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*trk*C, a receptor for neurotrophin-3, is widely expressed in the developing nervous system and in non-neuronal tissues

Lino Tessarollo, Pantelis Tsoulfas, Dionisio Martin-Zanca*, Debra J. Gilbert, Nancy A. Jenkins, Neal G. Copeland and Luis F. Parada†

Molecular Embryology Section and Mammalian Genetics Laboratory, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201, USA

*Present address: Instituto de Microbiologia Bioquimica CSIC, Departamento de Microbiologia, Facultad de Ciencias, 37008 Salamanca, Spain
†Author for correspondence

SUMMARY

The *Trk* family of tyrosine kinases encodes receptors for nerve growth factor-related neurotrophins. Here we present a developmental expression study of *trk*C, which encodes a receptor for neurotrophin-3 (NT-3). Like the related genes, *trk* and *trk*B, *trk*C is expressed primarily in neural lineages although the pattern is complex and includes non-neuronal cells. Direct comparison with *trk* and *trk*B developmental expression patterns permits the following observations. (1) *trk*C is expressed in novel neural tissues where other *Trk* genes are silent. (2) Some tissues appear to coexpress *trk*B and *trk*C receptors in the embryo and in the adult. (3) *trk*C expression can be detected in the gastrulating embryo. These data provide insights into the role of *Trk*-family receptors and nerve growth factor-related neurotrophins during development and suggest that, in addition to regulating neuronal survival and differentiation, the neurotrophin/*Trk* receptor system may have broader physiological effects. Finally, interspecific mouse backcrosses have been used to map the location of each of the *Trk* genes on mouse chromosomes. Alignment with available chromosomal maps identify possible linkage between the *Trk* genes and known neurological mutations.

Key words: *trk*C; tyrosine kinase; neural development, neurotrophin; NT-3; mouse nervous system

INTRODUCTION

The *Trk* gene family encodes receptor tyrosine kinases (RTKs) that include *trk*, *trk*B and *trk*C (for review see: Chao, 1992). The best characterized of these genes is human *trk*, which was originally described as a dominantly acting oncogene (Martin-Zanca et al., 1986) and recognized to encode a RTK (Martin-Zanca et al., 1989). However, the normal biological role of gp145$^{proto trk}$ remained enigmatic until expression studies provided the first clues regarding possible function (Martin-Zanca et al., 1990). Biochemical analysis demonstrated that the gp140$^{proto trk}$ RTK interacts directly with Nerve Growth Factor (NGF; Kaplan et al., 1991a,b; Klein et al., 1991), is required for NGF function (Loeb et al., 1991) and forms part of the high affinity NGF receptor (Klein et al., 1991; Hempstead et al., 1991). Furthermore, the NGF-related neurotrophins have been identified as ligands for the *Trk*-related RTK-encoding genes *trkB* and *trkC* (Berkemeier et al., 1991; Ip et al 1992; Soppet et al., 1991; Squinto et al., 1991; Lamballe et al., 1991; Tsoulfas et al., 1993). The interaction between neurotrophins and their respective receptors stimulates receptor tyrosine kinase activity and elicits different biological responses depending on the cellular environment of receptor expression. For example, in the PC12 rat pheochromocytoma cell line, neurotrophic factors promote neuronal survival and differentiation upon binding to the different members of the *Trk* receptor family (Kaplan et al., 1991a; Loeb et al., 1991; Squinto et al., 1991; Tsoulfas et al., 1993; D. Soppet and L. F. P., unpublished data). In contrast, stimulation of *Trk* receptors by neurotrophins induces proliferation in transfected NIH-3T3 fibroblasts (Glass et al., 1991; Barbacid et al., 1991). Other examples of RTKs exhibiting pleiotropic effects depending on the particular site of expression are the *let*-23 and c-*kit* genes. In *Caenorhabditis elegans*, genetic evidence indicates that the *let*-23 gene, which encodes a putative RTK of the epidermal growth factor (EGF) receptor subfamily, can control two opposing pathways in vulval differentiation and functions in at least five others tissues (Aroian and Sternberg, 1991). In the mouse, the c-*kit* RTK acts differently in cells originating from diverse embryonic lineages such as primordial germ cells, melanocytes and hematopoietic stem cells (Geissler et al., 1981, 1988). Similarly, findings that NGF has specific effects in the rat seminiferous epithelium (Parvinen et al., 1992) and B-lymphocytes (Otten et al., 1989) suggest that, in addition to its neurotrophic actions, this factor and presumably its related neurotrophins, brain-derived neurotrophic factor (BDNF; Leibrock et al., 1989), neurotrophin-3 (NT-3; Enfors et al., 1990; Hohn et al., 1990;

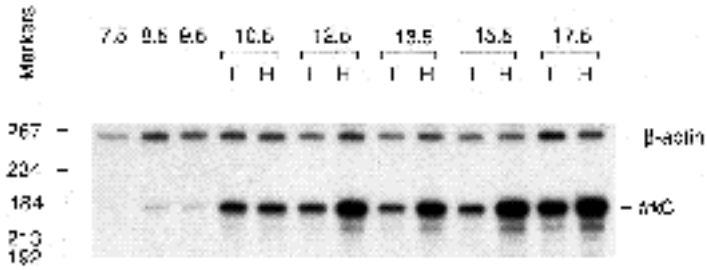

Fig. 1. RNAse protection analysis of *trk*C expression in mouse embryos from 7.5 to 17.5 days of gestation. Approximately 10 μg of total RNA from embryonic trunks (T) and heads (H) were analyzed using a ^{32}P-labeled *trk*C-specific probe (see Materials and methods). A β-actin probe was used in the same hybridization mixture with *trk*Cas an internal control for RNA quantitation. Co-electrophoresed DNA size markers are indicated at the left.

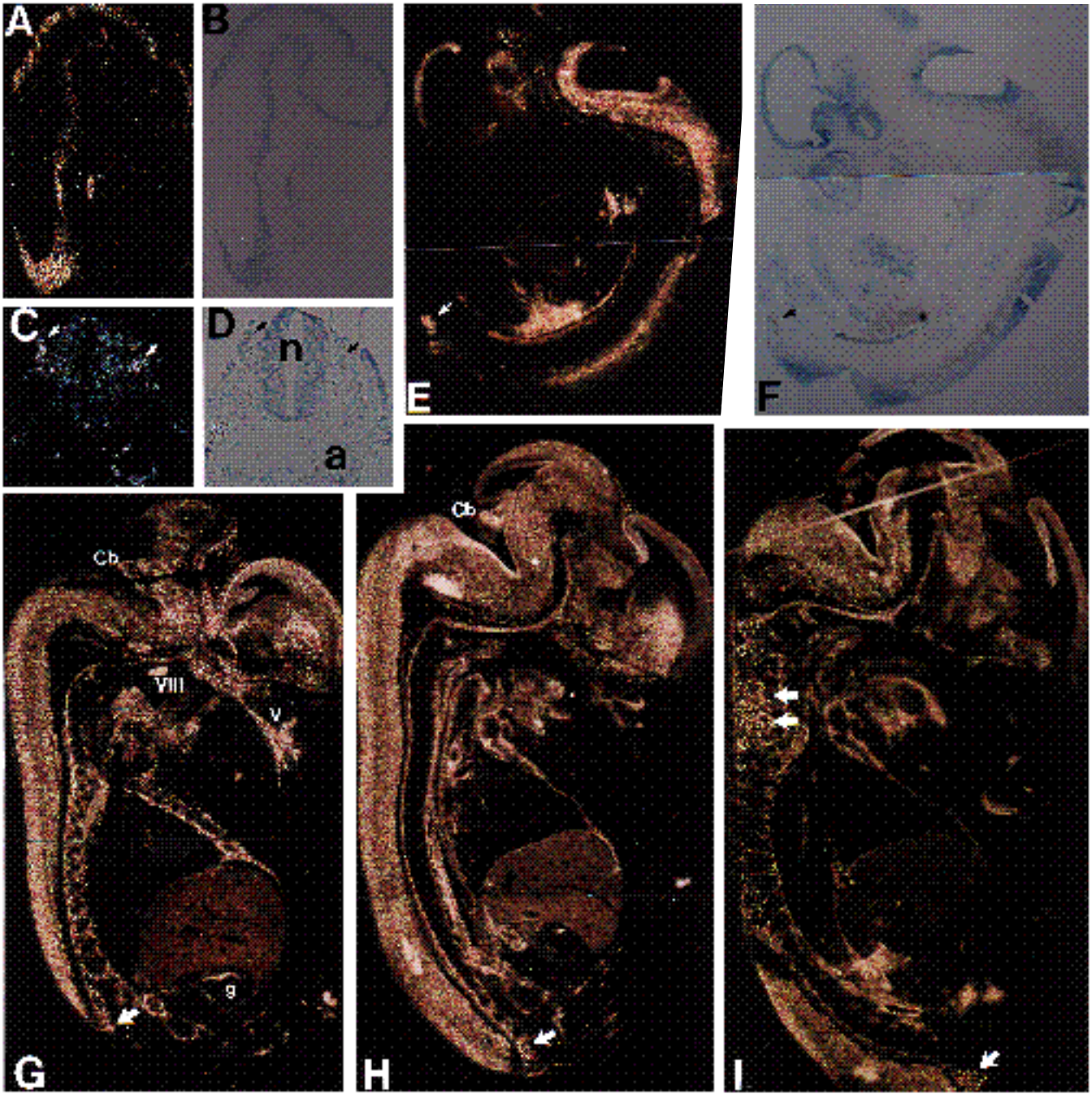

Fig. 2. *trk*C expression in 9.5, 11.5 and 13.5 day embryos. (A,C) Dark-field and (B,D) light-field views of a sagittal (A,B) and a frontal (C,D) section of a 9.5 day embryo hybridized with a *trk*C-specific probe. (E) Dark-field and (F) light-field optics of an 11.5 day mouse embryo sagittal section. (G,H,I) Dark-field views of serial adjacent sections from a 13.5 day embryo. Arrows in A-D indicate the forming DRG and migratory neural crest cells whereas in E-I they indicate the DRG (single arrow) and the sympathetic (double arrows). n, neural tube; a, dorsal aorta; cerebellum (Cb), trigeminal (V) and vestibulo-cochlear (VIII) ganglia, stomach (g).

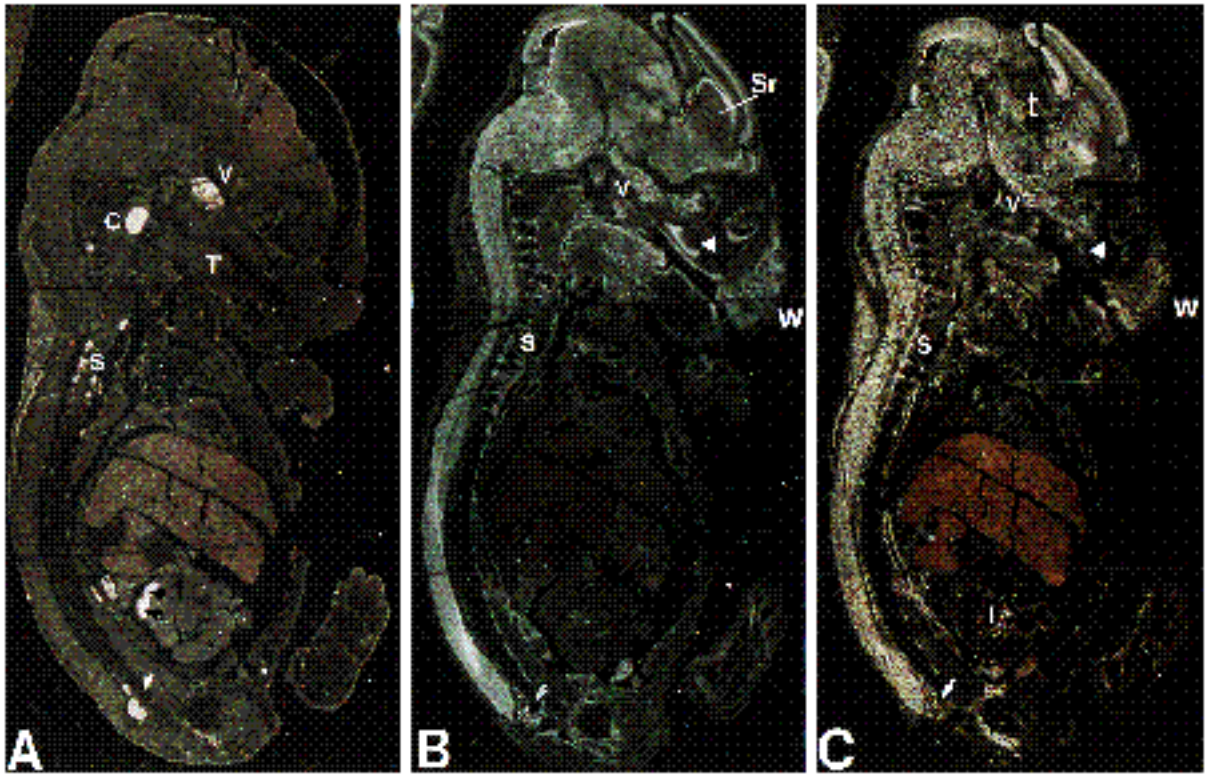

Fig. 3. Comparative expression of *Trk*-family genes. Dark-field views of serial adjacent sagittal sections of a 17.5 day mouse embryo hybridized with a *trk* (A), *trk*B (B) or *trk*C (C)-specific probe. White triangles indicate the tooth papilla; arrows, DRG; Sr, striatum; t, thalamus; T, tongue; V, fifth cranial ganglion; C, superior cervical ganglion; S, sympathetic ganglia; W, whisker pad; i, intestine. The black triangles in panel A point to an artifact.

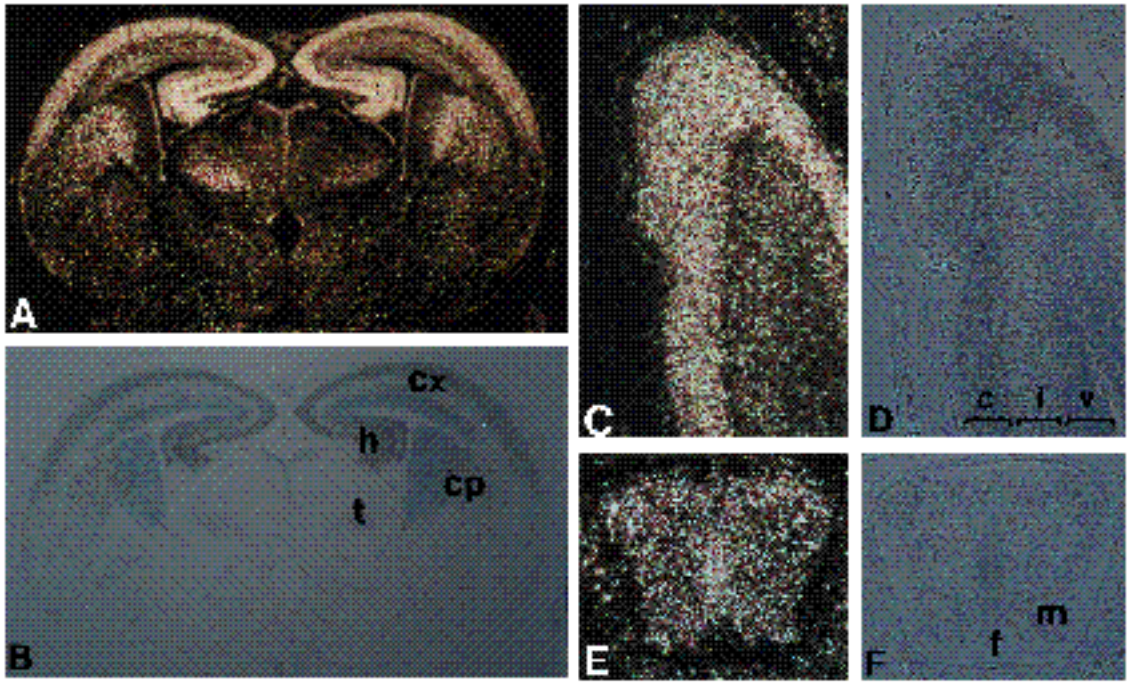

Fig. 4. *trk*C expression in embryonic CNS. (A) Dark-field and (B) bright-field of a coronal section from 17.5 day embryonic brain. (C) Dark-field and (D) bright-field magnification of 17.5 day embryonic cortex. (E) Dark-field and (F) bright-field view of a section through the mid-lumbar region of a 15.5 day embryo spinal cord. cx, cortex; c, cortical layer; i, intermediate layer; v, ventricular layer; cp, caudate putamen; h, hippocampus; t, thalamus; m, ventral horn (motor neuron region of the spinal cord); f, floor plate.

Table 1. Summary of *trk*C expression

	E9.5	E11.5	E13.5	E15.5	E17.5
(A) During mouse development					
CNS	Prosencephalon Mesencephalon	Telencephalon Diencephalon	See panel B	See panel B	See panel B
	Rhomboencephalon	Mesencephalon Rhomboencephalon	Cerebellum	Cerebellum	Cerebellum
	Neural tube	Neural tube	Spinal cord	Spinal cord	Spinal cord
PNS	Neural crest cells	DRG V trigeminal VII-VIII facio-acoustic complex IX superior and inferior glossopharyngeal X superior ganglion	DRG V trigeminal VIII vestibulo-coclear ganglia Intestine coeliac ganglion	DRG V trigeminal VIII vestibulo-coclear ganglia Intestinal ganglia Sympathetic	DRG V trigeminal VIII vestibulo-coclear ganglia Enteric ganglia Sympathetic
Others	Aortic arch arteries	Dorsal aorta Urogenital mesenchyme	Aorta Arteries Urogenital mesenchyme	Aorta Lung arteries Adrenal Submandibular gland Cervical brown adipose Foot pad Whisker pad	Aorta Lung arteries Adrenal Submandibular gland Cervical brown adipose Foot pad Whisker pad
(B)In the CNS					
	E13.5	E15.5	E17.5	10 day	6 wk
	Neocortex Basal telencephalon Hippocampal neo Striatum Diencephalon Pons Medulla Cerebellum Facial nucleus	Neocortex Cortical plate Intermediate layer Striatum Pons Medulla Cerebellum External germinal layer Purkinje cell layer	Neocortex Cortical plate Intermediate layer Striatum Pons Medulla Cerebellum External germinal layer Purkinje cell layer	Cortex Caudate putamen Fornix Thalamic nuclei Hyppocampus Medulla Olfactory nucleus Cerebellum Granule cell layer Purkinje cell layer	Cortex Hypotalamus Thalamic nuclei Hyppocampus Olfactory nucleus Cerebellum Granule cell layer Purkinje cell layer

Jones and Reichardt, 1990; Kaisho et al., 1990; Maisonpierre et al., 1990a; Rosenthal et al., 1990), *Xenopus* NT-4 (xNT-4, Hallböök et al., 1991; Ip et al., 1992) and human NT-5 (hNT-5; Berkemeier et al., 1991), may have broader physiological effects.

The rat *trk*C locus is complex, encoding multiple distinct receptors (Tsoulfas et al., 1993). These *trk*C isoforms bind NT-3, a widely expressed neurotrophin (Maisonpierre et al., 1990b), in chemical cross-linking and equilibrium binding analyses (Lamballe et al., 1991, Tsoulfas et al., 1993).

In this study, we have investigated the expression of *trkC* in the mouse embryo and adult CNS. We find *trk*C transcripts throughout the nervous system but also in non-neural tissues. Furthermore, we contrast the expression of the *trk*C gene with that of *trk* and *trk*B. Finally, we have mapped the chromosomal location of the three *Trk* loci and show that they are unlinked and map in the vicinity of previously existing neurological mutations. This information may lead to a direct association of *Trk* genes with the phenotypes elicited by known spontaneous mutations in the mouse.

MATERIALS AND METHODS

RNA preparation and RNAse protection analysis

RNA was extracted using RNAzol (Cinna/Biotecx) following the manufacturer's recommendations. RNAse protection experiments were performed as previously described (Tessarollo et al.,1992) using the RPA kit (Ambion). A genomic *trk*C-specific probe that spans 196 nucleotides of the extracellular domain (aa 336-401 of the rat sequence; Tsoulfas et al., 1993) and including 64 downstream intronic nucleotides was used to generate an antisense RNA probe employed in RNAse protection analysis. A 250 base β-actin c-RNA probe (Alonso et al., 1986) was included in the same reaction as a means of assessing relative levels of RNA present in each hybridization.

In situ hybridization

In situ hybridization protocols were as described (Martin-Zanca et al. 1990) with the following modifications. Dissected embryos were fixed overnight in 4% paraformaldehyde, dehydrated with alcohols and xylenes, and embedded in paraffin. Embryos were sectioned at 5 μm thickness and mounted on gelatin-coated slides. Slides were deparaffinized in xylene and rehydrated in graded (100-30%) ethanol solutions. After fixing in 4% paraformaldehyde, the tissues were pretreated with proteinase K (20 μg/ml) (Boehringer Mannheim), refixed and immersed in triethanolamine buffer containing acetic anydrate and dehydrated. Sections were hybridized with antisense cRNA probes (5×10^5 cts/minute) in a buffer containing 50% formamide, 0.3 M NaCl, 20 mM Tris-Cl (pH 7.4), 1× Denhardt's solution, 0.5 mg/ml yeast tRNA and 10 mM DTT at 50°C for 20 hours. After hybridization, washes were performed in 4× SSC and 10 mM DTT at 50°C. The slides were then incubated for 30 minutes at 37°C with RNase A (20 μg/ml) and RNase T_1 (2 μg/ml) followed by a 30 minute incubation at 55°C in 50% formamide, 0.2× SSC, 10 mM DTT, washed twice for 30 minutes in 0.2× SSC, 1% sodium pyrophosphate (w/v), 10 mM DTT and dehydrated. The slides were dipped in Kodak emulsion NTB-2 and exposed for up to 10 days at 4°C. The slides were then developed in Kodak D-19, fixed as recommended by manufacturer and stained in 0.2% toluidine blue. All photomi-

croscopy was done on a Zeiss Axiophot microscope. Sense and anti-sense probes labeled with ^{35}S were prepared by standard procedures (Krieg and Melton, 1987) by using UTP as the labeled nucleotide. The *trkC* antisense probe was synthesized from a ~2 kb rat *trk*C cDNA (NRT-8) as described by Tsoulfas et al. (1993). The *trk* and *trkB* probes are described in detail by Martin-Zanca et al. (1990) and Klein et al. (1989), respectively.

Interspecific backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 progeny were obtained; a random subset of these N_2 mice were used to map the *Trk* loci (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed as described (Jenkins et al., 1982). All blots were prepared with Zetabind nylon membrane (AMF-Cuno). Hybridization probes were labeled with [α^{32}P] dCTP using a nick translation labeling kit (IL-9) (Boehringer Mannheim) or random priming (*trk*, *trk*B, *trk*C) (Amersham Corporation). Washing was done to a final stringency of 0.2-0.8× SSCP, 0.1% SDS, 65°C.

The three mouse *Trk* loci, *trk*, *trk*B and *trk*C, have been designated *Ntrk1*, *Ntrk2* and *Ntrk3*, respectively, to conform with the current human locus designations for these genes. The *trk* probe was a 1.5 kb *Eco*RI genomic fragment from the mouse *trk* extracellular domain (Martin-Zanca et al., 1990). The *trk* probe hybridization to a 6.4 kb fragment in *Bgl*II-digested C57BL/6J (B6) DNA and 5.0 and 1.3 kb fragments in *M. spretus* (S) DNA. The two *M. spretus*-specific fragments cosegregated and were followed in backcross mice. The *trk*B probe was a 938 bp corresponding to amino acid 1-308 of mouse *trk*B sequences (Klein et al., 1989). The *trk*B probe hybridized to 3.8, 2.5, 1.1, 0.9 and 0.35 kb fragments in *Taq*I-digested B6 DNA and 3.5, 3.1, 2.5, 1.4, 1.1 and 0.35 kb fragments in S DNA. The 3.5, 3.1 and 1.4 kb *M. spretus*-specific fragments cosegregated and were followed in backcross mice. The *trk*C probe was a 288 bp cDNA corresponding to amino acid 282-378 of the mouse *trkC* gene (Tsoulfas et al., 1993). The *Trk*C probe hybridized to a 1.3 kb fragment in *Eco*RI-digested B6 DNA and a 3.6 kb fragment in S DNA. The 1.3 kb *M. spretus*-specific fragment was followed in backcross mice.

A description of most of the probes and RFLPs for loci used to position the *Trk* loci on the interspecific map have been reported. The loci in include: fibrinogen, gamma polypeptide (*Fgg*), connexin-40 (*Cnx-40*) and nerve growth factor beta (*Ngfb*) on chromosome 3 (Mucenski et al., 1988; Cox et al., 1991; Haefliger et al., 1992); neuroendocrine convertase-1 (*Nec-1)* on chromosome 13 (Copeland et al., 1992); and insulin-like growth factor 1 receptor (*Igflr*), feline sarcoma oncogene (*Fes*) and tyrosinase (*Tyr*) on chromosome 7 (Lunsford et al., 1990; Copeland et al., 1992). One locus, interleukin-9 (*IL-9*) on chromosome 13 has not been previously reported for our panel. The IL-9 probe (pP40.2B4) was a 550 bp mouse cDNA (Van Snick et al., 1989). The IL-9 probe hybridized to an 18.0 kb fragment in *Sph*I-digested B6 DNA and a 5.4 kb fragment in S DNA. The 5.4 kb *M. spretus*-specific fragment was followed in backcross mice.

Recombination distances were calculated as described (Green, 1981) using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS

To determine the temporal expression pattern for *trk*C during murine development, a *trk*C-specific probe was used in RNAse protection assays employing a β-actin internal control as described in Materials and methods. 10 μg of total mouse embryo RNA were assayed and, although a faint protected band is first observed on day 7.5, *trk*C gene expression is clearly detected at embryonic day 8.5 coinciding with the timing of neural tube formation (Fig. 1). Relative to the internal β-actin control, a substantial increase in *trk*C mRNA expression occurs around embryonic day 10.5 (25-35 somites).

The *trk* and *trk*B genes are expressed predominantly in the nervous system (Martin-Zanca et al., 1990; Klein et al., 1990a). Having determined that *trk*C transcripts are present in the embryo from the earliest stages of neural induction, we next performed RNA in situ hybridization of postimplantation embryo sections from day 7.5 through day 17.5 and in neo-nate and adult central nervous system (CNS; see Materials and methods). For comparison, in the course of this study, we hybridized adjacent embryo sections with *trk*, *trk*B and *trk*C probes.

The results obtained from our in situ experiments on *trk*C expression are summarized in Table 1. The expression of this gene is highly complex. We first observed *trk*C hybridization as a weak signal in 7.5 day egg cylinders with a pattern indicating expression in the early neuroectoderm (not shown). Whole-mount in situ experiments are in progress to extend these observations. *trk*C gene expression is evident throughout the neuroepithelium of 9.5 (12-30 somites) day embryos (Fig. 2A-D). At these early stages, we also observe *trk*C transcripts in regions where neural crest cells are known to localize. These include forming dorsal root ganglia (DRG) and regions adjacent to the neural tube and the dorsal aorta (Fig. 2C,D; arrows), locations identified as migratory routes used by cells of neural crest origin (see below).

During organogenesis, additional sites of active *trkC* are found. Fig. 2E-F shows a sagittal section from an 11.5 day embryo (35 somites). *trk*C expression is exhibited throughout the CNS (telencephalon, diencephalon, mesencephalon, rhombencephalon and neural tube) and comparison with an adjacent *trk*B hybridized section (not shown) indicates overlapping expression in the mantle (postmitotic) layer of the spinal cord, whereas the ependymal (mitotic) layer expresses only *trkB.* The *trk*B and *trk*C genes are coexpressed in the branchial arch arteries; however, more caudal *trk*B transcripts are present in the mesonephric region while *trk*C is expressed in the midgut and genital ridge associated mesenchyme (see Fig. 2E-F).

At 13.5 days of development, the *trk*C gene is expressed throughout the neural network (Fig. 2G-I). At this stage, *trk*C mRNA is present in neural tissues where *trk* and *trk*B gene activity has not been previously detected (Martin-Zanca et al., 1990; Klein et al., 1989, 1990a). One striking example is the expression of *trk*C in neural-crest-derived cells of the developing enteric nervous system (Figs 2H, 7). In contrast, *trk*C and *trk*B transcripts are present in the trigeminal (V^{th} cranial) ganglion (Fig. 2H,I) where punctate hybridization reflects expression in a small subset of cells (Fig. 2I). The three afferent branches of the trigeminal ganglion (ophthalmic, maxillary and mandibular) can be readily traced by abundant *trk*C hybridization, presumably reflecting expression in the neural crest-derived

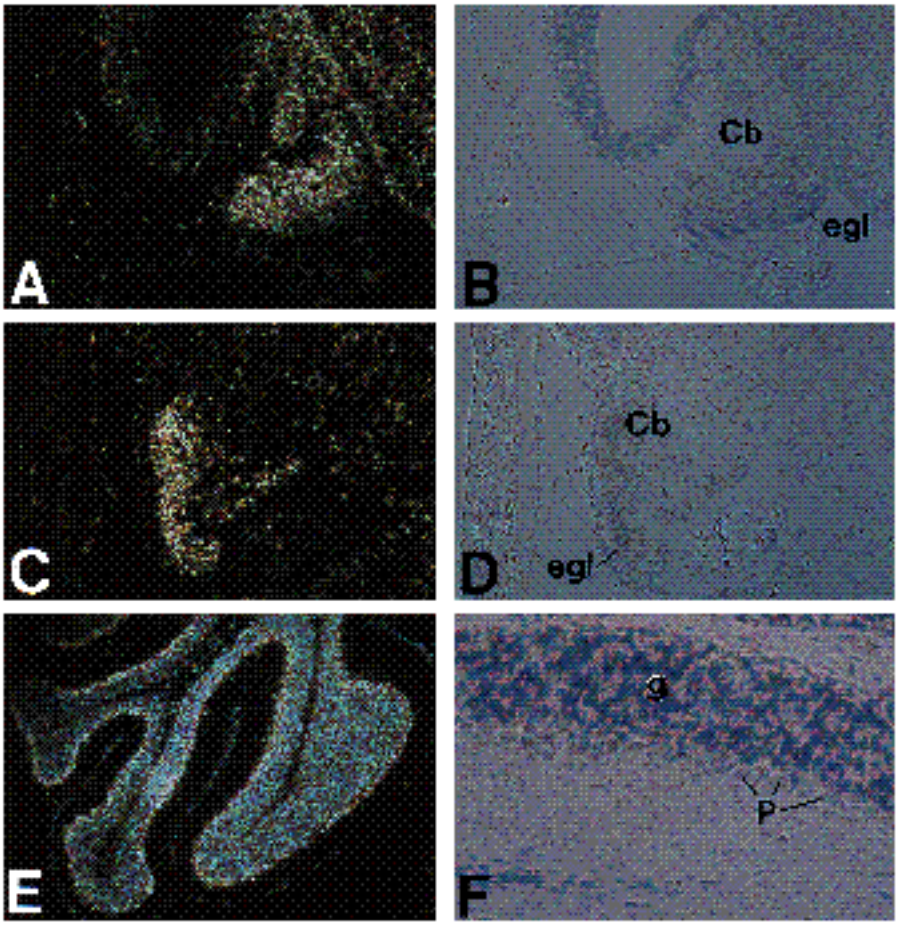

Fig. 5. *trk*C expression in the developing mouse cerebellum. Dark-field (A,C) and bright-field (B,D) of a sagittal section through a 13.5 (A,B); 17.5 (C,D) mouse embryonic cerebellum; dark-field view (E) and bright-field photomicrograph (F) of 10 day postnatal mouse cerebellum sections hybridized with a *trk*C probe. Cb, cerebellum; g, granular layer; P, Purkinje cells; egl, external germinal layer.

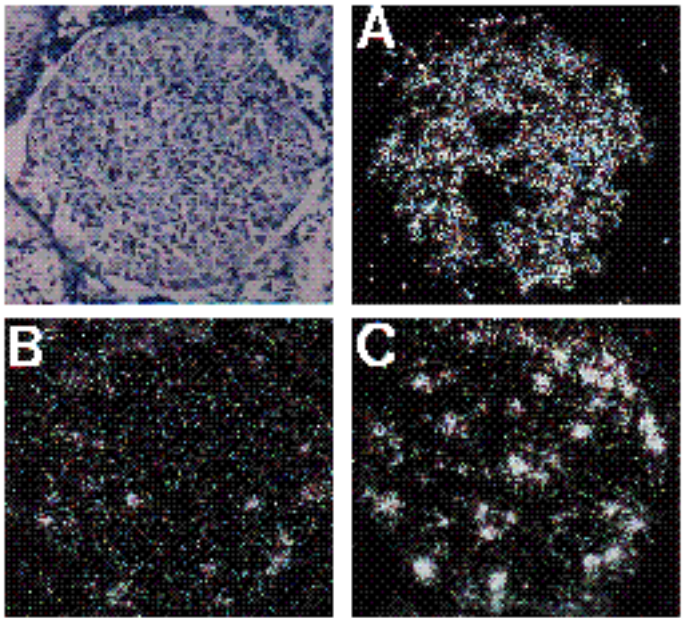

Fig. 6. Expression of the *Trk* gene family members in 17.5 embryonic DRG. Dark-field views of serial adjacent sections through a E17.5 DRG hybridized with a *trk* (A), *trk*B (B) and *trk*C (C)-specific probe.

Schwann cells that migrate and differentiate along the trigeminal processes (Fig. 2G).

Several insights can be drawn from a direct comparison of *trk*, *trk*B and *trk*C expression at later stages in development. Fig. 3 shows in situ hybridization of day 17.5 sections, from a single fetus, which were hybridized with probes to the three related genes. *trk* expression is distinctive in its tight regulation primarily limited to trigeminal (V), superior cervical (C), sympathetic (S) and dorsal root ganglia (arrows; Fig. 3A; Martin-Zanca et al., 1990). In comparison, *trk*B is expressed in these ganglia and at high levels in the CNS and additional structures including the whisker pad (W), tongue (T) and tooth buds (white arrowhead; Fig. 3B). This complex expression pattern is consistent throughout mid-gestation (days 14-18; L. F. P., unpublished observations). Expression of the *trk*C gene mirrors that of *trk*B in many regions of the embryo (Fig. 3B,C). Like the *trk*B gene (Klein et al 1990a; Parada et al., 1992), *trk*C is expressed throughout the nervous system including many of the same cranial and spinal ganglia (V, VII, VIII, X, DRG). The *trk*C and *trk*B genes are also apparently co-expressed in several non-neural structures including the tongue, the whisker pad (vibrissae) mesenchyme and forming teeth (Fig. 3B,C). In the tooth, *trk*B is expressed in the outer dermal papilla while *trk*C transcripts appear confined to the core of the dermal papilla (compare Fig. 3B and C).

*trk*C transcripts are also found in several additional

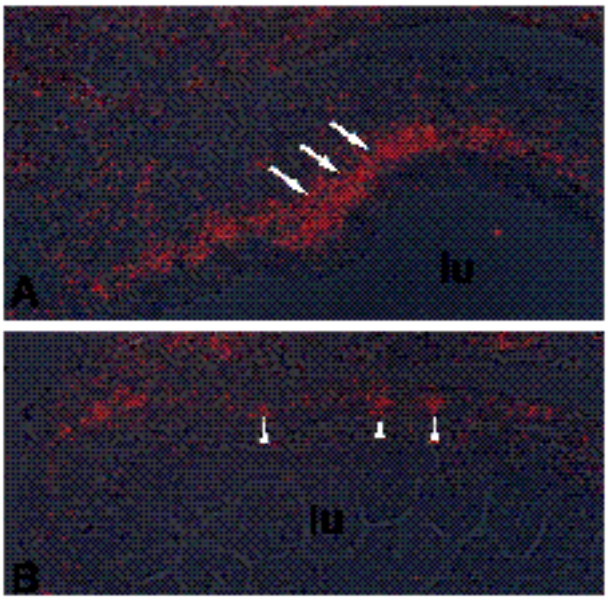

Fig. 7. *trk*C expression in the developing gut. Bright-dark-field view of sections through E13.5 embryonic stomach (A) and E17.5 intestine (B). lu indicates the stomach (A) and intestine (B) lumen; arrows in panel A point at migratory neural crest cells; arrowheads (B) indicate the forming myenteric plexus.

neural crest-derived structures including enteric ganglia (see Fig. 7) and in other non-neural cells (see below).

Embryonic CNS

The pattern of *trk*C gene expression is summarized in Table 1 and examples of in situ hybridization in the embryonic CNS are shown in Figs 2, 3C and 4. Interestingly, this gene shows both overlapping and exclusive expression profiles in the cephalic CNS when compared to the *trk*B gene. In the telencephalic cortical plate, both *trk*C and *trk*B transcripts are highly represented whereas in the intermediate layer only *trk*C mRNA was observed. In the ventricular layer, *trk*B expression is high and *trk*C expression is low (Figs 3B,C, 4A-D). In the striatum (Sr), *trk*C mRNA is most abundant centrally while *trk*B transcripts predominate along the ependymal layer. Finally, the thalamic nuclei (t) appear to express either *trk*B or *trk*C (Fig. 3).

A cross section through the caudal spinal cord of a 15.5 day embryo reveals *trk*C transcripts throughout the dorsal-ventral axis of the mantle layer including the ventral horn where motor neurons are located (Fig. 4E,F). No evidence of expression was seen in the floor plate at any developmental stages examined.

*trk*C transcripts are also abundant in the embryonic metencephalon (cerebellum). Fig. 5 provides examples of *trk*C expression in cerebellum at embryonic days 13.5, 17.5 and postnatal day 10. Transcripts are seen throughout embryonic stages with high levels in the immature but post-mitotic Purkinje cells (Fig. 5A-D; Altman and Bayer, 1985). No expression was observed in the external germinal layer (egl). In all postnatal stages examined, including adult, *trk*C mRNA levels are very high in the cerebellar granule layers and in Purkinje neurons (Fig. 5E,F and not shown).

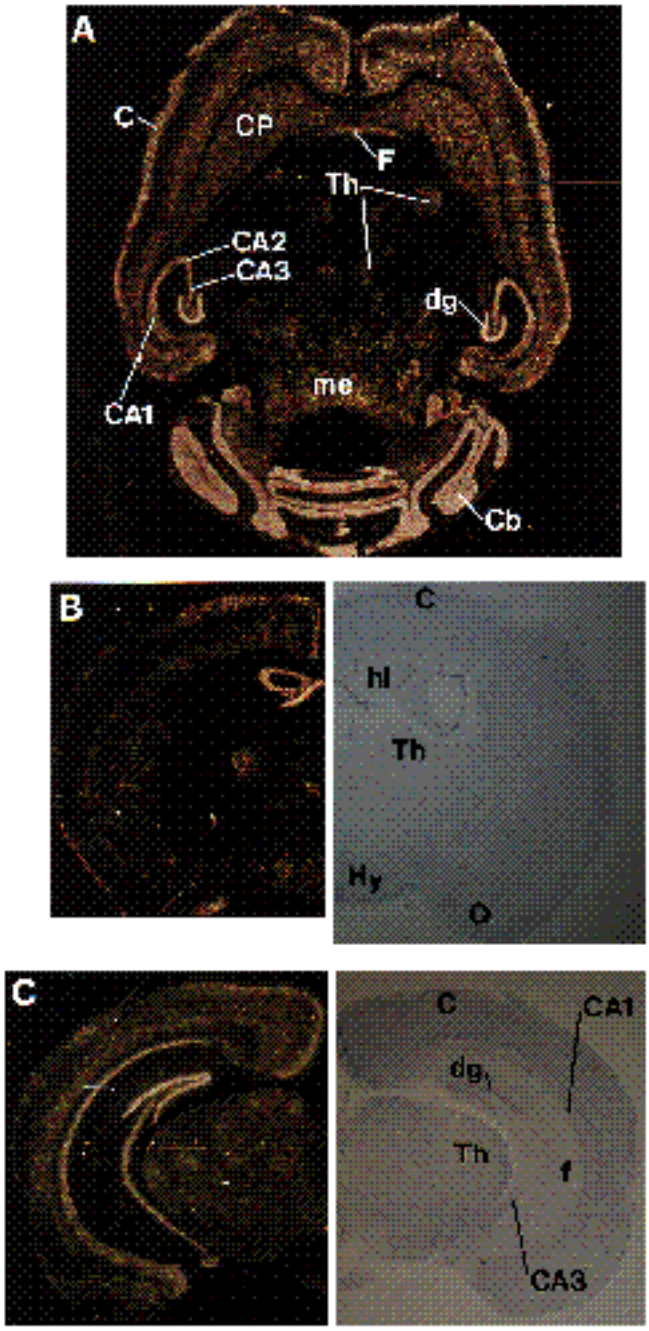

Fig. 8. *trk*C expression in early postnatal and adult mouse brain. (A) Dark-field view of an horizontal section throughout a 10 day postnatal mouse brain. (B,C) Dark-field and bright-field views of coronal sections of 6 weeks old mouse brain hybridized with a *trk*C-specific probe. C, cortex; CP, caudatus putamen; F, fornix; Th, thalamic nuclei; CA1, CA2, CA3: stratum pyramidali; dg, dentate gyrus; me, medulla; Cb, cerebellum; hi, hippocampus; Hy, hypothalamus; O, olfactory nucleus; f, fimbria hippocampi.

Peripheral nervous system

The neural crest gives rise to a large component of the peripheral nervous system (PNS). *trk*C transcripts are found in many PNS structures and also in cells that appear to be migratory, neural crest cells (Table 1, Fig. 2A,C,E, and unpublished results). As previously noted (Figs 2, 3), all

Trk genes are expressed to a greater or lesser extent in neural crest-derived embryonic cranial, spinal and sympathetic ganglia. Fig. 6 shows in situ hybridization of three adjacent DRG sections from a 17.5 day embryo. These sections (5 μm) provide direct comparison of *Trk* gene expression and, in agreement with a report by Carroll and co-workers (1992), show that a majority of neurons express *trk* (Fig. 6A), while *trk*B (Fig. 6B) and *trk*C (Figs 6C, 2I) transcripts are present in a reduced subset of neurons. Very similar distribution of the three genes is seen in trigeminal ganglion (Fig. 3; see also Martin-Zanca et al., 1990; and Klein et al., 1990a).

The ganglia of the enteric nervous system are formed from migratory neural crest cells originating in the vagal crest region (Le Douarin and Teillet, 1973). These cells hybridize with *trk*C probes (Fig. 7A) as exemplified by hybridization of embryonic day 13.5 stomach where *trk*C-expressing migratory neural crest cells can be observed intercalated within the mesenchyme. Several days later, *trk*C transcripts can be localized to the forming ganglia that appear throughout the muscle and submucosal layers of the intestine (Fig. 7B). We do not observe *trk*C expression in the epithelium (Fig. 7). Thus, *trk*C expression is found in diverse PNS structures some of which do not appear to express other *Trk*-family receptors (Martin-Zanca et al., 1990; Klein et al., 1990a).

Adult CNS expression

*trk*C gene expression remains abundant in the adult mouse CNS. The cerebral cortex exhibits high levels of transcripts particularly in the superficial layers and in the deep layers with reduced expression in the intermediate layers (Fig. 8). *Trk*C transcripts are also abundant in the caudate putamen from embryonic stages (Fig. 4A,B), a pattern that remains unchanged in the adult CNS (Fig. 8B). Similarly, thalamic nuclei maintain *trk*C expression throughout development and in the adult (Fig. 8A,B). Thalamic nuclei do not appear to express *trk*B transcripts in adult brain (Klein et al., 1990a).

In the hippocampus of adult brain, the pyramidal cell layer as well as the neurons of the dentate gyrus exhibit high levels of *trk*C mRNA (Fig. 8A-C). In the cerebellum, both the Purkinje and the granule cell layer express high levels of *trk*C transcripts. Thus, expression of the *trk*C gene observed in the mature brain is consistent with that exhibited during embryonic development.

*Trk*C expression outside the nervous system

As can be appreciated from Figs 2 and 3, *trk*C-encoding transcripts are abundant throughout the embryo including sites that are not necessarily consistent with expression in neural cells (see Table 1). Note the presence of *trk*C transcripts in vibrissae and dental papillae (Fig. 3). We also find *trk*C transcripts in cells of the submandibular gland (Fig. 9A,B) and in the mesenchyme surrounding mesonephric and urogenital ducts (Fig. 9C,D). High levels of *trk*C transcripts can also be localized to the brown adipose tissue dorsal to the cervical spinal cord (Fig. 9D,E); the cortex of the metanephros; and adrenal gland (Fig. 9F,G). Finally, abundant *trk*C expression is found along the subendothelial mesenchyme of arteries throughout the developing embryo (Figs 9H,I and 2, 3).

Chromosomal mapping

We next wished to identify the chromosomal location of the three *Trk* genes to determine whether they are linked or map to separate chromosomal locations and to assess the possibility that these three genes might be associated with previously reported neurological mutations in the mouse. The chromosomal location of *trk*, *trk*B and *trk*C was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *Mus spretus*)F_1 × C57BL/6J] mice that have been typed for over 1100 loci distributed among all mouse chromosomes (Copeland and Jenkins, 1991; see Materials and methods).

Each of the *Trk* genes mapped to a different mouse autosome (Fig. 10), indicating that they have become well dispersed during chromosome evolution. To determine whether any of the *Trk* genes mapped near a known mouse mutation with a phenotype that might be consistent with a defect in a *Trk* gene, we aligned our interspecific maps of chromosomes 3, 13 and 7 with composite linkage maps that report the map location of many uncloned mouse mutations (compiled from GBASE, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME). Interestingly, all three genes mapped near loci that exhibit neurological phenotypes.

trk mapped in the vicinity of the spontaneous neurological mutation spastic (*spa*) located on mouse chromosome 3 (Chai, 1961; Van Heyningen et al., 1975; Wilson et al., 1986; reviewed in Green, 1989). *trk*B mapped in the vicinity of the spontaneous neurological mutation Purkinje cell degeneration (*pcd*) located on chromosome 13 (Mullen et al., 1976; O'Gorman, 1985; reviewed in Green, 1989). Finally, *trk*C mapped in the vicinity of a gene on mouse chromosome 7 that has been shown to affect susceptibility of inbred mice to audiogenic seizures. (Neumann and Collins, 1991).

DISCUSSION

Recent studies from a number of laboratories, showing that *trk* and *trk*B are expressed mainly in the nervous system and encode functional receptors for the neurotrophins, have been important for advancing our understanding of the mechanisms that regulate neural development.

In the present study, we show that *trk*C, a third member of the *Trk* gene family (Lamballe et al., 1991; Tsoulfas et al., 1993), is expressed in a broad component of the nervous system and in non-neural tissues, including cell types where other *Trk* genes are not active.

*trk*C is expressed early in development

*trk*C expression was first observed in the gastrulating embryo, coordinate with the temporal expression pattern of its ligand NT-3, the more abundant neurotrophin detected early in development (Maisonpierre et al., 1990b). This is the earliest example of an active *Trk* gene reported to date, raising the possibility that the *trk*C RTK may mediate important functions in early neuroepithelium formation.

Neural crest

Kalcheim and co-workers (1992) have reported a proliferative activity of NT-3 in migratory neural crest cells. In the present study, we provide evidence for NT-3 receptor expression in migratory neural crest cells, thus supporting the notion that *Trk* receptors and their ligands may mediate alternative functions to trophic signals during development (for review, see Barbacid et al., 1991).

The *trk*C gene is expressed in many neural crest derivatives including cranial, dorsal root and sympathetic ganglia. In trigeminal and dorsal root ganglia, all *Trk* genes are active. *trk* mRNA is perhaps most abundant and confined to neurons in these ganglia. We note that in addition to expression in sympathetic ganglia late in development, *trk* expression can also be detected in isolated neurons of the basal forebrain, hindbrain and in other locations of the late embryonic and adult brain (Holtzman et al., 1992; L. F. P., unpublished observations). Like *trk*B, *trk*C is expressed in fewer neurons in sensory ganglia. However, in the trigeminal ganglion, the afferent projections are outlined by expression of these genes suggesting that *trk*B and *trk*C are expressed in neural crest-derived Schwann cells that migrate along the axonal projections to their sites of differentiation.

Another notable site of *trk*C-specific expression is the enteric neural crest and ganglia. Through *trk*C hybridization, it is possible to trace the migration of vagal neural crest cells into the gut region, suggesting that neurotrophins may have important functions in the migratory and trophic potential of enteric neurons.

trkC is also expressed in a group of non-neural tissues that, in quail-chick studies (Le Douarin, 1982) or in radioisotopic labeling experiments in the amphibian embryo (Chibon, 1964), have suggested neural crest origin. Among these are the artery walls derived from aortic arches, tooth papillae (Chibon, 1970) and salivary glands. Using RNAse protection and northern analyses, we also observe *trk*C transcripts in the thymus (Tsoulfas et al., 1993), another tissue whose connective tissue component is derived from the neural crest.

Brown adipose tissue, particularly in the cervical region dorsal to the spinal cord, also expresses high levels of *trk*C transcripts (Fig. 9E,F). Because of the high silver grain density caused by strong expression, we are unable to identify precisely the expressing cell types within this tissue. Electron microscopy studies indicate that brown adipose is particularly well innervated and Schwann cells have been observed sheathing unmyelinated nerve fibers in the lobules, close to the vessels, and between adipocytes (Bargmann et al., 1968; Linck et al., 1973). Therefore, *trk*C expression in this tissue may reflect the presence of transcripts in neural cells and not in adipocytes. However, in chick, the subcutaneous adipose of the face and the ventral neck region is derived from the neural crest (Le Douarin, 1982), while the precise origin of the brown adipose tissue has not been well established (Néchad, 1986). Thus, it is also possible that this adipose tissue may be of neural crest origin and retain *trk*C expression.

Trk genes are expressed in a broad spectrum of neural crest derivatives implying that neurotrophins may function in cells where activity has not previously been associated. The *trkB* and *trkC* expression profiles exhibited in migratory neural crest cells along the descending aorta suggest that *trk*B and *trk*C products may represent good markers for subsets of neural-crest-derived cells.

CNS

In the CNS, the *trk*B and *trk*C genes are coexpressed and differentially expressed in intriguing patterns. We observed coexpression of these genes in the hippocampus (pyramidal and granule neurons), in the cerebellum (Purkinje cells), in the ventral spinal cord and in regions of the cerebral cortex (L. T. and L. F. P., unpublished data). In contrast, thalamic nuclei appear to express *trk*C transcripts preferentially in the adult. Similarly in the cerebellum, *trk*C expression is abundant in the granular layer where little or no *trk*B transcripts could be detected (see Klein et al., 1990b).

The neurotrophin theory has been well substantiated in the sensory nervous system through the study of NGF-related neurotrophins and through the availability of well-defined primary culture systems (Barde, 1989). The search for trophic molecules acting on motor neurons has proven more elusive. Culturing of motor neurons is technically difficult and, although tissue extracts have been reported to promote motor neuron survival, only one molecule in particular, CNTF (Sendtner et al., 1992a) has been identified to promote survival of these cells in vivo. Until recently, efforts to identify a motor neuron response to the NGF family of neurotrophins have not been successful. Very recently BDNF has been shown to rescue motor neurons in vivo in rat and chick (Yan et al., 1992; Oppenheim et al., 1992; Sendtner et al., 1992b). The present results indicate the existence of *trk*C and *trk*B (Klein et al., 1990a) transcripts during embryogenesis in the ventral horn of the spinal cord and in other regions where motor neurons are localized. These results therefore suggest the need for a renewed effort at understanding neurotrophin function in motor neurons.

Chromosomal location

In mouse, the three *Trk* family members map to different mouse autosomes and are located in regions containing known neurological mutations. For example, the mouse *trk* gene maps near the *spa* mutation on chromosome 13. *spa* homozygotes show spastic symptoms although no anatomical abnormalities have been described. Glycine receptor deficiencies have been reported in these mice (White and Heller, 1982; White, 1985), but it remains to be determined whether these deficiencies are the cause or a secondary effect of the *spa* mutation.

Likewise, *trkB* maps on chromosome 3, in a region where the *pcd* mutation has previously been mapped. *pcd* homozygotes show a moderate ataxia beginning at 3 to 4 weeks of age. They are smaller than their normal littermates but live a fairly normal life span although adult males are infertile. In these mice, Purkinje cells begin to degenerate by 15 to 18 days of age followed by a slower degeneration of the photoreceptor cells of the retina and mitral cells of the olfactory bulb. Later (50-60 days of age) discrete populations of thalamic neurons also degenerate. Studies of fusion chimeras between *pcd/pcd* and +/+ embryos suggest that

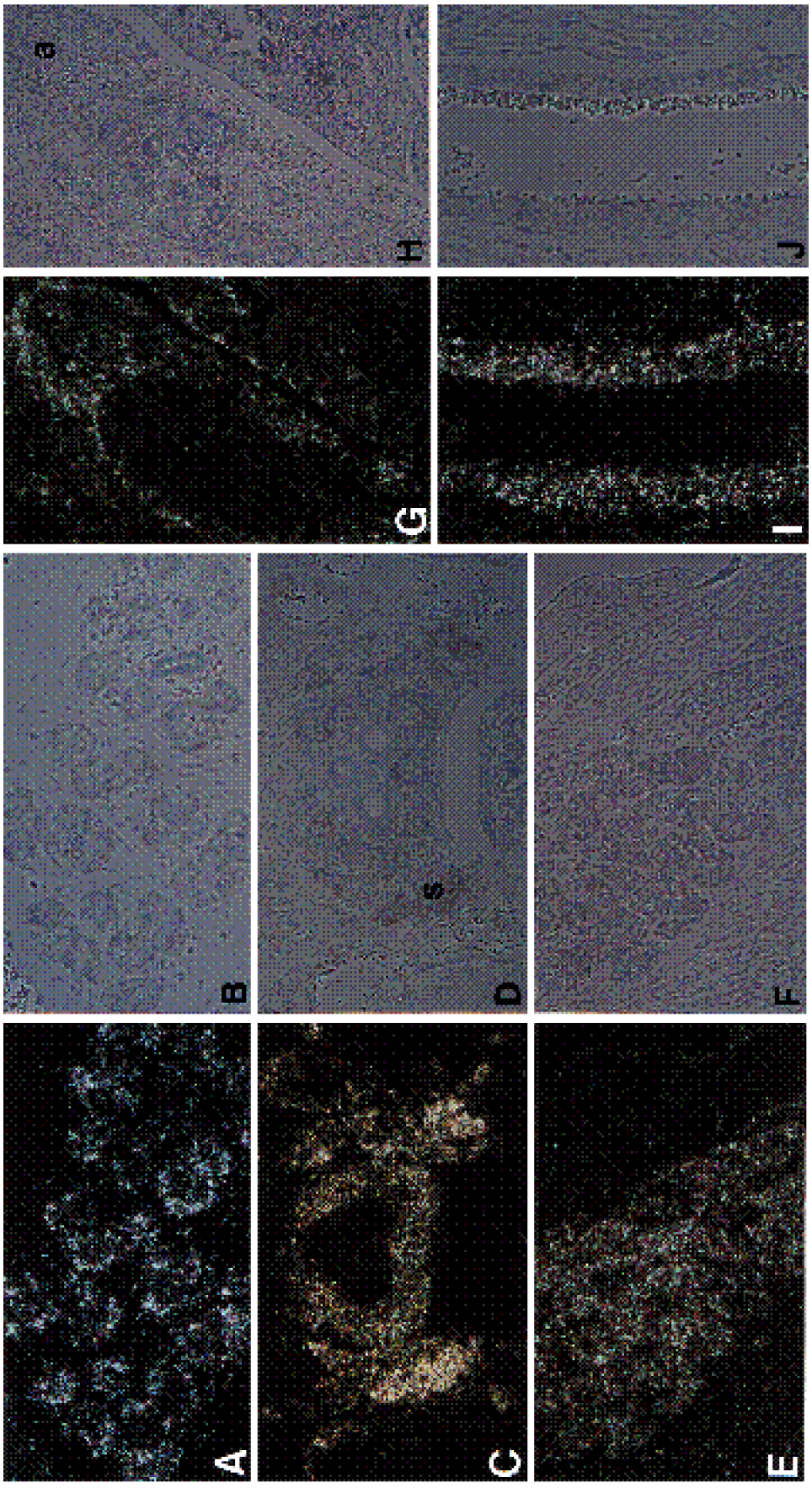

Fig. 9. *trkC* expression in non-neuronal E15.5 embryonic tissues. (A) Dark-field and (B) light-field of the submandibular salivary gland. (C) Dark-field and (D) bright-field of a section through the genital region; s, sympathetic ganglia. (E) Dark-field and (F) light-field view of cervical brown adipose tissue. (G) Dark-field and (H) light-field of a section throughout the kidney and the adrenal gland (a). (I,J) Dark-field and light-field magnification of a sagittal section through the dorsal aorta.

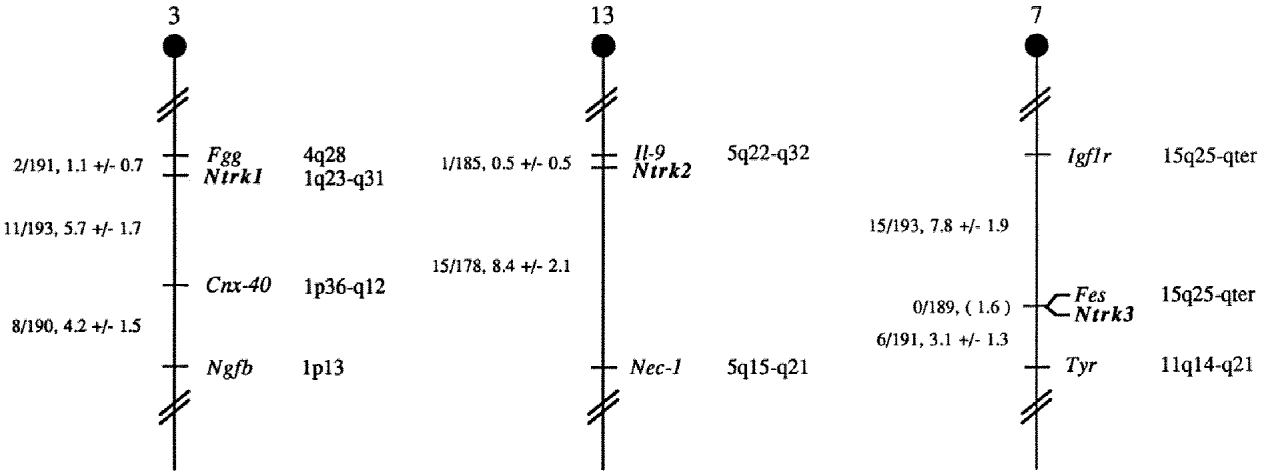

Fig. 10. Linkage maps showing the chromosomal locations of *Ntrk* loci in mouse. The *Ntrk* loci were mapped by interspecific backcross analysis. The number of recombinant N_2 animals over the total number of animals typed plus the recombination frequencies, expressed as genetic distance in centimorgans (± 1 s.e.), is shown for each pair of loci on the left of the chromosome maps. Where no recombinants were found between loci, the upper 95% confidence limit of the recombination distance is given in parentheses. The position of loci in human chromosomes, determined in previously reported studies, are shown to the right of the chromosome maps. References for the human map positions of loci mapped in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

pcd acts directly within the Purkinje cells themselves (Mullen, 1977). The *trk*B gene is expressed in Purkinje cells, in retinal ganglion cells, in motor neurons and in a spectrum of other neural tissues. Thus the *pcd* phenotype could be accounted for by mutations in the *trk*B locus.

The *trk*C locus maps in a region of chromosome 7 that has been associated with increased tendency to exhibit auditory defects in mice (Neumann and Collins, 1991). These auditory defect susceptibilities are ill-defined at this time but it is possible that the *trk*C locus may contribute to such abnormalities since the NT-3 gene and its receptor (data not shown) is expressed in the sensory components of the developing ear during embryogenesis (Pirvola et al., 1992). As germline mutations are induced in the *Trk* genes via homologous recombination in embryonic stem cells, it should be possible to perform allelism studies to determine whether any of these mutations do in fact result from defects in *Trk* genes.

The present mouse mapping studies can also be used to predict where the *Trk* genes will map in humans. For example, *trk*B maps between *Il-9* and *Nec-1* which have both been mapped to the long arm of human chromosome 5 (Fig. 10), suggesting that *trk*B will reside on the long arm of human chromosome 5 as well. Likewise, *trk*C does not recombine with *Fes*, which has been mapped to 15q25-qter. Finally, *trk* has been mapped by two groups to the long arm of human chromosome 1 (Miozzo et al., 1990; Morris et al., 1991). This placement is consistent with the mouse mapping studies shown in Fig. 10.

CONCLUSION

We have studied the expression of the *trk*C gene during development and in the adult CNS, and compared these data with that of the other identified *Trk* gene family members (Martin-Zanca et al., 1990; Klein et al., 1990a). These genes map to unlinked chromosomal locations and are primarily, though not exclusively, expressed in the nervous system. Understanding the expression patterns of *Trk* genes provides important information regarding potential sites of neurotrophin action and identification of cells that preferentially coexpress combinations of *Trk* receptors or which uniquely express one specific receptor. These data suggest that experimental strategies must be defined to explore the physiological significance of *Trk* receptor coexpression and of their possible function in previously unidentified sites of activity including motor neurons and non-neural cells. Finally knowledge of the sites of *Trk*-gene expression will provide important insights for the analysis of mice that lack *Trk*-gene function due to mutations generated by homologous recombination in embryonic stem cells.

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