The single AmphiTrk receptor highlights increased complexity of neurotrophin signalling in vertebrates and suggests an early role in developing sensory neuroepidermal cells

Èlia Benito-Gutiérrez[1], Christian Nake[2], Marta Llovera[2], Joan X. Comella[2,*] and Jordi Garcia-Fernàndez[1,*,†]

[1]Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Avinguda Diagonal, 645, E-08028, Barcelona, Spain
[2]Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina, Universitat de Lleida, Avinguda Rovira Roure 44, E-25198, Lleida, Spain

*These authors contributed equally to this work
†Author for correspondence (e-mail: jordigarcia@ub.edu)

Accepted 24 February 2005

Development 132, 2191-2202
Published by The Company of Biologists 2005
doi:10.1242/dev.01803

Summary

Neurotrophins (Nt) and their tyrosine kinase Trk receptors play an essential role in the development and maintenance of the complex vertebrate nervous system. Invertebrate genome sequencing projects have suggested that the Nt/Trk system is a vertebrate innovation. We describe the isolation and characterisation of the amphioxus Trk receptor, *AmphiTrk*. Its ancestral link to vertebrate Trk receptors is supported by phylogenetic analysis and domain characterisation. The genomic structure of *AmphiTrk* strongly suggests that a *ProtoTrk* gene emerged by means of exon-shuffling prior to the cephalochordate/vertebrate split. We also examined the physiological response of AmphiTrk to vertebrate neurotrophins, and found that despite 500 million years of divergence, AmphiTrk transduces signals mediated by NGF, BDNF, NT3 and NT4. Markedly, AmphiTrk is able to activate survival and differentiation pathways, but fails to activate the PLCγ pathway, which is involved in synaptic plasticity in higher vertebrates. *AmphiTrk* is expressed during amphioxus embryogenesis in sensory neural precursors in the epidermis, which possesses single migratory cells. We propose that the duplication and divergence of the Nt/Trk system, in tandem with recruitment of the PLCγ pathway, may have provided the genetic basis for a key aspect of vertebrate evolution: the complexity of the nervous system.

Key words: Amphioxus, Exon shuffling, Vertebrate transition, Nervous system, Neurotrophic activity

Introduction

Neurotrophins (Nt) and their tyrosine kinase Trk receptors are gene families deeply involved in the development of complex traits within the vertebrate nervous system, where they regulate neuronal survival, axonal growth and guidance, synaptic plasticity and long-term potentiation events (Huang and Reichardt, 2001; Poo, 2001). In mammalians, four distinct neurotrophins (NGF, BDNF, NT3 and NT4) activate three different Trk receptors (TrkA, TrkB and TrkC) with a significant degree of specificity, leading to a variety of finely controlled biological processes (Ibañez, 1998).

A deep understanding of the basal role of the Nt/Trk system is hampered by the presence of multiple members in vertebrates. The study of homologous gene families in simpler systems, devoid of high genetic redundancy, often has led to a better understanding of complex functions in vertebrates (Chao, 2000). Nevertheless, the neurotrophic field currently lacks non-vertebrate representatives (Hallböök, 1999). Genome sequencing projects have shown that invertebrate model systems, such as *Drosophila* or *C. elegans*, do not possess either Nt or Trk homologues (Adams et al., 2000; The C. elegans Sequencing Consortium, 1998). The formerly claimed *Drosophila* Trk receptor (Pulido et al., 1992) has recently been discarded as a Trk homologue, and re-named *off-track* (Winberg et al., 2001). Until now, the closest invertebrate Trk-related molecule was the molluscan LTrk receptor, but its extracellular domain features a non-vertebrate structure (van Kesteren et al., 1998). Furthermore, the absence of Nt and Trk in the genome of the ascidian *Ciona intestinalis* has recently led to the contention that Nt/Trk signalling system may well be a vertebrate innovation (Dehal et al., 2002).

The origin of vertebrates dates back 530-550 million years from a lancelet-like relative (Zimmer, 2000). Present-day lancelets (amphioxus) are in the appropriate place to illuminate the critical transition towards vertebrate complexity. Amphioxus (Cephalochordata) possesses a vertebrate-like body plan, but lacks many of the complex features of vertebrates, including a complex nervous system. Remarkably, the simplicity of the amphioxus body plan is mirrored by the simplicity of its genome: it escaped the extensive gene duplication events that took place coincidentally with the origins of vertebrates and the early stages of vertebrate evolution (Furlong and Holland, 2002).

Whether Nt/Trk signalling mechanisms are compulsory for evolving complex nervous systems, and whether their evolutionary appearance coincided with the origin of vertebrates, remains a subject of debate (Jaaro et al., 2001). We show that Trk receptors are not a vertebrate innovation, and

originated prior to the cephalochordate/vertebrate split. We report the isolation, molecular characterisation and expression of the single amphioxus Trk receptor, AmphiTrk. Its pro-orthology to vertebrate Trk receptors is strongly supported by both phylogenetic analysis and full-length protein domain structure. Its genomic structure suggests that the Trk gene emergence occurred by means of exon shuffling. We also investigate the physiological response of AmphiTrk to vertebrate neurotrophins. Developmental expression suggests an ancestral function for the Trk family in the formation of an ectodermal peripheral nervous system.

Materials and methods

Isolation of AmphiTrk

Degenerate oligonucleotides corresponding to sequences GDFGMSR and WMPPESI were designed based on conserved regions within the tyrosine kinase domain of vertebrate Trk receptors. PCR reactions (50 μl) were run on 50-100 ng of *Branchiostoma floridae* embryonic cDNA. PCR conditions were: 35 cycles of denaturation at 94°C for 20 seconds, annealing at 50-55°C for 30 seconds and 20 seconds of elongation at 72°C. PCR products were ligated into plasmid and sequenced. Two identical 90 bp fragments with high similarity to vertebrate Trk receptors were used to isolate full-length cDNAs via screening of an embryonic *B. floridae* cDNA library, generously given by J. Langeland. A *B. floridae* genomic library (Ferrier et al., 2000) was screened using the same probe for cDNA isolation. Further screenings with specific cDNA fragments allowed the isolation of a contiguous 24 kb region containing the complete transcribed sequence of *AmphiTrk*. For Southern blotting, 10 μg of genomic DNA from a single individual was digested with *Eco*RI or *Xba*I, and hybridised at medium stringency conditions with probes amplified from exons 4-5 or exon 10 of *AmphiTrk*.

Sequence and phylogenetic analysis

Public domain sequence tools were used to characterise the *AmphiTrk* gene and the peptide domains (http://www.cbs.dtu.dk/services/SignalP, http://www.expasy.ch/tools). Putative vertebrate and invertebrate orthologue sequences were retrieved from the GeneBank database. Alignment with AmphiTrk sequence was performed using ClustalX and bootstrapped trees were calculated by neighbour-joining method over 1000 replicas. Cladograms were visualised using TreeView V.1.5.3. For tyrosine kinase domain phylogenetic analysis, human and mouse ROR1 receptors were used as outgroups.

Functional assays

To express *AmphiTrk* in mammalian cultured cells, the complete coding region was cloned into a pCDNA3 vector. The chimaeric receptor rTrkA-AmphiTrk, including an HA epitope in its 5′ end, was generated by replacement of the intracellular domain of the rat TrkA (kindly provided by M. Zanca) with that of AmphiTrk through PCR amplification. Plasmids (3 μg) were transiently transfected into cultured PC12 nnr5 cells (Green et al., 1986), as described by Egea et al. (Egea et al., 1999). rTrkA and pcDNA3 were used as controls. DMEM medium containing 10% foetal calf serum (FCS) was changed 5 hours after transfection and cultures were left overnight. Cells were then serum-deprived for 12 hours before stimulation for 5 minutes with 10, 50 or 100 ng/ml of NGF (Sigma), BDNF, NT3 or NT4 (Alomone Laboratories). Cultures were then rinsed with ice-cold PBS and solubilised in lysis buffer (2% SDS, 125 mM Tris, pH 5.8). Protein was quantified with a BioRad-DC assay system. Total cell lysates were western blotted and immunodetected with anti-phospho-ERK or anti-phospho-Akt (Cell Signalling Technology). To control the protein content in each lane, membranes were stripped and re-probed with an anti-α-tubulin antibody (Sigma). For PLCγ immunoprecipitation, pc12nnr5 cells were stimulated as above with 100 ng/ml of NGF. Total cell lysates (500 μg) were incubated overnight with 1.5 μg of an anti-PLCγ-conjugated antibody. After immunoprecipitation with Protein G-sepharose beads, blots were immunodetected with anti-phospho-Tyr 4G10 (Cell Signalling Technology), stripped and re-probed with anti-PLCγ (BD Transduction Laboratories) as a control for immunoprecipitation efficiency. For neurite outgrowth assays, PC12nnr5 cells were plated onto polyornithine- and collagen-precoated 35-mm plates (1×10^6 cells/plate). Twenty-four-hour-old cell cultures were co-transfected with enhanced yellow fluorescent protein (EYFP) and the chimera rTrkA/AmphiTrk, AmphiTrk, rTrkA or the pcDNA3 vector as described above. Following overnight incubation, medium was changed and supplemented with NGF (50 ng/ml), and then renewed after three days. After 6 days, transfected cells were examined and those whose neurite lengths were twice the cell body diameter were counted as differentiated.

In situ hybridisation

Ripe *B. floridae* adults were collected from Old Tampa Bay (FL, USA) and induced to spawn by electric stimulation. Embryos and adults were obtained and fixed as described in Holland and Holland (Holland and Holland, 1993). Sense and antisense DIG-labelled probes were generated by in vitro transcription of the cDNA full-length coding region. Whole-mount in situ hybridisation and subsequent sectioning was performed in accordance with Benito-Gutiérrez et al. (Benito-Gutiérrez et al., 2005).

A novel method for in situ hybridisation of adult sections was specially developed, whereby fixed adults were dehydrated through an ethanol series, cut in three pieces and then prepared for embedding in paraplast (Sigma) by xylene series; xylene:ethanol (1:1), xylene, xylene:paraplast (1:1). Blocks were solidified and settled at 4°C for 12 hours prior to sectioning. Slides were immersed in 10% HCl for 5 minutes, washed with DEPC-water and immersed in acetone for 2 minutes before drying. They were then immediately covered with 2% silane-acetone (Sigma), dried and autoclaved. Serial sections (6 μm) were deposited on silane-coated slides, and settled at 45°C for 6 hours. Tissue slides were immersed in xylene and sections were rehydrated through an ethanol series. Tissue was digested using 1 μg/ml proteinase K in PBS for 30 minutes at 37°C and the reaction stopped by immersion in 0.2% glycine. Sections were refixed in 4% PFA-PBS for 20 minutes, and then immersed in 0.1 M triethanolamine with a posterior addition of 0.25% acetic anhydride. Slides were washed with PBS and then pre-hybridised for 3 hours at 60°C in 100 μg/ml heparin, 5×SSC, 0.1% Tween-20, 5 mM EDTA, 1×Denhardt's. A DIG-labelled probe (100 ng/ml) in pre-hybridisation buffer was added and incubated at 60°C for 14 hours. After hybridisation, slides were washed in 50% formamide/5×SSC/1% SDS (twice for 15 minutes each) and 50% formamide/2×SSC/1% SDS (twice for 15 minutes each), treated with 2 mg/ml RNAseA and 100 U/ml RNAseT1 in 2×SSC/0.1% Tween20 at 37°C for 30 minutes, and washed with 0.2×SSC/0.1% Tween20 (twice for 20 minutes each). DIG staining was performed following supplier recommendations (Roche). Slides were refixed in 4% PFA-PBS, immersed in 0.1% sodium azide/PBS and mounted in Mowiol.

In vivo DiI labelling

Hatching neurulae, which are extremely motile, were anesthetised by placing them in a reduced drop of seawater, causing oxygen deprivation. A glass capillary tube was filled with Fast DiI oil (D-3899, Molecular Probes) and immersed in seawater causing the dye to crystallise at the tip. Using a micromanipulator the crystallised dye was applied to the ventral surface of the immobilised hatching neurulae. Immediately after dye deposition, embryos were transferred to seawater, regaining movement and developing normally. At late neurula stage, embryos were fixed and photographed under a fluorescence rhodamine filter.

Results

Isolation and sequence analysis of the single amphioxus Trk receptor

Using a combination of degenerate PCR, cDNA and genomic screenings, we isolated the single gene coding for a tyrosine kinase Trk receptor, *AmphiTrk*, in the amphioxus *Branchiostoma floridae*. The cDNAs coded for a single open reading frame of 797 amino acids, with high similarity to vertebrate TrkA, TrkB and TrkC receptors (Fig. 1). All three degenerate PCR, medium-stringency cDNA and genomic screenings consistently produced the same sequence. To further confirm *AmphiTrk* was the only Trk gene in the amphioxus genome, Southern blots of genomic DNA from a single individual were hybridised with probes derived from exons encoding extracellular or intracellular domains (Fig. 2). One or two bands were detected in each lane, consistent with *AmphiTrk* being a single copy gene.

The in silico characterisation of AmphiTrk revealed a canonical vertebrate domain structure at every extracytoplasmic, transmembrane and intracytoplasmic level (Fig. 1A). Visually, the extracellular region of AmphiTrk shares a common architecture with the extracytoplasmic domain of vertebrate Trk receptors (Fig. 1A). It consists of two consecutive leucine-rich repeats (Fig. 1B, italics) flanked by cysteine-rich clusters (Fig. 1B, bold), followed by two immunoglobulin (Ig)-like domains (Fig. 1B, underlined). Leucine-rich motifs and cysteine-rich clusters have been reported to participate in ligand binding: TrkB splicing variants lacking leucine-rich motifs are unable to bind any TrkB ligands (Ninkina et al., 1997). However, the major interface for neurotrophin binding is the second Ig-like domain, where specific residues come into direct contact with the ligand (Wiesmann et al., 1999). Both Ig-like domains are of the C2 type in vertebrates and amphioxus, and the most C-terminal pole, which belongs to the second Ig-like domain, contains asparagine residues with structural roles for ligand-receptor interactions. AmphiTrk also possesses such residues (N355 and N364, Fig. 1B, dots).

On the cytoplasmic side, AmphiTrk includes a tyrosine kinase (TK) domain that contains all the key residues necessary to carry out its function as a catalytic receptor. Like its vertebrate counterparts, it contains the signature pattern of class II tyrosine kinase receptors [DIYSTDYYR (Fig. 1B, grey background)]. Within this short amino acid sequence, three tyrosine residues (Y676, Y680 and Y681; Fig. 1B, black background) constitute the putative auto-phosphorylation activation loop. A presumptive ATP-binding region is located at the N-terminal pole of the TK domain and contains a conserved lysine (K544, Fig. 1B, black background) responsible for binding ATP. In addition, two potential phosphorylation sites for cAMP/cGMP-dependent kinase proteins, lie in positions comparable to those in vertebrates: KIS at the juxtamembrane intracytoplasmic part preceding the TK domain; and RKFT following the activation loop within the

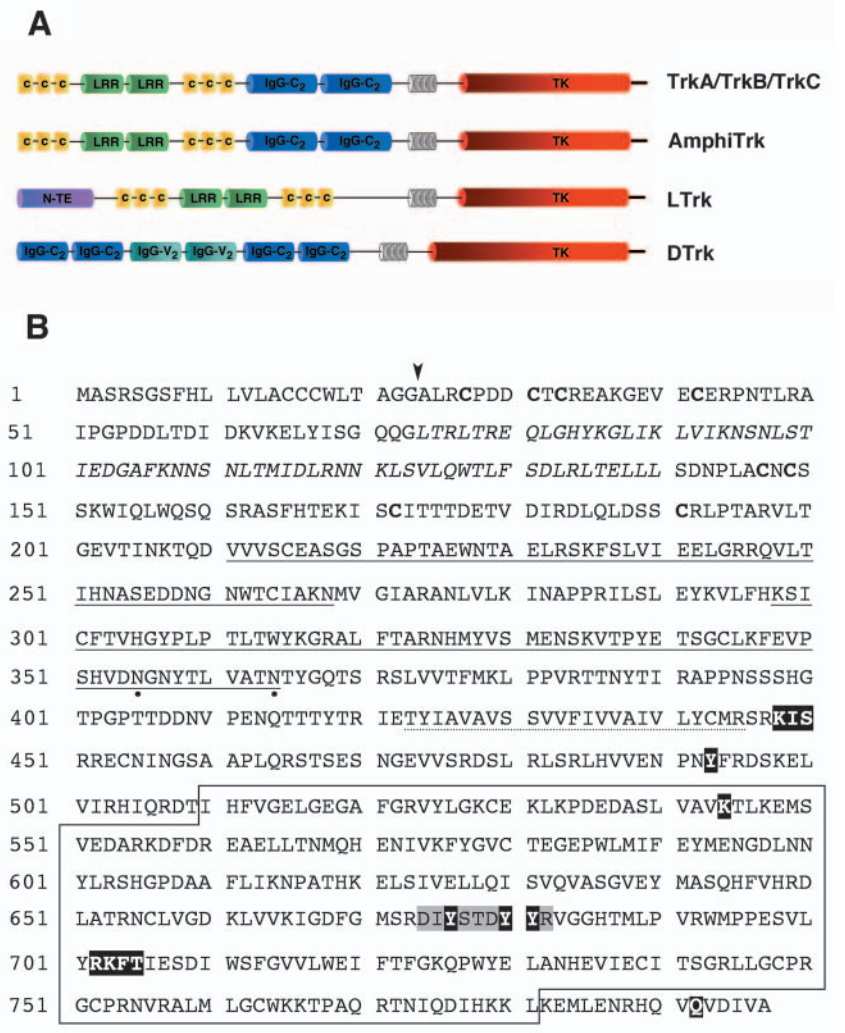


Fig. 1. (A) Domain structure comparison of AmphiTrk, vertebrate Trk receptors (TrkA, TrkB and TrkC) and representative invertebrate Trk-like proteins from *Lymnaea stagnalis* (LTrk) and *Drosophila melanogaster* (DTrk). Modules are colour coded: yellow, Cys-rich clusters; green, leucine-rich domains; blue, type C2 IgG domains; grey, transmembrane domain; red, tyrosine-kinase domain; purple, N-terminal extension; turquoise; type V2 IgG domains. (B) AmphiTrk amino acid sequence. Amino acid positions are numbered on the left. The putative signal sequence cleavage site is indicated by an arrowhead. Leucine-rich motifs are in italics and flanking cysteine clusters in bold. Both Ig-like domains are underlined; conserved asparagines with structural roles for ligand binding are indicated by dots. The transmembrane region is underlined by a dotted line. The first phosphorylation site by cAMP/cGMP-dependent kinase proteins, KIS, is shown with a black background, as is the tyrosine responsible for Shc recruitment. Within the tyrosine kinase domain (boxed), the lysine responsible for ATP binding and the second phosphorylation site by cAMP/cGMP-dependent kinase proteins, RKFT, are shown by a black background. The autophosphorylation sequence (DIYSTDYYR) is highlighted in grey and the autophosphorylated tyrosines are shown by a black background. Glutamine located in the same position as the vertebrate docking site for PLCγ is shown by a black background. *AmphiTrk* sequences have been deposited in the GeneBank under Accession Numbers AY902361-AY902364

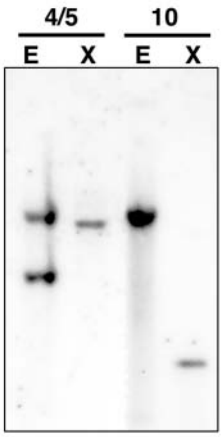


Fig. 2. Southern blot of a *Branchiostoma floridae* individual genomic DNA, digested with *Eco*RI (E) or *Xba*I (X). Hybridisations with probes derived from exons 4/5 or exon 10 show that *AmphiTrk* is a single copy gene.

TK domain (Fig. 1B, black background). In vertebrates, the former motif is a binding site for SNT, a protein involved in neuronal differentiation and neurite outgrowth pathways (Peng et al., 1995). Also present in amphioxus is the docking site for Shc, an adaptor protein which in vertebrates activates the Ras-Raf-Erk and PI3kinase-AKT signalling pathways involved in neuronal survival and differentiation events. It is identically placed, preceding the tyrosine kinase domain (Y493, Fig. 1B, black background). A distinctive feature of AmphiTrk resides at the furthest C terminus of the protein, outside the TK domain: a glutamine residue (Q792, Fig. 1B, black background). It occupies the position of a tyrosine (Y785), which in mammals serves as the docking site for PLCγ, whose transduction pathway leads to initiation and maintenance of long-term potentiation events (Huang and Reichardt, 2003).

Genomic structure

The genomic structure of the *AmphiTrk* gene was elucidated by screening a *Branchiostoma floridae* genomic library. Isolated genomic clones yielded an overlapped region of 21.4 kb, containing the complete transcriptional unit and flanking regions. The *AmphiTrk* transcriptional unit consists of 13 coding exons. Analysis of exonic domain distribution, and comparisons with the genomic structure of human TrkA, TrkB and TrkC, revealed a similar, but appreciably less fragmented, structure (Fig. 3A,B).

The first five exons exhibit the same domain arrangement as human Trk receptors. The signal peptide and the N-terminal cysteine-rich cluster are coded by the first exon, while the leucine-rich repeats are spread among exons 2, 3 and 4, leaving the C-terminal cysteine-rich cluster in exon 5. In contrast to human Trk proteins, each Ig-like domain in *AmphiTrk* is coded by a single exon (exons 6 and 7). In vertebrate Trk proteins, the first Ig-like domain is encoded by two exons. The extracellular juxtamembrane region is mainly encoded by exon 8 and the following transmembrane region by exon 9. The latter comprises the KIS motif and residue Y493, both located in the adjacent cytoplasmic part. This region around the transmembrane domain is structured differently in human Trk receptors, where analogous exons are split in two pairs (9/10 and 11/12). Interestingly, human exon 9 encodes a short amino acid sequence alternatively spliced in several of the isoforms described in vertebrate species. The presence or absence of this short amino acid sequence is tissue specific and affects ligand preference. Human and rat TrkAI isoforms, which lack six amino acids in this region (coded by exon 9), are expressed in non-neuronal tissues and bind only NGF, while TrkA-II isoforms, which contain the six amino acid insertion, are localised in neuronal tissues and can interact with both NGF and NT3 (Barker et al., 1993). The absence of this mini-exon in *AmphiTrk* suggests either that ligand flexibility is absent in amphioxus Trk, or that only one ligand molecule is present in amphioxus.

The TK domain and the intracytoplasmic C-terminus are distributed over four exons (10, 11, 12 and 13). Exons 11, 12 and 13 are indistinguishable between human and amphioxus in terms of structure and key residue distribution. However, exon 10 of *AmphiTrk* is split in two exons in humans (13 and 14), with a breakpoint centrally located in the ATP binding residue K544.

Although we cannot formally rule out the loss of some introns in the amphioxus lineage, it is more likely that four new introns were generated early in vertebrate evolution, because in *AmphiTrk* particular domains are encoded by fewer numbers of exons than occur in vertebrates. In summary, our data suggest that fragmentation occurred after the generation of a new modular protein, constructed through the shuffling of domain-encoding exons and predating the cephalochordate/vertebrate split.

Phylogenetic analysis

To establish AmphiTrk phylogenetic relationship with its vertebrate relatives and invertebrate Trk-related receptors, three different trees were generated. Trk receptors from rat, human and chicken were taken to avoid a biased mammalian representation; no other vertebrate species were included because of the incompleteness of available sequences. *Drosophila* Trk and *Limnaea* LTrk were used as Trk-related invertebrate representatives, as no other similar receptors have been reported in non-vertebrate clades.

All trees had similar topology and positioned AmphiTrk, with high bootstrap values at the base of, and equally related to, all three vertebrate Trk receptors (Fig. 4). When full-length

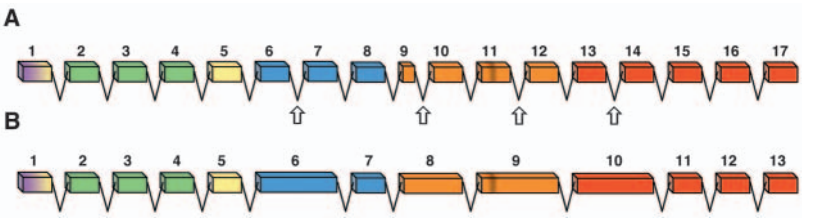


Fig. 3. Comparison of the genomic structure of *AmphiTrk* and human Trk genes. (A) Exonic domain distribution of human *TRKA*, *TRKB* and *TRKC*. Newly generated introns in human Trk receptors are indicated by arrows. (B) Exonic domain distribution of *AmphiTrk*. Domains are colour coded: purple, signal peptide; green, leucine-rich domains; yellow, cys-rich clusters; blue, type C2 Ig-like domains; orange, transmembrane surrounding regions; brown, transmembrane domain; red, tyrosine-kinase domain.

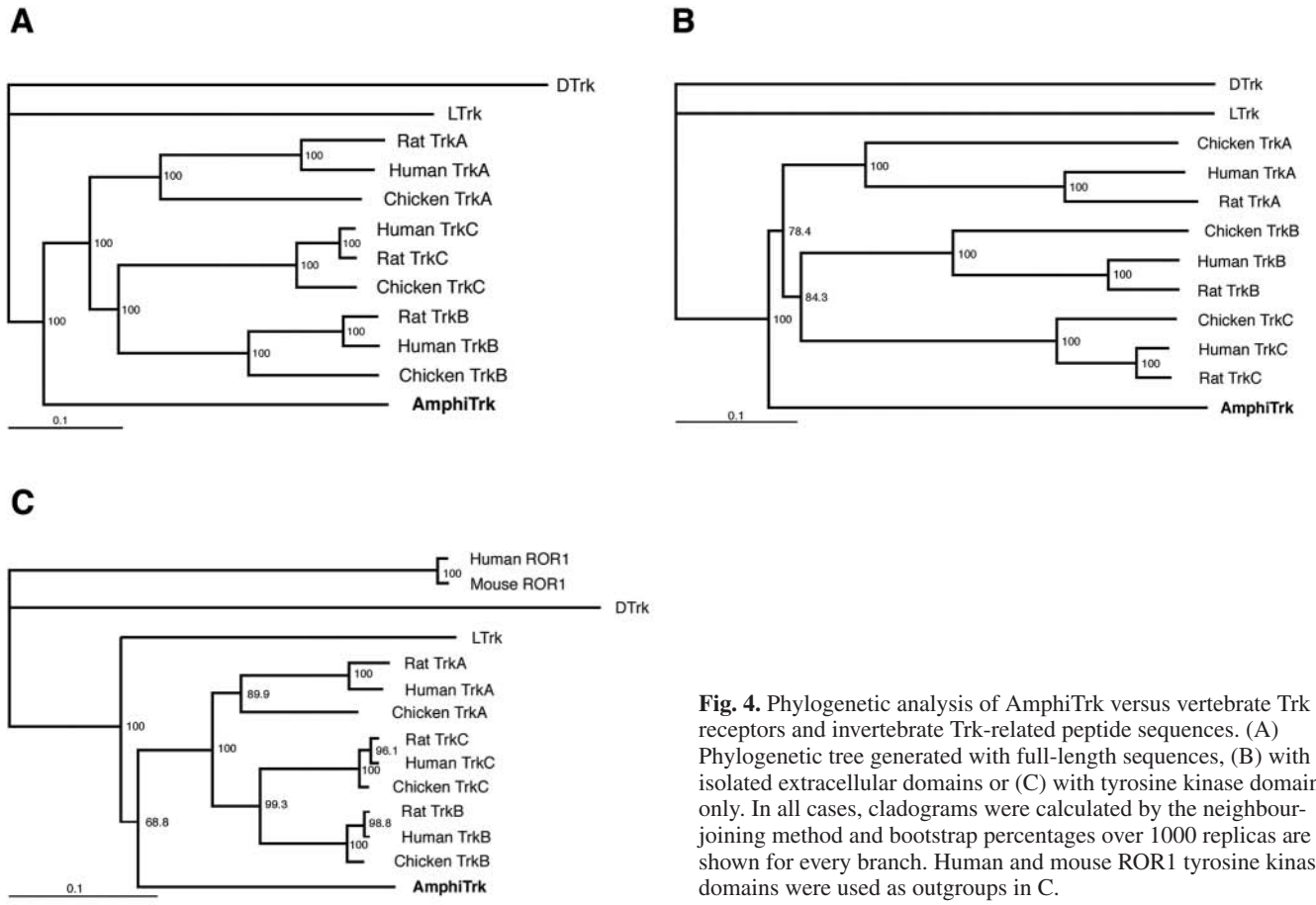


Fig. 4. Phylogenetic analysis of AmphiTrk versus vertebrate Trk receptors and invertebrate Trk-related peptide sequences. (A) Phylogenetic tree generated with full-length sequences, (B) with isolated extracellular domains or (C) with tyrosine kinase domains only. In all cases, cladograms were calculated by the neighbour-joining method and bootstrap percentages over 1000 replicas are shown for every branch. Human and mouse ROR1 tyrosine kinase domains were used as outgroups in C.

proteins were used, a clade formed by AmphiTrk and vertebrate TrkA, TrkB and TrkC excluded LTrk and DTrk (Fig. 4A). In the tree generated using only the extracellular domain, a similar topology was obtained (Fig. 4B). Although minor regions of the extracellular domains of DTrk and LTrk were conserved, these fell outside the group formed by AmphiTrk and vertebrate Trks. We also generated a tree using only the tyrosine kinase domain (Fig. 4C), wherein human and mouse ROR1 TK domains were taken as outgroups. In all analyses, AmphiTrk position was consistent with it being a direct descendant of the pre-duplicative, vertebrate-like, Trk, strengthening its condition as a primitive neurotrophic receptor.

Responsiveness of AmphiTrk to vertebrate neurotrophins

To examine AmphiTrk ability to respond through mammalian ligand binding, the receptor was transiently expressed in cultured PC12 nnr5 cells (PC12 derived, $Trk^{-/-}$) (Green et al., 1986). AmphiTrk-expressing PC12 nnr5 cells were acutely stimulated with increasing concentrations of NGF, BDNF, NT3 or NT4. Total cell lysates were western blotted to detect phosphorylated forms of Erk1/2 and AKT, which is indicative of AmphiTrk activation (Fig. 5A,D). Phosphorylation of AKT and Erk1/2 was stronger in cells stimulated by either of the neurotrophins than in non-stimulated cultures, in a dose-dependent manner. This suggests that AmphiTrk is not only able to bind to all vertebrate neurotrophins, but can also activate the vertebrate Erk1/2 and AKT transduction pathways. To determine whether AmphiTrk signalling strength, through mammalian neurotrophins, was most influenced by its extracellular domain, we tested its catalytic abilities. This was accomplished by transferring the AmphiTrk intracellular domain under mammalian extracellular control. The chimaeric receptor rTrkA-AmphiTrk (Fig. 5E) was generated by using the extracellular and transmembrane domains of an HA-tagged rat TrkA receptor and replacing its intracellular domain with that of AmphiTrk. Proceeding as above, downstream phosphorylations of AKT and Erk1/2 was detected through stimulation with NGF. The results obtained for rTrkA-AmphiTrk were comparable with those for rTrkA (Fig. 5F), indicating that the intracellular domain of AmphiTrk is able to mimic completely the function of the endogenous vertebrate receptor.

To functionally approach the sequence analyses suggestion for the inability of AmphiTrk to activate the PLCγ pathway, we analysed phosphorylation of PLCγ following NGF stimulation in rTrkA-AmphiTrk and rTrkA transfected cell cultures (Fig. 5G). Tellingly, the intracellular domain of AmphiTrk failed to induce phosphorylation of PLCγ.

In addition, we studied the ability of AmphiTrk and the chimaeric rTrkA-AmphiTrk receptor to induce neurite outgrowth through NGF activation in cultured PC12 nnr5 cells (Fig. 5H,K). rTrkA-AmphiTrk was found to induce neurite outgrowth following stimulation with NGF at comparable levels with those measured in rTrkA. However, although native

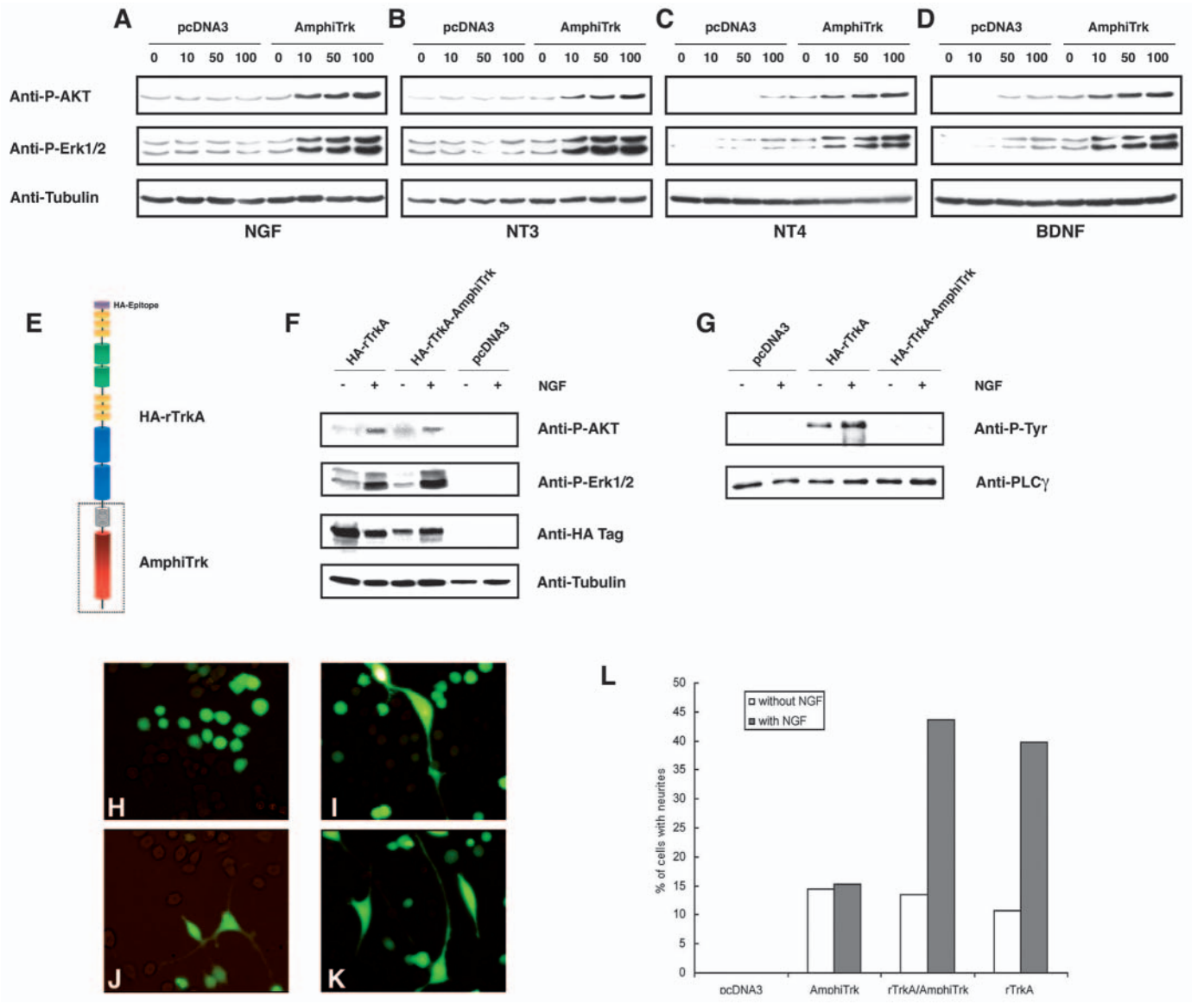


Fig. 5. Interaction of AmphiTrk with mammalian neurotrophins. (A-D) Western blots of AmphiTrk induced phosphorylation of AKT and Erk1/2 following stimulation with mammalian neurotrophins in transfected cell cultures. Concentrations of neurotrophins are indicated above the blots (ng/ml) (E) Schematic representation of the chimaeric rat TrkA receptor containing the intracellular domain of AmphiTrk (HA-rTrkA-AmphiTrk). (F) Western blot of HA-rTrkA-AmphiTrk induced phosphorylation of AKT and Erk1/2 after stimulation with NGF. Detection of the HA epitope was carried out to confirm expression of the chimaeric receptor and the native rat TrkA, which was used as a positive control. (G) Detection of PLCγ phosphorylation after stimulation with NGF (100 ng/ml) and immunoprecipitation with anti-PLCγ. No phosphorylation of PLCγ was detected through HA-rTrkA-AmphiTrk. An anti-PLCγ antibody was used to control immunoprecipitation efficiency. Stimulation of cultures transfected with the empty vector (pCDNA3) was performed as a negative control in A-D,F,G. Tubulin detection was used to control the loading of the lanes in A-D,F,G. (H-K) Neurite outgrowth assays. Neurite outgrowth was induced by NGF stimulation and visualised by co-transfection with an enhanced yellow fluorescent protein (EYFP). (H) Empty vector (pCDNA3), (I) AmphiTrk, (J) chimaeric HA-rTrkA-AmphiTrk and (K) rTrkA. (L) Percentages of cells that developed neurites under the absence or presence of NGF stimulation.

AmphiTrk was able to induce neurite outgrowth as well, no clear increase under NGF incubation was observed (Fig. 5L).

Developmental expression of AmphiTrk

AmphiTrk expression throughout embryonic development was examined by whole-mount in situ hybridisation. *AmphiTrk* transcripts are first detected at early neurula stage in about a dozen scattered individual epidermal cells of the ventral midline (Fig. 6A). As development proceeds, the number of expressing cells increases fourfold, and the signal moves dorsally to mediolateral positions on both sides of the embryo (Fig. 6B,K). Previous observations of adults and larvae have shown the presence of widespread ectodermal sensory cells in amphioxus (Bone and Best, 1978). More recently, scanning electron microscope (SEM) observations of mid-late neurula embryos show some of these cells to be morphologically differentiated by this stage (Mazet et al., 2004). Gene expression patterns of the pan-neuronal marker *AmphiElav*, in

tandem with other morphological evidence, suggest that the origin of these sensory neurons stems from general epidermal cells in the developing embryo (Satoh et al., 2001; Benito-Gutiérrez et al., 2005). *AmphiTrk*-expressing cells possess a distinctive fusiform morphology with respect to the surrounding rounded epidermal cells (Fig. 6J). Indeed, their localisation, in the ventrolateral epidermis of the embryos, is similar to that reported for sensory neurons in late neurulae and larvae, and their scattered distribution is similar to that of neurons expressing *AmphiElav*. Hence, *AmphiTrk* positive cells are probably sensory neurons belonging to the peripheral nervous system. Given that no morphologically differentiated sensory neurons have been found in early neurula stages (Mazet et al., 2004), the present results suggest that *AmphiTrk* is expressed by these sensory neurons during earlier stages of differentiation.

The puzzling laterodorsalisation of the *AmphiTrk* signal through neurulation raises the issue of whether epidermal movements occur at these stages or simply represent gene expression shifts. As signal intensity among individual *AmphiTrk*-expressing cells is homogeneous throughout, combinatorial down- and upregulation of transcripts within distinct cells seems unlikely. To determine whether the shifting position of the *AmphiTrk* signal correlated with cell migration, in vivo DiI labelling experiments were performed (Fig. 6L,O). To then distinguish whether movement was due to an entire epidermal layer dragging or to individual cell migrating upwards, we deposited DiI crystals, which preferentially adsorb to neural cells (Holland and Yu, 2002), on the ventral surface of hatching neurulae (Fig. 6L arrows), allowing them to develop until they reached late neurula stage. Consequently, individual fluorescent cells in mediolateral positions were detected in these late neurulae (arrows in Fig. 6N and in the merged image, Fig. 6O). This indicates that individual cells, in the ventral midline at early neurula stages, incorporated DiI and then migrated to dorsal territories. Although our data cannot reliably conclude that *AmphiTrk*-labelled cells are those that are migrating, detailed localisation in cross-sections, either within the epidermis or just beneath it (Fig. 6E,H, arrows and arrowheads, respectively), is consistent with dorsal migration through mesenchymal territories. This behaviour suggestively mimics that of the epithelial-mesenchymal transition and the migration of vertebrate neural crest cells.

In larval stages, *AmphiTrk* is expressed asymmetrically in the left dorsolateral quadrant of the developing Hatschek's pit (Fig. 6P, arrow), a neurosecretory structure thought to be homologous to the vertebrate adenohypophysis (Gorbman et al., 1999). In early larva, weak labelling is also visible at the most anterior tip of the embryo (Fig. 6P, arrowhead). This probably corresponds to the sensory cell clusters marking the future corpuscles of the Quatrefags, whose axonal processes contribute to rostral nerves.

Expression in adults

AmphiTrk expression in adults was tested by in situ hybridisation of sections (Fig. 7). *AmphiTrk* transcripts were detected along the nerve cord and diffuse labelling was still visible in the Hatschek's pit (Fig. 7B, asterisk). The anterior limit of *AmphiTrk* expression was located at the beginning of the proper nerve cord, caudal to the Joseph cells group, and next to the posteriormost dorsal part of the cerebral vesicle.

Conspicuous labelling was recognisable around the ventral midline of the neural tube (Fig. 7A,F). *AmphiTrk*-expressing cells were clustered at the ventral periventricular grey and deeper ventral white matter, forming a characteristic V-shaped pattern, which was maintained along the entire nerve cord. Based on previous examination of the amphioxus ventral nerve cord, *AmphiTrk*-expressing cells seem to be located within this compartment, where massive synaptic inputs arrive from a variety of sensory and interneuronal sources (Lacalli and Kelly, 2003). The ventral compartment motoneurons extend from the primary motor centre to far beyond the nerve cord, coincident with *AmphiTrk* expression. However, the number of ventral motoneurons was apparently lower than the number of labelled cells in our sections, indicating that *AmphiTrk* transcripts may also be present in glial-like cells and motoneuron-associated neurons (including interneurons). This is consistent with the wide range of neuronal cell types expressing Trk receptors within the vertebrate central nervous system. Intriguingly, at some levels, perikarya included in the V vertex touch the surrounding membrane of the notochord (Fig. 7F, arrow), a structure that is under nervous control in adult amphioxus (Stach, 1999).

In the dorsal part of the nerve cord, only a few labelled cells were intermittently visible on the right or left side of the central canal (Fig. 7A,D). Such a segmented pattern is consistent with the paired motoneurons of the dorsal compartment (Lacalli, 2002). They are positioned near the somitic boundaries, and as do the dorsal *AmphiTrk*-expressing cells, they show a slight offset, that parallels the left/right asymmetry of the somites. These iterative patterns through the serial sections are concurrent with the weak labelling found in Hatschek's pit-including sections (Fig. 7B). Large columnar cells in the dorsalmost part of the Hatschek's pit are diffusely labelled in only the most posterior portions (Fig. 7B, asterisk). Based on previous examinations of this structure, *AmphiTrk* transcripts may localise in the infundibulum, which is composed of axonal, endocrine and other cell types (Gorbman et al., 1999).

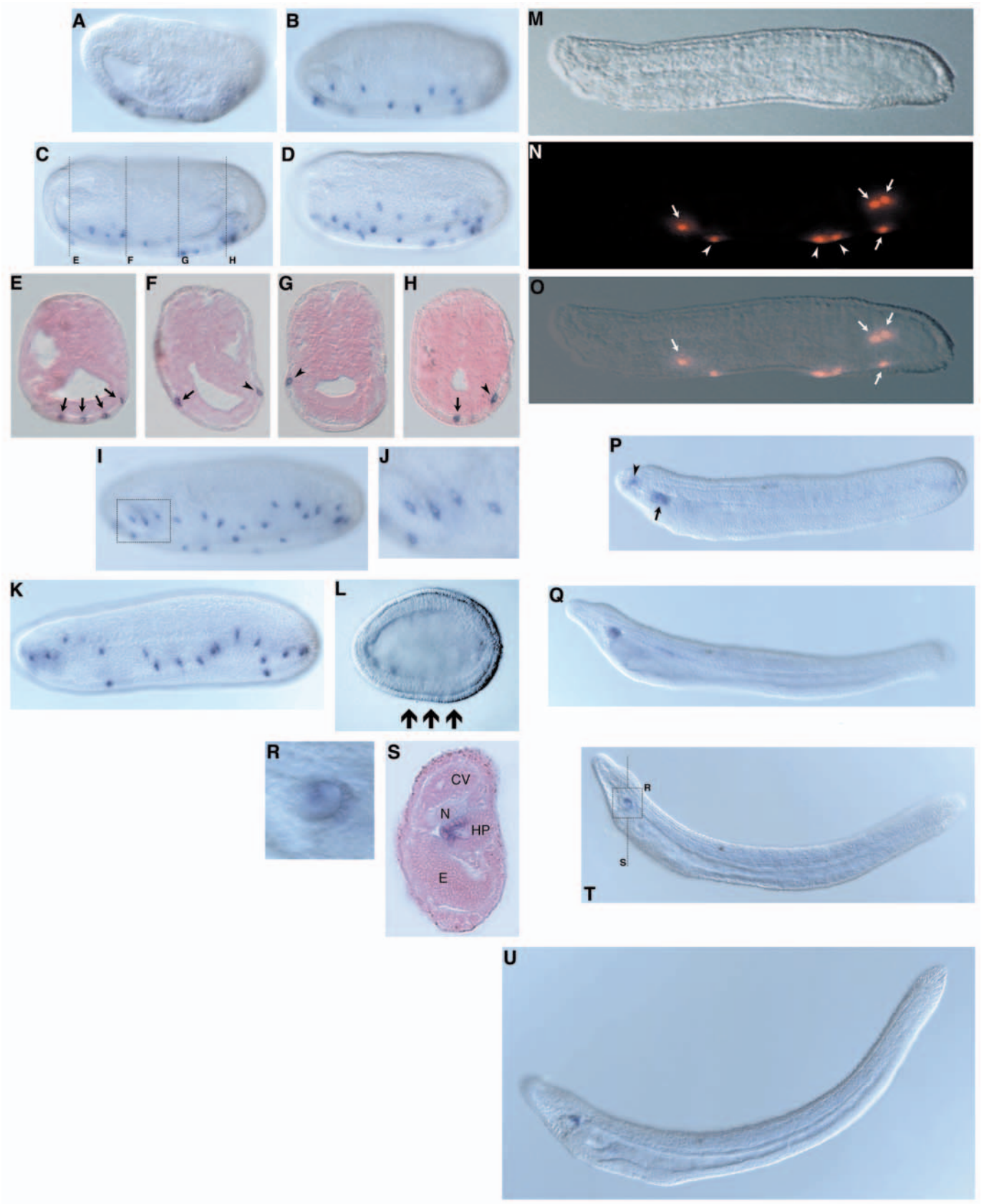
Discussion

Invention of the Trk receptor by exon shuffling in the lineage leading to vertebrates

The isolation of the bona fide amphioxus Trk receptor demonstrates that the Trk family was not an invention of vertebrates, dating its origin back to the cephalochordate/vertebrate split. Our phylogenetic data argue that *AmphiTrk* is the pro-orthologue of vertebrate Trk receptors, as well as a direct descendant of the pre-duplicative pioneer *ProtoTrk* gene. High bootstrap values confirm the primitive nature of *AmphiTrk*, even when extracellular or intracellular domains are weighed separately, or together as a full-length protein. The rift between AmphiTrk and invertebrate Trk-related receptors is remarkable and strengthened by phylogenetic analyses and domain structure. The *Drosophila* Trk is, in fact, a plexin-binding protein and has been recently renamed Doff-Track (Winberg et al., 2001). LTrk has an extracellular part lacking the key Ig-like domains characteristics of vertebrate Trks, and has a peculiar N-terminal extension. However, AmphiTrk precisely possess all domains characteristic of vertebrate Trk receptors in the correct position.

Recent advances in comparative genomics suggest that the

increase in genome size is paralleled by a general decrease in genome compactness and an increase in the number and size of introns (Patthy, 1999). *AmphiTrk* genomic structure reveals a more compacted domain distribution when compared with that of human Trks. Noticeably, the central core of the molecule may have been a target for the generation of four new



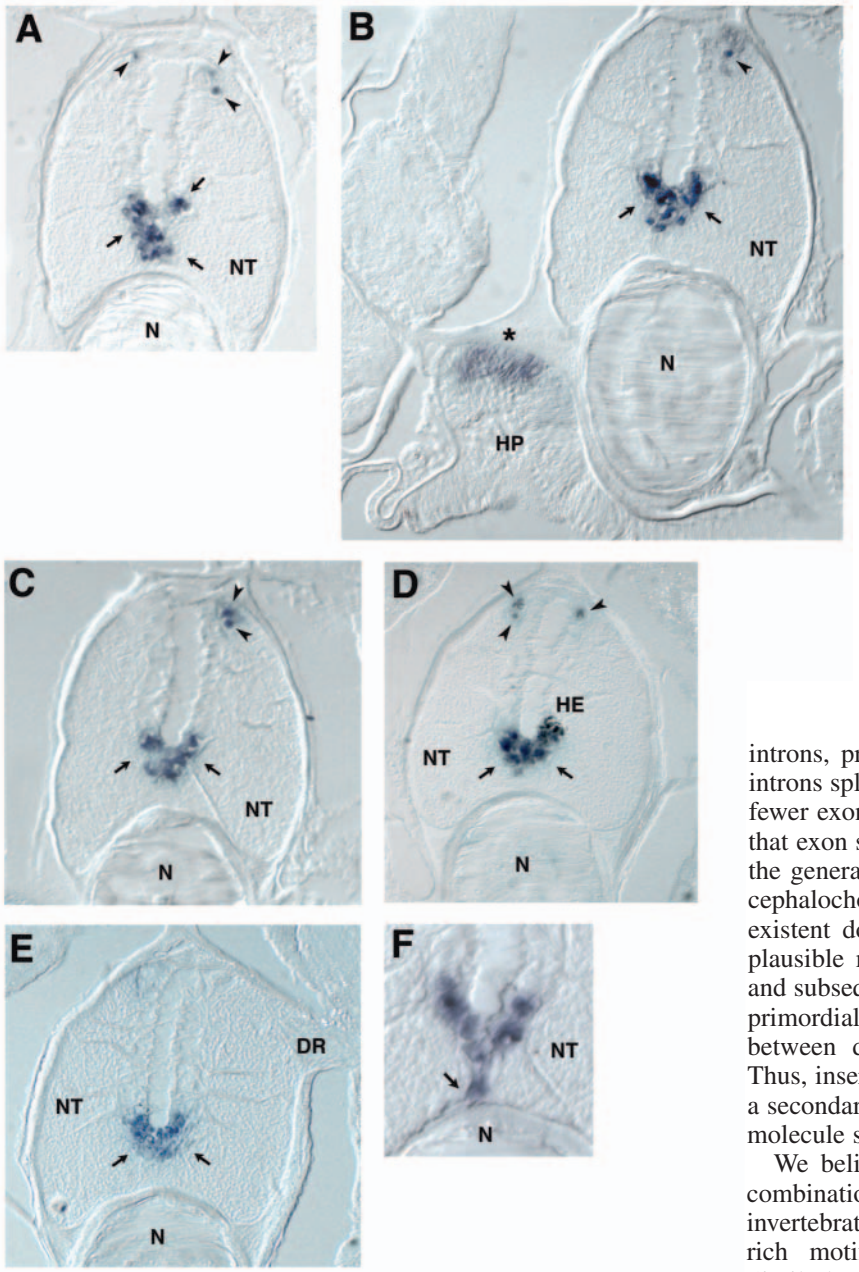


Fig. 7. *AmphiTrk* expression in the adult central nervous system. Pictures are lettered from most anterior (A) to most posterior (E) section. (A) The anteriormost limit of *AmphiTrk* expression is located at the beginning of the proper nerve cord, where strong signal is visible around the ventral part of the central canal (arrows). Coincidentally, some cells are labelled in both sides of the dorsal part of the neural tube (arrowheads), at the level where the primary motor centre initiates. (B) Section including the posterior part of the Hatschek's pit, showing *AmphiTrk* expression in the most dorsal part (asterisk). Arrows and arrowhead indicate *AphiTrk*-expressing cells in the ventral and dorsal compartments, respectively. (C-E) *AmphiTrk* expression is maintained along the neural tube around the ventral side of the central canal (arrows). Iteratively, some cells simultaneously express *AmphiTrk* in the dorsal part of the neural tube, albeit with a slight right-left offset (arrowheads). (F) At some points along the neural tube, *AmphiTrk*-expressing cells extend ventrally, touching the surrounding membrane of the notochord (arrows). N, notochord; NT, neural tube; HP, Hatschek's pit; HE, Hesse eyecup; DR, dorsal root.

introns, present in humans but not in amphioxus. These introns split domains that are coded by a single exon or by fewer exons in the amphioxus gene. This finding suggests that exon shuffling was the crucial mechanism involved in the generation of a ProtoTrk modular protein prior to the cephalochordate/vertebrate split. Rearrangement of pre-existent domains, rather than creation of new ones, is a plausible mode for generating molecules able to expand and subsequently evolve to complexity. When domains are primordially encoded by single exons, the exchange between different genomic regions may be facilitated. Thus, insertion of introns within single exons may well be a secondary trait, result of the divergence from an original molecule successfully selected by evolution.

We believe that AmphiTrk novelty resides in its new combination of protein modules already present in lower invertebrate genomes. Certainly, LTrk possesses leucine-rich motifs sandwiched between clustered cysteines. Similarly, the tyrosine kinase domain itself arose before the divergence of animals from other higher eukaryotes (King

Fig. 6. Developmental expression of *AmphiTrk*. In all lateral views, the anterior is towards the left and the dorsal is towards the top. (A) Earliest *AmphiTrk* expression was detected in individual epidermal cells of the ventral midline of the early neurula. (B) The number of expressing cells began to increase at 14 hours of development. (C,D) Left- and right-hand views of the same neurula at 16 hours of development. (E-H) Cross-sections at different levels of the embryos shown in C and D (dotted lines). Positive cells are located within the epidermal layer (arrows) or just beneath it (arrowheads). (I) At 18 hours of development *AmphiTrk*-expressing cells in ventrolateral positions extend towards dorsal directions. (J) Magnified view of the area inside the rectangle in I, showing the fusiform shaped positive cells and the cytoplasmic distribution of *AmphiTrk* transcripts. (K) At late neurula stages, dorsalisation of the *AmphiTrk* signal was more evident. (L-O) In vivo DiI labelling assays. (L) Initial DiI crystal deposition in the ventral epidermis of the hatching neurula (arrows). (M) Cultured late neurula after DiI labelling shown in L. (N) Rhodamine filtered view of M, showing DiI labelled cells (arrows and arrowheads). (O) Merged image of M and N showing labelled cells that migrated individually towards dorsal positions (arrows). (P) In the early larval stage *AmphiTrk* was upregulated during Hatschek's pit formation (arrow) and weak expression was detected in a rostral patch of cells (arrowhead). (Q) At 26 hours of development, expression was just visible in the developing Hatschek's pit. (R) Magnified view of the area inside the dotted square in T, showing the dorsoposterior expression of *AmphiTrk* during Hatschek's pit formation. (S) Cross-section at the level of the dotted line in T, showing staining limited to the left-sided dorsolateral part of the Hatschek's pit primordia. (T,U) *AmphiTrk* expression was maintained in the Hatschek's pit at 28 hours (T) and 30 hours (U) of development. HP, Hatschek's pit; N, notochord; E, endostyle; CV, cerebral vesicle.

and Carrol, 2001). Nevertheless, assembly of the central core most probably occurred just predating the cephalochordate/vertebrate split, as the genome of the lower chordate *Ciona intestinalis* does not contains a Trk-like gene (Dehal et al., 2002). Conversely, we cannot totally rule out secondarily loss of Trk in tunicates, even though the lack of Trk receptors in the nearly completed genome of a sea urchin species (http://sugp.caltech.edu) strongly strengthens the argument for a nascent Trk gene close to the cephalochordate/vertebrate split.

Acquisition of functional complexity

Neurotrophin signalling through Trk receptors elicits many biological effects. Beyond its crucial role in neuronal survival and differentiation during development, neurotrophin signalling is critically involved in axonal regulation and dendritic outgrowth, synapse formation and function, and cell migration. Among the numerous ways of controlling these processes, differential splicing and distinct preferences for ligand binding seem to be two decisive mechanisms (Segal, 2003).

We have no evidence for the presence of *AmphiTrk* splicing variants. *AmphiTrk* does not possess the separated miniexon, which in vertebrates confers a loose Trk-binding ability to the distinct neurotrophins. Therefore, promiscuous ligand-receptor relationships may be absent in amphioxus, or regulated by other means. The latter, however, seems unlikely as the pre-duplicative nature of the amphioxus genome suggests the AmphiTrk ligand to be a single amphioxus neurotrophin. AmphiTrk peptide characterisation reveals all the features defining a functional vertebrate Trk receptor. The sequence analysis was further confirmed by experiments in cultured rat cells, where AmphiTrk activated the Ras-Raf-Erk and PI3kinase-AKT signalling pathways in response to neurotrophins. AmphiTrk responded similarly to NGF, BDNF, NT3 and NT4. This indicates that after 500 million years of divergence, the extracellular domain of AmphiTrk can still molecularly bind and recognise post-duplicative vertebrate neurotrophins. Our functional data suggests that the biological functions of AmphiTrk may include neuronal survival and differentiation during development, as well as a likely role in axonal elongation and dendritic outgrowth, processes that are associated with the signalling pathways mentioned above (Patapoutian and Reichardt, 2001). Remarkably, the inability of AmphiTrk to activate the PLCγ pathway underscores its primitive nature as a Trk receptor, because this pathway is believed to drive the most complex responses generated by Trk-Nt interactions in mammalians (Koponen et al., 2004).

The increase in the numbers of neurotrophin and Trk receptors does not always imply the increase in nervous system complexity, as exemplified by the lineage-specific duplications in teleost fishes (Hallböök, 1999). However, as we suggest here, vertebrate Trk receptors were originated from a single *ProtoTrk* gene through the wide genome duplication events that were linked to the invertebrate/vertebrate transition. Consistent with amphioxus position in the transition from simple to complex nervous systems, our results suggest that a *ProtoTrk*, *AmphiTrk*-like, ancestral gene may well have provided the genetic basis for acquisition of functional complexity. The recruitment of the PLCγ pathway, the expansion of the gene family, the appearance of alternative splicing, the co-evolution of ligands and receptors, and the finely graded control of promiscuity may have been instrumental in the development of the vertebrate complex nervous system and in the acquisition of higher neuronal functions.

Evolutionary developmental insights from expression patterns

The variety of actions driven by the vertebrate Nt/Trk system are further manifested by their complicated gene expression patterns during development and adulthood. Furthermore, interpretation of gene knockout phenotypes in mice is hampered by the partial overlapping and redundancy among the three Trk receptors. The amphioxus simple nervous system offers an uncomplicated context in which to study the expression of a single Trk receptor, and may serve to provide insights into the basic function of this gene family in vertebrates.

During embryogenesis, *AmphiTrk* expression is restricted to the developing peripheral nervous system. Our results suggest that *AmphiTrk* is involved in sensory neuronal fate commitment and differentiation. *AmphiTrk* transcripts are detected earlier than epidermal differentiated primary neurons are identified in SEM observations, and also earlier than neurons are revealed by the pan-neural marker *AmphiElav* (Satoh et al., 2001; Mazet et al., 2004; Benito-Gutiérrez et al., 2005). As the number of *AmphiElav*-expressing cells is higher than the number of positive cells shown by our whole-mount experiments, we suspect that *AmphiTrk* is specifying only a subset of the neurons later identified by *AmphiElav*. Conversely, the reduced number of primary neurons shown by SEM observations suggests that *AmphiTrk* also plays a role in differentiating other types of sensory neurons, i.e. secondary neurons without an axonal process embedded in the epidermal layer.

Interestingly, TrkB$^{-/-}$TrkC$^{-/-}$ (*Ntrk2/Ntrk3* – Mouse Genome Informatics) double-mutant mice show severe sensory defects (Silos-Santiago et al., 1997), and mice deprived of NGF/TrkA signalling during embryogenesis exhibit reduced sensitivity to painful stimuli (Fariñas, 1999). Neuronal losses of primary neurons in these null mice are localised in the sensory ganglia, structures absent in amphioxus. Nevertheless, prevention of TrkB and TrkC signalling in knockout mice for NT4, BDNF and NT3 leads to a severe deficiency in cutaneous sensory neurons, e.g. D-hair receptors, slow adapting mechanoreceptors and cutaneous mechanoreceptors, respectively (Stucky et al., 1998), all of them secondary neurons. Considering that *AmphiTrk* may well function in similar fashion to all the three vertebrate Trk receptors, these data support the idea that *AmphiTrk* is expressed by both primary and secondary neurons in the peripheral nervous system.

Some innovative vertebrate hallmarks can be correlated to the acquisition of a complex nervous system. Neural crest and placodes are exclusive vertebrate features clearly absent in amphioxus. It is widely accepted that the genetic machinery of neural crests was already present in amphioxus and, by extension, in the ancestor of vertebrates. However, the ability of neural crest cells to migrate individually seem to have arisen during vertebrate evolution (Trainor et al., 2003). We have evidence to show the presence of individually migrating cells in the amphioxus embryonic ectoderm (the first time this has been reported), which appealingly mimics that of neural crest

cells in vertebrates. Interestingly, vertebrate Trk receptors are expressed in migrating neural crest cells (Airaksen and Meyer, 1996). Moreover, many placode and neural crest derivatives express Trk receptors in vertebrates (Baker et al., 2002; Huang and Reichardt, 2001). Among these derivatives, adenohypophysis, which has its homologous in the amphioxus Hatscheck's pit, originates from nonneurogenic placodes, and a wide variety of mechano- and chemosensory structures, including the olfactory epithelium, from neurogenic placodes (Holland and Holland, 2001). *AmphiTrk* is expressed during the formation of Hatscheck's pit throughout larval stages, and into adulthood. Transcript locations in the infundibulum are comparable with those of the vertebrate Trk receptors in the anterior pituitary gland (Aguado et al., 1998). The corpuscles of Quatrefags are specialised organs, presumably with sensory roles, whose structure has been likened to the vertebrate olfactory placode. This structure, which lacks a counterpart in any other chordate, expresses *AmphiTrk* for a short time during the early larval stage.

Conspicuous labelling for *AmphiTrk* is evident in the adult central nervous system. As the pathways activated by *AmphiTrk* seem to be limited to survival and differentiation actions, as suggested by our functional studies in cell cultures, we cannot discard more complex roles for *AmphiTrk* in adults. Vertebrate neurotrophin signalling through Trk receptors modulates not only dendritic growth, but also the number of synapses as well as the efficacy of synaptic transmission (Bibel and Barde, 2000). Thus, *AmphiTrk* may have a similar role within the CNS of adults. Although AmphiTrk is unable to drive PLCγ-mediated actions, this does not prevent AmphiTrk from having a role in regulating neurotransmitter release within the neural tube.

We thank Nick Holland, Seb Shimeld and Mario Pestarino for help, advice and encouraging discussions. We also thank Robin Rycroft for editorial assistance. This research was funded by the Fundació La Marató de TV3, by grant BMC2002-03316 (MCYT, Spain), by the Generalitat de Catalunya (Distinció per la Promoció de la Recerca Universitaria to J.X.C. and J.G.F.) and by the European Community's Human Potential Programme HPRN-CT-2002-00263 'Neurogenome'. C.N. was supported by a postdoctoral contract from the above named EC programme.

References

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. et al. (2000). The genome sequence of Drosophila melanogaster. *Science* **287**, 2185-2195.

Aguado, F., Majó, G., Gómez de Aranda, I. and Ferrer, I. (1998). Trk neurotrophin receptor family immunoreactivity in rat and human pituitary tissues. *Neurosci. Lett.* **243**, 13–16.

Airaksen, M. S. and Meyer, M. (1996). Most classes of dorsal root ganglion neurons are severely depleted but not absent in mice lacking neurotrophin-3. *Neuroscience* **73**, 907-911.

Baker, C. V. H., Stark, M. R. and Bronner-Fraser. M. (2002). Pax3-expressing trigeminal placode cells can localize to trunk neural crest sites but are committed to cutaneous sensory fate. *Dev. Biol.* **249**, 219-236.

Benito-Gutiérrez, E., Illas, M., Comella, J. X. and Garcia-Fernàndez, J. (2005). Outlining the nascent nervous system of *Branchiostoma floridae* (amphioxus) by the pan-neural marker AmphiElav. *Brain Res. Bull.* (in press).

Bibel, M. and Barde, Y. A. (2000). Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes Dev.* **14**, 2919-2937.

Bone, Q. and West, C. G. (1978). Ciliated sensory cells in amphioxus (Branchiostoma). *J. Mar. Biol. Ass. UK* **58**, 479-486.

Chao, M. V. (2000). Trophic factors: an evolutionary cul-de-sac or door into higher neuronal function? *J. Neurosci. Res.* **59**, 353-355.

Dehal, P., Satou, Y., Campbell, R. K., Chapman, J., Degnan, B., de Tomaso, A., Davidson, B., di Gregorio, A., Gelpke, M., Goodstein, D. M. et al. (2002). The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins. *Science* **298**, 2157-2167.

Egea, J., Espinet, C. and Comella, J. X. (1999). Calcium influx activates extracellular-regulated kinase/mitogen-activated protein kinase pathway through a calmodulin-sensitive mechanism in PC12 cells. *J. Biol. Chem.* **274**, 75-85.

Fariñas, I. (1999). Neurotrophin actions during the development of the peripheral nervous system. *Mic. Res. Tech.* **45**, 233-242.

Ferrier, D. E., Minguillón, C., Holland, P. W. H. and Garcia-Fernàndez, J. (2000). The amphioxus Hox cluster: deuterostome posterior flexibility and Hox14. *Evol. Dev.* **2**, 284-293.

Furlong, R. and Holland, P. W. H. (2002). Were vertebrates octoploid? *Phil. Trans. R. Soc. Lond. B* **357**, 531-544.

Gorbman, A., Nozaki, M. and Kubokawa, K. (1999). A brain-Hatschek's pit connection in amphioxus. *Gen. Comp. Endocrinol.* **113**, 251-254.

Green, S. H., Rydel, R. E., Connolly, J. L. and Greene, L. A. (1986). PC12 cell mutants that possess low- but not high-affinity nerve growth factor receptors neither respond to nor internalize nerve growth factor. *J. Cell Biol.* **102**, 830-843.

Hallböök, F. (1999). Evolution of the vertebrate neurotrophin and Trk receptor gene families. *Curr. Opin. Neurobiol.* **9**, 616-621.

Holland, L. Z. and Holland, N. D. (2001). Evolution of neural crest and placodes: amphioxus as a model for the ancestral vertebrate. *J. Anat.* **199**, 85-98.

Holland, N. D. and Holland, L. Z. (1993). Embryos and larvae of invertebrate deuterostomes. In *Essential Developmental Biology, A Practical Approach* (ed. C. D. Stern, and P. W. H. Holland), pp. 21-32. Oxford: IRL Press.

Holland, N. D. and Yu, Jr-K (2002). Epidermal receptor development and sensory pathways in vitally stained amphioxus (*Branchiostoma floridae*). *Acta Zool.* **83**, 309-319.

Huang, E. J. and Reichardt, L. F. (2001). Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci.* **24**, 677-736.

Huang, E. J. and Reichardt, L. F. (2003). Trk receptors: roles in neuronal signal transduction. *Annu. Rev. Biochem.* **72**, 609-642.

Ibañez, C. F. (1998). Emerging themes in structural biology of neurotrophic factors. *Trends Neurosci*. **21**, 438-444.

Jaaro, H., Beck, G., Conticello, S. G. and Fainzilber, M. (2001). Evolving better brains: a need for neurotrophins. *Trends Neurosci.* **24**, 79-85.

King, N. and Carrol, S. B. (2001). A receptor Tyrosine kinase from choanoflagellates: molecular insights into early animal evolution. *Proc. Natl. Acad. Sci. USA* **98**, 15032-1537.

Koponen, E., Voikar, V., Riekki, R., Saarelainen, T., Rauramaa, T., Rauvala, H., Taira, T. and Castren, E. (2004). Transgenic mice overexpressing the full-length neurotrophin receptor TrkB exhibit increased activation of the TrkB-PLCgamma pathway, reduced anxiety, and facilitated learning. *Mol. Cell Neurosci.* **26**,166-181.

Lacalli, T. C. (2002). The dorsal compartment locomotory control system in amphioxus larvae. *J. Morphol.* **252**, 227-237.

Lacalli, T. C. and Kelly, S. J. (2003). Ventral neurons in the anterior nerve cord of amphioxus larvae. I. An inventory of cell types and synaptic patterns. *J. Morphol.* **257**, 190-211.

Mazet, F., Masood, S., Luke, G. H., Holland, N. D. and Shimeld, S. M. (2004). Expression of *AmphiCoe*, an Amphioxus COE/EBF Gene, in the developing central nervous system and epidermal sensory neurons. *Genesis* **38**, 58-65.

Ninkina, N., Grashchuck. M., Buchman, V. L. and Davies, A. M. (1997). TrkB variant with deletions in the leucin-rich motifs of the extracellular domain. *J. Biol. Chem.* **272**, 13019-13025.

Patapoutian, A. and Reichardt, L. F. (2001). Trk receptors, mediators of neurotrophin action. *Curr. Opin. Neurobiol.* **11**, 272-280.

Patthy, L. (1999). Genome evolution and the evolution of exon-shuffling – a review. *Gene* **238**, 103-114.

Peng, X., Greene, L. A., Kaplan, D. R. and Stephens, R. M. (1995). Deletion of a conserved juxtamembrane sequence in Trk abolishes NGF-promoted neuritogenesis. *Neuron* **15**, 395-406.

Poo, M. (2001). Neurotrophins as synaptic modulators. *Nat. Rev. Neurosci.* **2**, 1-9.

Pulido, D., Campuzano, S., Koda, T., Modolell, J. and Barbacid, M. (1992).

DTrk, a Drosophila gene related to the Trk family of neurotrophin receptors, encodes a novel class of neural cell adhesion molecule. *EMBO J.* **11**, 391-404.

Satoh, G., Wang, I., Zhang, P. and Satoh, N. (2001). Early development of amphioxus nervous system with special reference to segmental cell organisation and putative cell precursors: a study based on the expression of pan-neuronal marker *Hu/elav*. *J. Exp. Zool.* **291**, 354-364.

Segal, R. A. (2003). Selectivity in neurotrophin signalling: theme and variations. *Annu. Rev. Neurosci.* **26**, 299-330.

Silos-Santiago, I., Fagan, A. M., Garber, M., Fritzsch, B. and Barbacid, M. (1997). Severe sensory deficits but normal CNS development in newborn mice lacking TrkB and TrkC tyrosine protein kinase receptors. *Eur. J. Neurosci.* **9**, 2045-56.

Stach, T. (1999). The ontogeny of the notochord of Branchiostoma lanceolatum. *Acta Zool. Stockholm* **80**, 25-33.

Stucky, C. L., DeChiara, T., Lindsay, R. M., Yancopoulos, G. D. and Koltzenburg, M. (1998). Neurotrophin 4 is required for the survival of a subclass of hair follicle receptors. *J. Neurosci.* **18**, 7040-7046.

The C. elegans Sequencing Consortium (1998). Genome sequence of the nematode C. elegans, a platform for investigating biology. *Science* **282**, 2012-2018.

Trainor, P. A., Melton, K. R. and Manzanares, M. (2003). Origins and plasticity of neural crest cells and their roles in jaw and craniofacial evolution. *Int. J. Dev. Biol.* **47**, 541-553.

van Kesteren, R., Fainzilber, M., Hauser, G., van Minen, J., Vreugdenhil, E., Smit, A. B., Ibáñez, C. F., Geraerts, W. P. M. and Bulloch, A. G. M. (1998). Early evolutionary origin of the neurotrophin receptor family. *EMBO J.* **17**, 2534-2542.

Wiesmann, C., Ultsch, M. H., Bass, S. H. and de Vos, A. M. (1999). Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor. *Nature* **401**, 184-188.

Winberg, M. L., Tamagnone, L., Bai, J., Comoglio, P. M., Montell, D. and Goodman, C. S. (2001). The transmembrane protein Off-Track associates with plexins and functions downstream of semaphorin signaling during axon guidance. *Neuron* **32**, 53-62.

Zimmer, C. (2000). In search of vertebrate origins, Beyond brain and bone. *Science* **287**, 1576-1579.