incubation for 1 h at room temperature. After washing with PBS containing 0.2 mg ml⁻¹ BSA, specific counts were measured. The IGOR program was used to determine K_d . Cell-based equilibrium binding analysis⁹ was used to confirm the specificity of GDNFR- α and NTNR- α for GDNF and NTN, respectively. Each binding experiment was repeated at least 4 times. For PIPLC analysis, 293 cells transiently transfected with either the full-length NTNR- α expression construct or an irrelevant plasmid were incubated with ~20,000 c.p.m. ¹²⁵I-hNTN in the presence of the indicated amounts of PIPLC for 30 min at room temperature. The cells were washed with ice-cold PBS containing 0.2 mg ml⁻¹ BSA, and cell-associated ¹²⁵I was measured.

Survival assays. E14 rat motor neurons were purified, plated and grown in duplicate wells in L15 medium with the N2 supplement and 2% horse serum²⁸. After 3 days in culture, only 30–40% of the motor neurons initially present survived in basal medium, whereas at saturating concentrations of GDNF or BDNF nearly all motor neurons remained alive. The increase in the number of surviving neurons in the presence of saturating concentrations of GDNF compared to basal medium was taken as 100% (maximal) survival; percentage of maximal survival under the indicated conditions is shown in Fig. 3b. PIPLC (2 μ g ml⁻¹) was added to the indicated samples 2 h before, as well as 12 h and 24 h after, addition of the indicated growth factors.

Tyrosine phosphorylation. To assay for tyrosine phosphorylation, cells were incubated for 1 h at 37°C with or without PIPLC and then exposed to NTN with or without soluble NTNR- α (10 μ g ml⁻¹) for 5–10 min at 37°C. Cells were then removed from the plates with 2 mM EDTA in PBS and lysed with ice-cold buffer (comprising, in mM 10 sodium phosphate (pH 7.0), 100 NaCl, 1% NP40, 5 EDTA, 100 sodium vanadate, 2 mM PMSE, and 0.2 units of aprotinin) and used for immunoprecipitation with antiserum raised against the 19 amino-acid C terminus of Ret, following by binding to protein A–Sepharose. The immunoprecipitated proteins were released by boiling in SDS sample buffer, separated on an 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and reacted with anti-phosphotyrosine antibody (Upstate Biotechnology). Detection was with an ECL western blotting detection system (Amersham Life Science).

Co-immunoprecipitation. To examine the formation of protein complexes, TGW-I-nu cell were exposed to 500 ng ml⁻¹ of NTN. Protein complexes were immunoprecipitated with Ret antibodies, transferred onto a nitrocellulose filter, and analysed with polyclonal antibody against NTN. Alternatively, HEK 293 cells were transiently transfected with expression vectors for Ret, an epitope-tagged NTNR- α , or a combination of an epitope-tagged NTNR- α and Ret. Cells were stimulated with NTN as indicated and lysed with brij 96 detergent (Fluka)¹⁶. Putative immune complexes were immunoprecipitated with a polyclonal antibody against Ret, transferred onto a nitrocellulose filter, and analysed with a monoclonal antibody against the epitope-tagged NTNR- α .

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Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase

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Neurturin (NTN)¹ is a recently identified homologue of glial-cell-line-derived neurotrophic factor (GDNF)². Both factors promote the survival of a variety of neurons^{1–5}, and GDNF is required for the development of the enteric nervous system and kidney^{6–8}. GDNF signals through a receptor complex consisting of the receptor tyrosine kinase Ret and a glycosyl-phosphatidylinositol (GPI)-linked receptor termed GDNFR- α ^{9–13}. Here we report the cloning of a new GPI-linked receptor termed NTNR- α that is homologous with GDNFR- α and is widely expressed in the nervous system and other tissues. By using microinjection to introduce expression plasmids into neurons, we show that coexpression of NTNR- α with Ret confers a survival response to neurturin but not GDNF, and that coexpression of GDNFR- α with Ret confers a survival response to GDNF but not neurturin. Our findings indicate that GDNF and neurturin promote neuronal survival by signalling through similar multicomponent receptors that consist of a common receptor tyrosine kinase and a member of a GPI-linked family of receptors that determines ligand specificity.

GDNFR- α	<u>MFLALLYLALPLADVLL-S--AEVSGLPGGDRL-----D</u> <u>NKASDQCL</u>	40
NTNR- α	<u>MILANAFECIVLFVDET</u> <u>LRSLAAPP</u> <u>S-PPGQD-LQGWRVPVD</u> <u>IRANKLCA</u>	48
GDNFR- α	KEQSSTKYRTLROCVAGKESNFSRATGLEA-KDECKSAMEALKQKS-LY	88
NTNR- α	AEGSSSRYSRTLROCLAGRDRN---TML-ANK-ECDAALEVL-QESPLY	91
GDNFR- α	NCRCKRGMKKEKNCLRIYWSMYQSL-QGNDLLEDSPYEPVNSRLSDIFRL	137
NTNR- α	DRCCKRGMKKEIQLQVYWSIHLGLAEGEEFYEASPYEPITSRLSDIFRL	141
GDNFR- α	APIVSV-EPVL-SKGNCLDAAKANLNDTKRFRSAYITPISSTS-NE	184
NTNR- α	ASIFSGMDPATNSKSNHCLDAAKANLNDNCKRLRSGYISTCSKEISATE	191
GDNFR- α	ICNKRCKHKALRLFFDKVPKHSYGMLEFCSCRDVACTERRQTIVPVCSY	234
NTNR- α	HCSSRRCKHKALRQFFDNVPSEYTYRLFCSCKDQCAEPRRTIVPVCSY	241
GDNFR- α	EDREKPNCLNLQESCKKNYICRSRLADFFTNCOPESSRVSSCLKENYADC	284
NTNR- α	EDKEKPNCLDLRNVCRADHLCRSRLADFHANCOASFQSLTSCPGDNYQAC	291
GDNFR- α	LLAYSGLIGTVMTPNYIDSSSLSV--APWCCSNGNDIDECRKFLNFFQ	332
NTNR- α	LGSYTGLIGFDMTPNYVDASTTITISPWCSCKGSGNLEEECEKFLRDFT	341
GDNFR- α	DNTCLKNAIQAFNGTVDNVW--QIPLPVQTTTATTTASRLKNTGSETT	380
NTNR- α	ENPCCLRNAIQAFNGTVDNLSPKNPSPI-TMLPKVEK-SPALPDDINDS	389
GDNFR- α	NNEIPTHNDSPACANLQAQKKRKSNEVSVDTECLNENAIGKDNTPGVSTS	430
NTNR- α	NTMYDTSI-ITTTTSIQ-EHGQKLKSKESQSLCYSETQLTTDTPDQKTF	437
GDNFR- α	HISSENSFALPTS-FYPSTPLILMTIALSLFLFLSSSVVL	469
NTNR- α	VDQKAAGSRHRAARILPAVPIVLLKLLL	465

Figure 1 Aligned sequences of chicken GDNFR- α and NTNR- α . Identical amino acids are marked with asterisks and the conserved cysteines are enclosed in vertical boxes. The N-terminal, hydrophobic, putative signal peptides are underlined; the C-terminal hydrophobic domain is double-underlined; and the putative binding/cleavage consensus sequences for GPI linkage are enclosed in horizontal boxes. GenBank accession numbers: GDNFR- α , U90541; NTNR- α , U90542.

We have screened an embryonic-day-10 (E10) chicken brain cDNA library at low stringency using the full coding region of mouse GDNFR- α as a probe, and isolated two sets of overlapping cDNAs clones encoding chicken GDNFR- α and a GDNFR- α homologue designated, for reasons given below, neurturin receptor α (NTNR- α) (Fig. 1). The predicted chicken GDNFR- α is a protein of 469 amino acids which has 80% identity with rodent GDNFR- α . NTNR- α is a protein of 465 amino acids that has 49% identity with chicken GDNFR- α , including conservation of all 30 cysteines. Both GDNFR- α and NTNR- α possess an amino-terminal, hydrophobic, putative signal peptide for secretion, and the characteristic carboxy-terminal feature of GPI-linked proteins: a C-terminal hydrophobic domain separated by a hydrophilic linker region from a cleavage/binding consensus sequence for GPI linkage (Ser-Thr-Ser for GDNFR- α , and Ala-Gly-Ser for NTNR- α)¹⁴. These features suggest that both GDNFR- α and NTNR- α are secreted proteins attached to the extracellular surface of the plasma membrane.

To ascertain the potential involvement of NTNR- α in the survival responses of developing neurons to neurotrophic factors and to compare its function with that of GDNFR- α , we used microinjection to introduce expression plasmids encoding NTNR- α and GDNFR- α into cultured neurons that do not survive with either GDNF or neurturin. From a survey of several different populations of neurons, we found that sympathetic neurons of the superior cervical sympathetic ganglion (SCG) of postnatal day 4 (P4) mice are not supported by either neurturin or GDNF in culture. Reverse

transcription-polymerase chain reaction (RT-PCR) analysis showed that these neurons do not express endogenous messenger RNAs encoding NTNR- α , GDNFR- α or Ret, whereas mouse nodose neurons, which survive in response to GDNF and neurturin, express these mRNAs (Fig. 2a). Furthermore, because postnatal mouse SCG neurons die rapidly in defined medium lacking neurotrophic factors, they are ideal for examining the involvement of ectopically expressed receptors in mediating the responses of neurotrophic factors.

Ectopic expression of either NTNR- α or GDNFR- α alone in SCG neurons has a negligible effect on their survival (Fig. 2b) and does not confer a survival response to either GDNF or neurturin. This is consistent with the idea that GPI-linked receptors cannot mediate responses to their ligands without the appropriate transmembrane signalling proteins, such as Ret in the case of GDNFR- α ^{9,10} and gp130 and LIFR- β in the case of CNTFR- α ¹⁵.

Because Ret is an essential signalling component of the GDNF receptor complex⁹⁻¹³, we co-injected neurons with expression plasmids for Ret and either NTNR- α or GDNFR- α to see whether neurons expressing both of these receptor components would exhibit specific survival responses to GDNF and neurturin. Ectopic expression of Ret alone promoted the survival of approximately 10% of SCG neurons in the absence of added factors (Fig. 2b). However, neither GDNF nor neurturin significantly increased the survival of Ret-expressing neurons ($P > 0.05$, t -test, $n = 6$), suggesting that Ret alone is not capable of mediating survival responses

Figure 2 a, RT-PCR detection of endogenous *c-Ret*, *GDNFR- α* and *NTNR- α* transcripts in mouse P4 SCG neurons (which do not respond to GDNF and neurturin) and P4 mouse nodose neurons (which do respond to these factors). The presence of similar amount of the total RNA in samples is demonstrated by amplification of the 437-bp fragment of *GAPDH* mRNA. **b**, Survival of P4 mouse

SCG neurons 48 h after injection with expression plasmids for *GDNFR- α* and *NTNR- α* and *Ret*, singularly and in combination. The injected neurons were incubated in medium containing either neurturin or GDNF. Results are means ($\pm$ s.e.) of the combined results of 8 separate experiments ($n \geq 4$ for each experiment condition).

Figure 3 Survival of E6 chicken nodose ganglion neurons grown with either neurturin or BDNF for 36 h. Half of the cultures were treated with PIPLC. Results show mean ($\pm$ s.e.) percentage survival ($n = 4$).

to GDNF and neurturin. However, neurons coexpressing *NTNR- α* plus *Ret* had a substantially enhanced survival response to neurturin which was significantly greater than that of neurons expressing *Ret* alone ($P = 0.003$, t -test, $n = 6$). Similarly, neurons coexpressing *GDNFR- α* plus *Ret* had a substantially enhanced survival response to GDNF which was significantly greater than that of neurons expressing *Ret* alone ($P = 0.0002$, t -test, $n = 8$). In contrast, neurons coexpressing *GDNFR- α* plus *Ret* showed no enhanced survival response to neurturin; there were no more neurons expressing *Ret/GDNFR- α* surviving with neurturin than neurons expressing *Ret*. Similarly, the number of neurons expressing *Ret/NTNR- α* surviving with GDNF was not significantly different from the number of neurons expressing *Ret* surviving with this factor ($P > 0.2$, t -test, $n = 8$). These results imply that *GDNFR- α* and *NTNR- α* are receptors for GDNF and neurturin, respectively, and that *Ret* is required for signalling and the survival response of neurons to both factors. Although *GDNFR- α* and *NTNR- α* in the presence of *Ret* conferred specific survival responses to GDNF and neurturin at concentrations up to 5 ng ml^{-1} , at 10-fold higher concentrations of these factors some cross-talk in responsiveness was observed (see Supplementary Information).

To investigate further whether a GPI-linked protein is normally required for the survival response of neurons to neurturin, we treated neurturin-responsive embryonic chicken nodose ganglion neurons with phosphoinositide-specific phospholipase C (PIPLC), an enzyme that specifically cleaves GPI linkages¹⁶. PIPLC treatment

reduced the number of neurons surviving with neurturin from over 70% to less than 10% (Fig. 3). This was not due to a neurotoxic effect of PIPLC, because the survival response of nodose neurons to brain-derived neurotrophic factor (BDNF), which signals through the transmembrane *TrkB* receptor tyrosine kinase¹⁷, was unaffected by PIPLC treatment (Fig. 3). Similarly, the neurturin survival response of P4 SCG neurons coexpressing *Ret* and *NTNR- α* was significantly reduced from $30.6 \pm 2.9\%$ to $9.2 \pm 3.0\%$ by PIPLC treatment ($P < 0.01$, t -test, $n = 4$). The survival of these PIPLC-treated neurons was not significantly different from the survival of neurons expressing *Ret* alone ($13.3 \pm 2.2\%$, $P > 0.05$). This indicates that PIPLC treatment had eliminated the specific neurturin survival response in these neurons, which in turn demonstrates that ectopically expressed *NTNR- α* is required for this response.

Northern blotting was used to study the expression of *GDNFR- α* and *NTNR- α* transcripts in chicken embryos at different developmental stages (Fig. 4). A 10-kilobase (kb) *GDNFR- α* transcript and 3.4-kb and 2.8-kb *NTNR- α* transcripts were expressed in the nervous system and several other tissues. Regional and developmental differences in the levels of these transcripts were apparent in these tissues.

In summary, we have identified a specific GPI-linked receptor for neurturin that is structurally related to *GDNFR- α* . We have shown that the survival responses of neurons to neurturin and GDNF require *NTNR- α* and *GDNFR- α* , respectively, together with a common transmembrane receptor tyrosine kinase, *Ret*. Our

Figure 4 Analysis of the expression of *GDNFR-α* and *NTNR-α* mRNAs by northern blotting. Similar amounts of total RNA from various embryonic tissues at E10 (top) and skin and regions of the central nervous system (bottom) were transferred to filters and hybridized sequentially with *GDNFR-α* and *NTNR-α* probes. Bands corresponding to the 10-kb *GDNFR-α* and 3.5-kb and 2.8-kb *NTNR-α* transcripts are indicated. Sp. Cord, spinal cord.

demonstration that *NTNR-α* and *GDNFR-α* are required for ligand discrimination in the physiological responses of neurons to neurturin and GDNF is consistent with the results of binding studies which show that *NTNR-α* and *GDNFR-α* are specific high-affinity receptors for neurturin and GDNF, respectively¹⁸. Furthermore, because we investigated the actions of mammalian neurturin and GDNF on avian receptors, our study also shows that the specific, functional interactions between these neurotrophic factors and their corresponding GPI-linked receptors have been conserved between two classes of vertebrates. Previous studies have identified several unrelated GPI-linked, ligand-binding components of multicomponent receptors, such as the CNTF receptor¹⁵ and the bacterial endotoxin receptor^{19,20}. However, we have established the existence of a family of GPI-linked proteins that discriminate between structurally related ligands in multicomponent receptors that share a common transmembrane signalling component. □

Methods

Receptor cloning and expression. An E10 chicken brain cDNA library in λZAPII was screened²¹ using a mouse GDNF cDNA probe at low stringency (hybridization at 59 °C, final wash at 65 °C in 2X SSC, 0.2% SDS). Five *GDNFR-α* and three *NTNR-α* cDNA independent clones were sequenced. Northern blot analysis²¹ used unique regions of the *GDNFR-α* and *NTNR-α* cDNAs as specific probes. For RT-PCR analysis of *GDNFR-α*, *NTNR-α* and *c-Ret* mRNA expression in cultured mouse neurons, total RNA was reverse transcribed and amplified²². The amounts of total RNA in samples were normalized by amplification of a 437-bp fragment of glyceraldehyde-3-phosphate dehydrogenase mRNA²². The primers were as follows: *c-Ret*, 5'-TGTATGTAGAC-CAGCCAGCT-3' and 5'-ACTATGCACAAAGCCTCCAG-3'; *GDNFR-α*, 5-

CATGTTCTAGCCACTCTGT-3' and 5'-TCCAGTAGGTCATTTCCCTG-3'; *NTNR-α*, 5'-ATGATCTTGGCAAACGCCCTTCTG-3' and 5'-GCTTCATA-GAACTCCTCACCCTC-3'. Amplification was carried out for 40 cycles of 95 °C for 45 s; 54 °C for 30 s; and 72 °C for 60 s.

Neuronal culture and microinjection studies. Purified P4 SCG neurons were grown in defined medium²³. After incubation for 12 h with NGF (2 ng ml⁻¹), the neurons were washed extensively to remove NGF and injected with expression plasmids into the nucleus (*GDNFR-α* cDNA and *NTNR-α* cDNA in pMEX, and *c-Ret* cDNA in pRC/CMV) as described²⁴. The cultures were supplemented with either GDNF or neurturin immediately after injection, and the number of surviving neurons was counted 48 h later and is expressed as a percentage of the number injected. To investigate the importance of GPI-linked proteins in neurturin responsiveness, neurturin-responsive E6 chicken nodose neurons were cultured in 11-mm diameter wells²⁵. BDNF (5 ng ml⁻¹) or neurturin (5 ng ml⁻¹) was added to the cultures 1.5 h after plating, and half of the cultures were supplemented with PIPLC (4 μg ml⁻¹) at the time of plating and after a further 12 and 24 h (ref. 9). The number of surviving neurons was counted at 36 h, and is expressed as a percentage of the number plated.

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Supplementary information is available on Nature's World Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

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