. A potential splice donor site is identified at the junction of the netrin and the divergent sequence. A clone that corresponds to the 3' end of human netrin-1 was isolated using reverse transcription and PCR with a 5' primer at position 999 of the human sequence and a 3' degenerate primer to the last 15 base pairs of the mouse netrin sequence. Three additional independent clones were isolated to confirm the sequence of the PCR reaction

product. The region of overlap between this new clone and the HBC-1 Eco clone is 46 base pairs and is identical in sequence. To verify the overlap, an additional clone was isolated using a 5' primer at position 818 and a 3' primer at position 1582 of the human netrin-1 sequence. Two independent clones isolated using these primers confirm the structure of the cDNA. Finally, the sequence encoding the C-terminal 5 amino acids was confirmed. A primer was designed to a region within the 3' untranslated region that is conserved between chicken and mouse netrin-1. A PCR product was generated using this primer and a 5' primer in the human sequence at position 1568 and the sequence was verified.

RT-PCR procedures were as follows: fetal brain RNA (19-23 weeks) was obtained from Clontech. RT-PCR was performed using the GeneAmp*Thermostable rTth Reverse Transcriptase RNA PCR Kit from Perkin Elmer. A hot start technique was used to denature the RNA by mixing 50 ng with 30 pmol degenerate primer deg-1, 1 µl 10X rTth Reverse Transcriptase Buffer (Perkin Elmer) and water in a total volume of 7.2 µl; this mixture was heated to 95° C for 2 minutes, followed by a 5 minute incubation at 70°C. The reaction was cooled to 60°C and reverse transcription was begun by adding a mixture containing 1 µl 10 mM $MnCl_2$, 1 µl rTth DNA polymerase (Perkin Elmer, 2.5 U), and 0.2 µl each 10mM dATP, dCTP, dGTP, and dTTP . The 60°C incubation was continued for 5 minutes, followed by two 5 minute incubations at 65°C and 70°C. The reaction was then chilled on ice.

To amplify the human netrin-1 fragment by PCR, a mixture containing 2 µl DMSO, 3 µl 25 mM $MgCl_2$, 4 µl 10X Chelating Buffer (Perkin Elmer) and 34.5 µl water was added to the reverse transcription reaction. 30 pmol of a human netrin-specific primer, h-net-5'999, was added (0.5 µl) to the mixture and PCR was carried out in an MJ Research PTC-200 Peltier Thermal Cycler using the "Calculated" temperature control method and the following conditions: 1) 95° C for 2 minutes; 2) 55° C for 25 seconds; 3) 95° C for 10 seconds; 4) Repeat steps 2-3, 34 times; 5) 60° C for 7 minutes; and 6) 4° C hold.

The reaction was analyzed by gel electrophoresis and transferred to nylon membrane. Netrin-specific products were detected by Southern hybridization using a mouse netrin probe corresponding to sequence from position 1342 to position 1875 of mouse netrin. A plug of agarose corresponding to the cross hybridizing fragment (migrating at approximately 850 bp) was extracted with a Pasteur pipet and reamplified as follows: the agarose plug was combined with a reaction mixture containing 1.25 µl formamide, 5 µl of 10X PCR Buffer II (Perkin Elmer), 5 µl of 25 mM $MgCl_2$, 30 pmol human netrin-specific primer h-net-5'999,

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30 pmol degenerate netrin primer deg-1, 5 U AmpliTaq* DNA Polymerase (Perkin Elmer), and sterile water in a total volume of 49 µl. PCR was carried out in an MJ Research PTC-200 Peltier Thermal Cycler using the "Block" temperature control method and the following conditions: 1) 95° C for 2 minutes; 2) 50° C for 1 minute; 3) 75° C for 1 minute, 30 seconds; 4) 95° C for 20 seconds; 5) Repeat steps 2- 4, 39 times; 6) 75° C for 10 minutes; and 7) 4° C hold.

The reaction was analyzed by gel electrophoresis and a band of DNA at approximately 850 bp was purified using the BioRad Prep-A-Gene* system, ligated with the TA cloning vector (InVitroGen), and transformed into DH5α competent bacterial cells. Colonies were analyzed for the correct size insert by colony PCR, grown overnight at 37° C in LB media containing 100µg/ml ampicillin, and DNA was prepared from the culture using a Qiagen Tip-100* column. The inserts were confirmed by restriction digests and the sequence was generated using an ABI 377 automated sequencer.

To confirm the last 15 nucleotides of the human netrin-1 clone, the 3' end of the clone was amplified from first strand cDNA using a 5' primer to known sequence and a 3' primer to sequence in the 3' untranslated region of the mouse netrin-1 clone. First, cDNA was synthesized as described in "Rapid Amplification of cDNA Ends," by Michael Frohman (In: PCR Primer: A Laboratory Manual, C.W. Dieffenbach and G.S. Dveksler, eds., Cold Spring Harbor Laboratory Press, 1995) using the protocol described in the section "3'-End cDNA Amplification" (pp. 388-389), and 1 µg fetal brain poly A+ RNA (Clontech). 1 µl of the diluted cDNA was amplified in a 50 µl reaction containing 67 mM Tris HCl, pH 9.0, 6.7 mM magnesium chloride, 16.6 mM ammonium sulfate, 0.17 mg/ml BSA, 10 % DMSO, 1.5 mM each dNTP, 30 pmol 5' primer "h-net 5'1449" and 30 pmol 3' primer "m-net 3' UT 2238". The primer "h-net 5'1449" corresponds to sequence beginning at position 1449 in the human netrin-1 clone. The primer "m-net 3' UT 2238" corresponds to sequence in the 3' untranslated region of mouse netrin-1, with the addition of an Xba I restriction site sequence at the 5' end. Taq DNA polymerase, 1 µl, (Perkin Elmer) was combined with 0.5 µl TaqStart Antibody (ClonTech) and 2.5 µl TaqStart Antibody Dilution Buffer, incubated at room temperature for 10 minutes, and added to the PCR reaction mixture. The reaction was amplified in an MJ Research PTC-200 Peltier Thermal Cycler using the "Calculated" temperature control method and the following conditions: 1) 95° C for 2 minutes; 2) 62° C for 30 seconds; 3) 57° C for 30 seconds; 4) 52° C for 10 seconds; 5) 72° C for 40 minutes;

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6) 94° C for 10 seconds; 7) 62° C for 1 minute; 8) 72° C for 3 minutes; 9) Repeat steps 6-8, 4 times; 10) 94° C for 10 seconds; 11) 57° C for 1 minute; 12) 72° C for 3 minutes; 13) Repeat steps 10-12, 4 times; 14) 94° C for 10 seconds; 15) 52° C for 1 minute; 16) 72° C for 3 minutes; 17) Repeat steps 14-16, 24 times; 18) 75° C for 10 minutes; 19) 4° C hold.

The reaction was diluted 1:20 in TE, and 1µl was re-amplified in a 50 µl reaction containing 67 mM Tris HCl, pH 9.0, 6.7 mM magnesium chloride, 16.6 mM ammonium sulfate, 0.17 mg/ml BSA, 10 % DMSO, 1.5 mM each dNTP, 30 pmol of the 5' primer "h-net 5'1702," 30 pmol of the 3' primer "m-net 3' UT 2238" and 0.25 ul Taq DNA Polymerase. The primer "m-net 5'1702" corresponds to sequence beginning at position 1568 in the human netrin-1 clone. The reaction was amplified in an MJ Research PTC-200 Peltier Thermal Cycler using the "Calculated" temperature control method and the following conditions: 1) 95° C for 2 minutes; 2) 94° C for 10 seconds; 3) 58° C for 1 minute; 4) 72° C for 3 minutes; 5) Repeat steps 2-4, 4 times; 6) 94° C for 10 seconds; 7) 54° C for 45 seconds; 8) 72° C for 3 minutes; 9) Repeat steps 6-8, 4 times; 10) 94° C for 10 seconds; 11) 50° C for 30 seconds; 12) 72° C for 3 minutes; 13) Repeat steps 10-12, 24 times; 14) 75° C for 5 minutes; and 15) 4° C hold.

The reaction was analyzed by gel electrophoresis and a 530 bp product was isolated from the gel using the Prep-A-Gene*System (BioRad). The product was ligated into pCR 2.1 (InVitroGen) overnight at 14° C. Transformants were cultured overnight in LB media containing 100 µg/ml ampicillin and DNA was purified from cultures using Easy Pure Plasmid Preps*(Super Mini, Primm Labs). DNA sequence was generated using an ABI 377 automated sequencer.

To confirm the 3' end sequence, two additional fragments spanning the 3' end of human netrin were generated by PCR using the m-net 5' 1702 primer and a 3' primer, h-net 3' 1959 corresponding to sequence in the 3' untranslated region of human netrin-1. Amplification with these primers generated products of approximately 390 base pairs in duplicate reactions. The fragments were amplified in an MJ Research PTC-200 Peltier Thermal Cycler using the "Calculated" temperature control method and the following conditions: 1) 95° C for 2 minutes; 2) 94° C for 10 seconds; 3) 58° C for 1 minute; 4) 72° C for 3 minutes; 5) Repeat steps 2-4, 4 times; 6) 94° C for 10 seconds; 7) 54° C for 45 seconds; 8) 72° C for 3 minutes; 9) Repeat steps 6-8, 4 times; 10) 94° C for 10 seconds; 11) 50° C for 30 seconds; 12) 72° C for 3 minutes; 13) Repeat steps 10-12, 19 times; 14) 75° C

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for 5 minutes; and 15) 4° C hold. The reactions were analyzed by gel electrophoresis to confirm their size, and directly sequenced using an ABI 377 automated sequencer.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: EXELIXIS PHARMACEUTICALS, INC. -AND- THE REGENTS OF THE UNIVERSITY OF CALIFORNIA

(ii) TITLE OF INVENTION: HUMAN NETRIN-1

(iii) NUMBER OF SEQUENCES: 2

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(F) ZIP: K1P 5Y6

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC* compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS*

(D) SOFTWARE: ASCII (text)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: CA 2,250,263

(B) FILING DATE: 19-APR-1997

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/635,137

(B) FILING DATE: 19-APR-1996

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*Trademark

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1848 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 34..1845

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGCGCGGCAG GGCCGGGGCA AGCTGGACGC AGC ATG ATG CGC GCA GTG TGG GAG 54

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                                        Met Met Arg Ala Val Trp Glu
                                          1               5

GCG CTG GCG GCG CTG GCG GCG GTG GCG TGC CTG GTG GGC GCG GTG CGC      102
Ala Leu Ala Ala Leu Ala Ala Val Ala Cys Leu Val Gly Ala Val Arg
          10                  15                  20

GGC GGG CCC GGG CTC AGC ATG TTC GCG GGC CAG GCG GCG CAG CCC GAT      150
Gly Gly Pro Gly Leu Ser Met Phe Ala Gly Gln Ala Ala Gln Pro Asp
     25                  30                  35

CCC TGC TCG GAC GAG AAC GGC CAC CCG CGC CGC TGC ATC CCG GAC TTT      198
Pro Cys Ser Asp Glu Asn Gly His Pro Arg Arg Cys Ile Pro Asp Phe
 40                  45                  50                  55

GTC AAT GCG GCC TTC GGC AAG GAC GTG CGC GTG TCC AGC ACC TGC GGC      246
Val Asn Ala Ala Phe Gly Lys Asp Val Arg Val Ser Ser Thr Cys Gly
                 60                  65                  70

CGG CCC CCG GCG CGC TAC TGC GTG GTG AGC GAG CGC GGC GAG GAG CGG      294
Arg Pro Pro Ala Arg Tyr Cys Val Val Ser Glu Arg Gly Glu Glu Arg
             75                  80                  85

CTG CGC TCG TGC CAC CTC TGC AAC GCG TCC GAC CCC AAG AAG GCG CAC      342
Leu Arg Ser Cys His Leu Cys Asn Ala Ser Asp Pro Lys Lys Ala His
         90                  95                  100

CCG CCC GCC TTC CTC ACC GAC CTC AAC AAC CCG CAC AAC CTG ACG TGC      390
Pro Pro Ala Phe Leu Thr Asp Leu Asn Asn Pro His Asn Leu Thr Cys
     105                 110                 115

TGG CAG TCC GAG AAC TAC CTG CAG TTC CCG CAC AAC GTC ACG CTC ACA      438
Trp Gln Ser Glu Asn Tyr Leu Gln Phe Pro His Asn Val Thr Leu Thr
 120                 125                 130                 135

CTG TCC CTC GGC AAG AAG TTC GAA GTG ACC TAC GTG AGC CTG CAG TTC      486
Leu Ser Leu Gly Lys Lys Phe Glu Val Thr Tyr Val Ser Leu Gln Phe
                 140                 145                 150

TGC TCG CCG CGG CCC GAG TCC ATG GCC ATC TAC AAG TCC ATG GAC TAC      534
Cys Ser Pro Arg Pro Glu Ser Met Ala Ile Tyr Lys Ser Met Asp Tyr
             155                 160                 165

GGG CGC ACG TGG GTG CCC TTC CAG TTC TAC TCC ACG CAG TGC CGC AAG      582
Gly Arg Thr Trp Val Pro Phe Gln Phe Tyr Ser Thr Gln Cys Arg Lys
         170                 175                 180

ATG TAC AAC CGG CCG CAC CGC GCG CCC ATC ACC AAG CAG AAC GAG CAG      630
Met Tyr Asn Arg Pro His Arg Ala Pro Ile Thr Lys Gln Asn Glu Gln
     185                 190                 195

GAG GCC GTG TGC ACC GAC TCG CAC ACC GAC ATG CGC CCG CTC TCG GGC      678
Glu Ala Val Cys Thr Asp Ser His Thr Asp Met Arg Pro Leu Ser Gly
 200                 205                 210                 215

GGC CTC ATC GCC TTC AGC ACG CTG GAC GGG CGG CCC TCG GCG CAC GAC      726
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Gly Leu Ile Ala Phe Ser Thr Leu Asp Gly Arg Pro Ser Ala His Asp
                220                 225                 230

TTC GAC AAC TCG CCC GTG CTG CAG GAC TGG GTC ACG GCC ACA GAC ATC      774
Phe Asp Asn Ser Pro Val Leu Gln Asp Trp Val Thr Ala Thr Asp Ile
            235                 240                 245

CGC GTG GCC TTC AGC CGC CTG CAC ACG TTC GGC GAC GAG AAC GAG GAC      822
Arg Val Ala Phe Ser Arg Leu His Thr Phe Gly Asp Glu Asn Glu Asp
        250                 255                 260

GAC TCG GAG CTG GCG CGC GAC TCG TAC TTC TAC GCG GTG TCC GAC CTG      870
Asp Ser Glu Leu Ala Arg Asp Ser Tyr Phe Tyr Ala Val Ser Asp Leu
    265                 270                 275

CAG GTG GGC GGC CGG TGC AAG TGC AAC GGC CAC GCG GCC CGC TGC GTG      918
Gln Val Gly Gly Arg Cys Lys Cys Asn Gly His Ala Ala Arg Cys Val
280                 285                 290                 295

CGC GAC CGC GAC GAC AGC CTG GTG TGC GAC TGC AGG CAC AAC ACG GCC      966
Arg Asp Arg Asp Asp Ser Leu Val Cys Asp Cys Arg His Asn Thr Ala
                300                 305                 310

GGC CCG GAG TGC GAC CGC TGC AAG CCC TTC CAC TAC GAC CGG CCC TGG      1014
Gly Pro Glu Cys Asp Arg Cys Lys Pro Phe His Tyr Asp Arg Pro Trp
            315                 320                 325

CAG CGC GCC ACA GCC CGC GAA GCC AAC GAG TGC GTG GCC TGT AAC TGC      1062
Gln Arg Ala Thr Ala Arg Glu Ala Asn Glu Cys Val Ala Cys Asn Cys
        330                 335                 340

AAC CTG CAT GCC CGG CGC TGC CGC TTC AAC ATG GAG CTC TAC AAG CTT      1110
Asn Leu His Ala Arg Arg Cys Arg Phe Asn Met Glu Leu Tyr Lys Leu
    345                 350                 355

TCG GGG CGC AAG AGC GGA GGT GTC TGC CTC AAC TGT CGC CAC AAC ACC      1158
Ser Gly Arg Lys Ser Gly Gly Val Cys Leu Asn Cys Arg His Asn Thr
360                 365                 370                 375

GCC GGC CGC CAC TGC CAT TAC TGC AAG GAG GGC TAC TAC CGC GAC ATG      1206
Ala Gly Arg His Cys His Tyr Cys Lys Glu Gly Tyr Tyr Arg Asp Met
                380                 385                 390

GGC AAG CCC ATC ACC CAC CGG AAG GCC TGC AAA GCC TGT GAT TGC CAC      1254
Gly Lys Pro Ile Thr His Arg Lys Ala Cys Lys Ala Cys Asp Cys His
            395                 400                 405

CCT GTG GGT GCT GCT GGC AAA ACC TGC AAC CAA ACC ACC GGC CAG TGT      1302
Pro Val Gly Ala Ala Gly Lys Thr Cys Asn Gln Thr Thr Gly Gln Cys
        410                 415                 420

CCC TGC AAG GAC GGC GTG ACG GGT ATC ACC TGC AAC CGC TGC GCC AAA      1350
Pro Cys Lys Asp Gly Val Thr Gly Ile Thr Cys Asn Arg Cys Ala Lys
    425                 430                 435

GGC TAC CAG CAG AGC CGC TCT CCC ATC GCC CCC TGC ATA AAG ATC CCT      1398
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Gly Tyr Gln Gln Ser Arg Ser Pro Ile Ala Pro Cys Ile Lys Ile Pro
440                 445                 450                 455

GTA GCG CCG CCG ACG ACT GCA GCC AGC AGC GTG GAG GAG CCT GAA GAC      1446
Val Ala Pro Pro Thr Thr Ala Ala Ser Ser Val Glu Glu Pro Glu Asp
                460                 465                 470

TGC GAT TCC TAC TGC AAG GCC TCC AAG GGG AAG CTG AAG ATT AAC ATG      1494
Cys Asp Ser Tyr Cys Lys Ala Ser Lys Gly Lys Leu Lys Ile Asn Met
            475                 480                 485

AAA AAG TAC TGC AAG AAG GAC TAT GCC GTC CAG ATC CAC ATC CTG AAG      1542
Lys Lys Tyr Cys Lys Lys Asp Tyr Ala Val Gln Ile His Ile Leu Lys
        490                 495                 500

GCG GAC AAG GCG GGG GAC TGG TGG AAG TTC ACG GTG AAC ATC ATC TCC      1590
Ala Asp Lys Ala Gly Asp Trp Trp Lys Phe Thr Val Asn Ile Ile Ser
    505                 510                 515

GTG TAT AAG CAG GGC ACG AGC CGC ATC CGC CGC GGT GAC CAG AGC CTG      1638
Val Tyr Lys Gln Gly Thr Ser Arg Ile Arg Arg Gly Asp Gln Ser Leu
520                 525                 530                 535

TGG ATC CGC TCG CGG GAC ATC GCC TGC AAG TGT CCC AAA ATC AAG CCC      1686
Trp Ile Arg Ser Arg Asp Ile Ala Cys Lys Cys Pro Lys Ile Lys Pro
                540                 545                 550

CTC AAG AAG TAC CTG CTG CTG GGC AAC GCG GAG GAC TCT CCG GAC CAG      1734
Leu Lys Lys Tyr Leu Leu Leu Gly Asn Ala Glu Asp Ser Pro Asp Gln
            555                 560                 565

AGC GGC ATC GTG GCC GAT AAA AGC AGC CTG GTG ATC CAG TGG CGG GAC      1782
Ser Gly Ile Val Ala Asp Lys Ser Ser Leu Val Ile Gln Trp Arg Asp
        570                 575                 580

ACG TGG GCG CGG CGG CTG CGC AAG TTC CAG CAG CGT GAG AAG AAG GGC      1830
Thr Trp Ala Arg Arg Leu Arg Lys Phe Gln Gln Arg Glu Lys Lys Gly
    585                 590                 595

AAG TGC AAG AAG GCC TAG                                             1848
Lys Cys Lys Lys Ala
600
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 604 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Met Arg Ala Val Trp Glu Ala Leu Ala Ala Leu Ala Ala Val Ala
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1               5                  10                  15
Cys Leu Val Gly Ala Val Arg Gly Gly Pro Gly Leu Ser Met Phe Ala
            20                  25                  30
Gly Gln Ala Ala Gln Pro Asp Pro Cys Ser Asp Glu Asn Gly His Pro
        35                  40                  45
Arg Arg Cys Ile Pro Asp Phe Val Asn Ala Ala Phe Gly Lys Asp Val
    50                  55                  60
Arg Val Ser Ser Thr Cys Gly Arg Pro Pro Ala Arg Tyr Cys Val Val
65                  70                  75                  80
Ser Glu Arg Gly Glu Glu Arg Leu Arg Ser Cys His Leu Cys Asn Ala
                85                  90                  95
Ser Asp Pro Lys Lys Ala His Pro Pro Ala Phe Leu Thr Asp Leu Asn
            100                 105                 110
Asn Pro His Asn Leu Thr Cys Trp Gln Ser Glu Asn Tyr Leu Gln Phe
        115                 120                 125
Pro His Asn Val Thr Leu Thr Leu Ser Leu Gly Lys Lys Phe Glu Val
    130                 135                 140
Thr Tyr Val Ser Leu Gln Phe Cys Ser Pro Arg Pro Glu Ser Met Ala
145                 150                 155                 160
Ile Tyr Lys Ser Met Asp Tyr Gly Arg Thr Trp Val Pro Phe Gln Phe
                165                 170                 175
Tyr Ser Thr Gln Cys Arg Lys Met Tyr Asn Arg Pro His Arg Ala Pro
            180                 185                 190
Ile Thr Lys Gln Asn Glu Gln Glu Ala Val Cys Thr Asp Ser His Thr
        195                 200                 205
Asp Met Arg Pro Leu Ser Gly Gly Leu Ile Ala Phe Ser Thr Leu Asp
    210                 215                 220
Gly Arg Pro Ser Ala His Asp Phe Asp Asn Ser Pro Val Leu Gln Asp
225                 230                 235                 240
Trp Val Thr Ala Thr Asp Ile Arg Val Ala Phe Ser Arg Leu His Thr
                245                 250                 255
Phe Gly Asp Glu Asn Glu Asp Asp Ser Glu Leu Ala Arg Asp Ser Tyr
            260                 265                 270
Phe Tyr Ala Val Ser Asp Leu Gln Val Gly Gly Arg Cys Lys Cys Asn
        275                 280                 285
Gly His Ala Ala Arg Cys Val Arg Asp Arg Asp Asp Ser Leu Val Cys
    290                 295                 300
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Asp Cys Arg His Asn Thr Ala Gly Pro Glu Cys Asp Arg Cys Lys Pro
305                 310                 315                 320

Phe His Tyr Asp Arg Pro Trp Gln Arg Ala Thr Ala Arg Glu Ala Asn
                    325                 330                 335

Glu Cys Val Ala Cys Asn Cys Asn Leu His Ala Arg Arg Cys Arg Phe
            340                 345                 350

Asn Met Glu Leu Tyr Lys Leu Ser Gly Arg Lys Ser Gly Gly Val Cys
        355                 360                 365

Leu Asn Cys Arg His Asn Thr Ala Gly Arg His Cys His Tyr Cys Lys
    370                 375                 380

Glu Gly Tyr Tyr Arg Asp Met Gly Lys Pro Ile Thr His Arg Lys Ala
385                 390                 395                 400

Cys Lys Ala Cys Asp Cys His Pro Val Gly Ala Ala Gly Lys Thr Cys
                    405                 410                 415

Asn Gln Thr Thr Gly Gln Cys Pro Cys Lys Asp Gly Val Thr Gly Ile
            420                 425                 430

Thr Cys Asn Arg Cys Ala Lys Gly Tyr Gln Gln Ser Arg Ser Pro Ile
        435                 440                 445

Ala Pro Cys Ile Lys Ile Pro Val Ala Pro Pro Thr Thr Ala Ala Ser
    450                 455                 460

Ser Val Glu Glu Pro Glu Asp Cys Asp Ser Tyr Cys Lys Ala Ser Lys
465                 470                 475                 480

Gly Lys Leu Lys Ile Asn Met Lys Lys Tyr Cys Lys Lys Asp Tyr Ala
                    485                 490                 495

Val Gln Ile His Ile Leu Lys Ala Asp Lys Ala Gly Asp Trp Trp Lys
            500                 505                 510

Phe Thr Val Asn Ile Ile Ser Val Tyr Lys Gln Gly Thr Ser Arg Ile
        515                 520                 525

Arg Arg Gly Asp Gln Ser Leu Trp Ile Arg Ser Arg Asp Ile Ala Cys
    530                 535                 540

Lys Cys Pro Lys Ile Lys Pro Leu Lys Lys Tyr Leu Leu Leu Gly Asn
545                 550                 555                 560

Ala Glu Asp Ser Pro Asp Gln Ser Gly Ile Val Ala Asp Lys Ser Ser
                    565                 570                 575

Leu Val Ile Gln Trp Arg Asp Thr Trp Ala Arg Arg Leu Arg Lys Phe
            580                 585                 590

Gln Gln Arg Glu Lys Lys Gly Lys Cys Lys Lys Ala
        595                 600
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WHAT IS CLAIMED IS:

1. An isolated netrin comprising the amino acid sequence of SEQ ID NO:2.

2. An isolated polynucleotide encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:2.

3. An isolated polynucleotide comprising SEQ ID NO:1 or a fragment thereof at least 100 nucleotides in length.

4. An isolated polynucleotide according to claim 3, comprising the nucleotide sequence of SEQ ID NO:1.

5. An isolated polynucleotide comprising a sequence fully complementary to SEQ ID NO:1 or a fragment thereof at least 100 nucleotides in length.

6. An isolated polynucleotide according to claim 5, comprising a sequence fully complementary to SEQ ID NO:1.

7. Use, in the manufacture of an agent, of a netrin according to claim 1 for modulating the growth, differentiation or morphology of a neuron.

8. A method of determining the presence of a human netrin gene in a sample comprising a nucleic acid, said method comprising contacting said sample with a polynucleotide according to claim 3 or 5 and detecting the presence of specific hybridization of said polynucleotide to said nucleic acid.

9. A method of determining the presence of a human netrin having the amino acid sequence of SEQ ID NO:2 in a sample, said method comprising contacting said sample with an antibody which specifically binds said netrin and detecting the presence of specific binding of said agent to said netrin.

10. A method of screening for candidate drugs, said method comprising the steps of:

contacting a prospective agent with a mixture comprising a netrin according to claim 1 and a netrin binding target under conditions wherein, but for the presence of said prospective agent, said netrin and said netrin binding target have a first association;

detecting a second association between said netrin and said netrin binding target;

comparing said first and second association;

wherein a difference between said first and second association identifies said prospective agent as a lead pharmaceutical compound which modulates the interaction of said netrin and said netrin binding target.