Developmental Expression of *trkC*, the Neurotrophin-3 Receptor, in the Mammalian Nervous System

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The mammalian *trkC* gene encodes a tyrosine protein kinase that serves as a functional receptor for neurotrophin-3 (NT-3). Here, we report that *trkC* is widely expressed in the developing and adult nervous system. Using *in situ* hybridization, we first detect *trkC* transcripts in the telencephalon and spinal cord of embryonic day 9.5 mouse embryos. In later embryonic development, *trkC* is expressed in various structures of the CNS including the caudatoputamen, septal nuclei, cerebellum, and brainstem. In the PNS, *trkC* hybridization appears to correlate, both temporally and spatially, with the outgrowth of axons toward their peripheral targets. *trkC* transcripts were also identified in the autonomous enteric nervous system as well as in some non-neural tissues such as the wall of the aorta and the acini of the submaxillary and sublingual glands. In the adult mouse, *trkC* gene expression is heterogeneously distributed throughout the brain, with highest levels in limbic and diencephalic structures. These results indicate that the *trkC* gene is widely expressed in the three identified branches of the mammalian nervous system and appears to correlate with the expression of NT-3, its cognate ligand. The apparent colocalization of *trkC* transcripts with NT-3 raises the possibility this neurotrophin exerts its trophic effects by a paracrine and/or autocrine mechanism.

[Key words: tyrosine kinase, *trk*, *trkB*, NGF, brain-derived neurotrophic factor, growth factors]

The generation of neurons during embryonic development as well as their survival in the adult animal is thought to be dependent upon availability of diffusible neurotrophic factors. These neurotrophins, however, can only exert their programmed trophic roles in those cells that express their cognate signaling receptors. Therefore, a detailed knowledge of the spatial and temporal pattern of expression of these trophic factors and their receptors should help us to understand better the ontogeny of the vertebrate nervous system.

One of the most important groups of neurotrophic factors in the nervous system is the NGF family. To date, four members of this gene family have been identified. They include NGF (Levi-Montalcini, 1987), brain-derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3

(NT-3) (Ernfors et al., 1990a; Hohn et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1990b; Rosenthal et al., 1990), and neurotrophin-4 (NT-4) (Berkemeier et al., 1991; Hallbook et al., 1991; Ip et al., 1992). These molecules show a remarkable similarity in amino acid sequences and overall structural features but have a distinct range of neurotrophic activities (reviewed in Barde, 1989; Hefti, 1992). In the PNS, NGF supports the growth and survival of sympathetic neurons as well as that of certain sensory neurons such as those present in the dorsal root ganglia (DRG) and the trigeminal ganglion. NGF, however, does not support neurons derived from the nodose ganglia. In the CNS, the neurotrophic activity of NGF is restricted to cholinergic neurons of the basal forebrain and the striatum. BDNF, like NGF, supports DRG neurons in the PNS and septal cholinergic neurons in the CNS. Moreover, BDNF has neurotrophic activity in CNS retinal ganglion cells, GABAergic neurons of the basal forebrain, and dopaminergic neurons of the substantia nigra, as well as in peripheral neurons derived from nodose ganglia (Lindsey and Rohrer, 1985; Johnson et al., 1986; Thanos et al., 1989; Alderson et al., 1990; Hyman et al., 1991; Knusel et al., 1991).

NT-3 mimics NGF in its ability to elicit fiber outgrowth in sympathetic ganglia and BDNF in its trophic activity on neurons from nodose ganglia. Unlike BDNF, NT-3 does not have neurotrophic activity on central cholinergic or dopaminergic neurons (Johnson et al., 1986; Knusel et al., 1991). More recently, Collazo et al. (1992) have reported that NT-3 can enhance the proliferation of embryonic hippocampal precursors. The neurotrophic properties of NT-4, a neurotrophin primarily expressed outside of the brain, are beginning to be explored. NT-4 can promote neuritic outgrowth in dorsal root and nodose, but not in sympathetic ganglia (Hallbook et al., 1991). Since NT-4 mediates its trophic effects through the same signaling receptors as BDNF (Ip et al., 1992; Klein et al., 1992), it is likely that both neurotrophins will exhibit the same pattern of activity in *in vitro* assays. However, their *in vivo* activity will depend on their spatial and temporal pattern of expression as well as that of their cognate receptor(s).

The distribution of NGF, BDNF, and NT-3 transcripts reveals a general overlap in several regions of the CNS, although significant differences have been observed. For example, high levels of BDNF mRNA can be found in the CA2, CA3, and hilus region of the hippocampus, while NT-3 mRNA has been predominantly detected in the CA1 and CA2 regions (Ernfors et al., 1990b). Moreover, there are significant variations in the temporal pattern of expression for these neurotrophins. Whereas NT-3 is highly expressed in the developing brain and its expression decreases in the adult, BDNF transcripts are relatively lowly abundant in the embryo and undergo a steady increase

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during the late stages of development until they reach levels of expression comparable to those observed for NT-3 in the adult brain (Maisonpierre et al., 1990a).

The neurotrophins of the NGF family bind to two classes of receptors that can be distinguished according to their binding affinities and biological properties. The low-affinity receptor p75^{NGFR} binds all members of the NGF family of neurotrophins with similar nanomolar affinities (Chao, 1992). However, accumulating evidence indicates that p75^{NGFR} is not involved in mediating the trophic effects of these neurotrophins (Klein et al., 1991a; Birren et al., 1992; Ibanez et al., 1992; Jing et al., 1992). Instead, they appear to mediate their respective trophic activities through the Trk family of tyrosine protein kinase receptors (reviewed in Meakin and Shooter, 1992; Barbacid, 1993). These receptors include the product of the *trk* proto-oncogene, gp140^{trk} (Martin-Zanca et al., 1989) and two related tyrosine protein kinases, gp145^{trkB} and gp145^{trkC}, encoded by the *trkB* (Klein et al., 1989; Middlemas et al., 1991) and *trkC* (Lamballe et al., 1991) genes, respectively. The Trk family of receptors is also responsible for providing functional specificity to the NGF neurotrophin family. Whereas NGF binds specifically to gp140^{trk}, the related gp145^{trkB} tyrosine protein kinase serves as a receptor for both BDNF and NT-4.

The third member of this tyrosine protein kinase family, gp145^{trkC}, is a receptor for NT-3. When ectopically expressed in NIH3T3 cells, gp145^{trkC} binds NT-3 with high affinity in the low picomolar range (Lamballe et al., 1991). More importantly, this interaction results in receptor activation, phosphorylation of downstream signaling elements such as phospholipase C γ , induction of DNA synthesis, and ultimately in the malignant transformation of these cells (Lamballe et al., 1991, 1993). NT-3 also binds to gp140^{trk} and gp145^{trkB} receptors and mediates similar responses in NIH3T3 cells, albeit with 10–100-fold less potency (Cordon-Cardo et al., 1991; Glass et al., 1991; Ip et al., 1993). It is unlikely, however, that gp140^{trk} and gp145^{trkB} mediate the *in vivo* activities of this neurotrophin. For instance, NT-3 can only mediate neuronal differentiation of PC12 cells when they express gp145^{trkC} (Ip et al., 1993; Lamballe et al., 1993). Moreover, NT-3 promotes the survival of *trkC*-, but not of *trk*- or *trkB*-expressing DRG neurons (Ip et al., 1993).

The present studies were first undertaken to determine the temporal and spatial appearance of *trkC* transcripts and second to describe the dynamic changes that are observed throughout development. Previous studies have indicated that expression of the *trk* and *trkB* genes is tightly regulated during embryogenesis (Klein et al., 1990b; Martin-Zanca et al., 1990). While *trk* sequences remain confined to sympathetic and sensory neurons of neural crest origin throughout development, those of *trkB* extend to multiple structures of the CNS and PNS. Our results with *trkC* indicate that this gene is also expressed in multiple structures in the developing CNS and PNS. Moreover, *trkC* appears to be the only member of the *trk* gene family to be expressed in the autonomous enteric nervous system (ENS). These observations should contribute to our understanding of the molecular mechanisms that regulate the development and maintenance of the mammalian nervous system.

Materials and Methods

In situ hybridization. *trkC* riboprobes were generated from pFL25, a pGEM-3Zf(+)–derived plasmid (Promega) that contains a 570 base pair AccI DNA insert of a mouse *trkC* cDNA clone. These sequences encode a 190 amino acid region of the extracellular domain of the mouse

gp145^{trkC} receptor, which corresponds to residues 111–300 of its porcine counterpart. These sequences do not exhibit significant homology to the related *trk* and *trkB* genes and therefore should be specific for the *trkC* gene (Lamballe et al., 1991). To generate the antisense riboprobe, pFL25 was linearized with SacI and transcribed from the SP6 promoter. The sense riboprobe used as a negative control was obtained by linearizing pFL25 with SphI followed by transcription from the T7 promoter. Probes were synthesized as described (Lamballe et al., 1991) using either ³⁵S-UTP (>1000 Ci/mmol; New England Nuclear), or ³³P-UTP (3000 Ci/mmol; New England Nuclear). Sections were obtained from either 4% paraformaldehyde-fixed paraffin-embedded C57Bl/6 mouse embryos (5- μ m-thick sagittal sections) or flash-frozen adult mouse brain (7- μ m-thick coronal sections). Paraffin-embedded sections were deparaffinized in xylene, rehydrated through a gradient of ethanol solutions, and fixed in 4% paraformaldehyde. Frozen sections were thaw-mounted onto glass slides treated with 3-aminopropyltriethoxysilane (Sigma), and then immersion-fixed in 4% paraformaldehyde. Tissues were treated with proteinase K (20 μ g/ml for paraffin-embedded sections and 40 ng/ml for frozen sections), refixed with 4% paraformaldehyde, immersed in triethanolamine/acetic anhydride solution for 10 min, and dehydrated. Sections were hybridized with 5×10^7 cpm/ml of the respective riboprobes (see above) under stringent conditions [50% formamide, 10% dextran sulfate, $1 \times$ Denhardt's solution, 0.5 mg/ml yeast tRNA, and 10 mM dithiothreitol (DTT)] for 16 hr at 52°C. Hybridized sections were washed in $5 \times$ saline-sodium citrate (SSC), 10 mM DTT at 52°C for 15 min, and then at 65°C for 30 min in a solution containing 50% formamide, $2 \times$ SSC, 10 mM DTT. Sections were washed once at room temperature and twice at 37°C in a solution containing 10 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 5 mM EDTA, for 10 min each. Sections were incubated for 30 min at 37°C in the same buffer containing 20 μ g/ml RNase A and 2 μ g/ml RNase T1 and washed in the same solution without RNases at 37°C for 15 min. Sections were finally washed in 50% formamide, $2 \times$ SSC, 10 mM DTT at 65°C for 20 min, in $2 \times$ SSC at room temperature for 15 min, and in $0.1 \times$ SSC for 15 min at room temperature. After dehydration, sections were dipped into NTB-2 nuclear track emulsion (Kodak) and exposed for 1–3 weeks at 4°C, developed, dehydrated, and coverslipped. Adjacent sections used for histological purposes were stained with 0.2% toluidine blue or cresyl violet.

Immunohistochemistry. Five-micrometer-thick paraffin-embedded sections were deparaffinized in xylene and hydrated through a series of graded ethanols. Following blocking of the sections with serum to eliminate nonspecific binding, they were incubated overnight at 4°C with a 1:50 dilution of anti-NFH, a monoclonal antibody directed against the high molecular weight neurofilament protein (a gift from P. Levitt, CABM, VMDNJ, Piscataway, NJ). After several rinses with PBS, sections were incubated for 30 min at room temperature with a 1:50 dilution of a biotinylated secondary antibody conjugated to HRP (Histostain-SP kit, Zymed Laboratories, Inc.). Sections were washed in PBS and reacted with a substrate-chromagen mixture from the Histostain-SP kit at room temperature for 15 min. Sections were rinsed with distilled water and counterstained using hematoxylin (Meyers). Following the counterstain, sections were coverslipped using a glycerol-polyvinyl mounting medium. In control experiments, the primary antibody was replaced by preimmune serum.

Results

trkC expression is first detected in E9.5 embryos

trkC gene expression during mouse development was visualized using ³⁵S- or ³³P-labeled *trkC* riboprobes synthesized from pFL25, a plasmid containing mouse *trkC* cDNA sequences encoding part of the extracellular domain of gp145^{trkC} (Lamballe et al., 1991). These cDNA sequences have been found to be present in each of the known *trkC* transcripts (Lamballe et al., 1993). Embryos were examined for *trkC* expression starting at embryonic day 7.5 (E7.5). No specific *trkC* labeling was detected until E9.5 (data not shown). At this time, approximately 25 somites can be identified and the embryo has a distinct head, body, and tail (Rugh, 1990). In the CNS, specific *trkC* hybridization was observed in regions of the developing telencephalon and spinal cord (Fig. 1A,B). Outside of the nervous system, no significant levels of *trkC* transcripts were detected. In control experiments

Figure 1. *trkC* expression in an E9.5 mouse embryo. *A–C*, Bright-field (*A*) and dark-field (*B*, *C*) views of a section of a twisted E9.5 mouse embryo demonstrating a sagittal section rostrally and coronal section caudally. The section was hybridized with antisense (*B*) and sense (*C*) *trkC* cRNA probes. Spinal cord (*sc*) and telencephalon (*tel*) are labeled. Scale bar, 180 μ m.

using a sense riboprobe, we did not detect specific hybridization in adjacent sections (Fig. 1*C*).

At E11.5, the CNS has fully differentiated into five well-described zones, including, from rostral to caudal, the telencephalon, diencephalon, mes- and metencephalon, and the myelencephalon. In addition, many interrelated organ systems outside of the CNS such as the circulatory and urogenital systems are beginning to form (Vogels et al., 1990; Perkins et al., 1991). Within the CNS, the highest levels of *trkC* expression were seen in the developing telencephalon, tectum (Fig. 2*A,B*), and optic stalk and recess (Fig. 2*D,E*). Similar levels of *trkC* expression were also found in the cervical, thoracic, and lumbar regions of the spinal cord (Fig. 2*A,B*). Lower levels of *trkC* hybridization were observed in the ventricular ridges that give rise to the caudatoputamen complex (Fig. 2*D,E*), the diencephalon, and its associated ganglionic eminence as well as in the mes- and metencephalic ventricular zone, which ultimately gives rise to neurons in the midbrain and cerebellum (Fig. 2*A,B,D,E*). No expression of the *trkC* gene was seen in the tufts of choroid plexus situated in the fourth ventricle (data not shown), a region rich in related *trkB* transcripts (Klein et al., 1990a). In the PNS, the spinal nerves express relatively low levels of *trkC* transcripts (Fig. 2*A,B*). In contrast, the DRGs depict high levels of *trkC* expression (Fig. 2*D,E,G,H*). Outside of the nervous system, *trkC* expression was observed in the pericardium surrounding the heart and in the walls of the aorta (Fig. 2*A,B*). Hybridization signal was also detected in the developing diaphragm and meso-

nephric regions (Fig. 2*A,B*). In control experiments, no significant hybridization could be observed when adjacent sections were hybridized with the corresponding sense cRNA probe (Fig. 2*C,F*).

trkC is widely expressed in midgestation embryos

At E13.5, many of the CNS structures seen in the adult begin to differentiate, including, but not limited to, the septal nuclei, specific thalamic nuclei, the cerebellum, the pituitary via Rathke's pouch, and several cranial ganglia. In the CNS, *trkC* expression is observed at relatively high levels in the telencephalon, septal nuclei, and spinal cord, and at lower levels in the developing hypothalamus, mesencephalon, and brainstem (Fig. 3*A,B*). In each of these structures, the labeling is relatively homogeneous, with no distinct subnuclei expressing substantially higher levels of *trkC* mRNA than its neighbor. Two exceptions were observed. In the tectum, high levels of *trkC* expression are seen throughout its superficial layers, while the more inferior layers contain little *trkC* mRNA (Fig. 3*D,E*). Second, the ventricular neuroepithelium that gives rise to the Purkinje and Golgi cells of the cerebellum demonstrates high levels of *trkC* expression in its ventral zone, with little hybridization signal noted in the more dorsal cerebellum (data not shown). Interestingly, the diencephalic ganglionic eminence, which expressed high levels of *trkC* in E11.5 embryos, does not exhibit detectable signal at E13.5 (Fig. 3*B*). In the PNS, we observed high levels of *trkC* expression in two cranial ganglia, including the trigeminal gan-

Figure 2. *trkC* expression in an E11.5 mouse embryo. *A–C*, Bright-field (*A*) and dark-field (*B*, *C*) photomicrographs of a midsagittal section through an E11.5 mouse embryo. *D–F*, bright-field (*D*) and dark-field (*E*, *F*) photomicrographs of a sagittal section lateral to that shown in *A*. Sections were hybridized with antisense (*B*, *E*) or sense (*C*, *F*) *trkC* cRNA probes. *G* and *H*, Enlarged views of developing DRGs shown in *D* and *E*, respectively. Labeled are aorta (*a*), anlage of the caudatoputamen complex (*cp_a*), diaphragm (*dia*), dorsal root ganglia (*drg*), ganglionic eminence of the diencephalon (*ge*), mesonephric region (*mes*), mes- and metencephalic ventricular zone (*mvz*), optic recess and stalk (*os*), pericardium (*pc*), spinal cord (*sc*), spinal nerves (*sn*), tectum (*tec*), and telencephalon (*tel*). Scale bars: *A–F*, 1200 μ m; *G* and *H*, 160 μ m.

Figure 3. *trkC* expression in an E13.5 mouse embryo. *A–C*, Bright-field (*A*) and dark-field (*B*, *C*) photomicrographs of a midsagittal section through an E13.5 mouse embryo. *D* and *E*, Enlarged view of tectum depicted in *A* and *B*, respectively. Note the heterogeneity of the labeling in the different subregions of this structure. *F* and *G*, Bright-field (*F*) and dark-field (*G*) photomicrographs of sagittal sections through the trigeminal and otic ganglia. Sections were hybridized with antisense (*B*, *E*, *G*) or sense (*C*) *trkC* cRNA probes. Labeled are choroid plexus (*cp*), diaphragm (*dia*), genital ridge (*gr*), hypothalamus (*hypo*), mesencephalon (*mes*), otic ganglion (*og*), septal nuclei (*sn*), spinal cord (*sc*), tectum (*tec*), thalamus (*thal*), and trigeminal ganglion (*gV*). Scale bars: *A–C*, 1000 μ m; *D* and *E*, 60 μ m; *F* and *G*, 50 μ m.

Figure 4. *trkC* expression in an E15.5 mouse embryo. *A–C*, Bright-field (*A*) and dark-field (*B*, *C*) photomicrographs of a midsagittal section through an E15.5 mouse embryo. Adjacent sections were hybridized with antisense (*B*) and sense (*C*) *trkC* cRNA probes. *D*, Dark-field micrograph of DRGs hybridized with *trkC* antisense riboprobe. These DRGs were photographed in a section sagittal to the one depicted in *A–C*. The majority of the *trkC* transcripts are localized at the periphery of the ganglia. Labeled are cerebellum (*cb*), genital ridge (*gr*), pontine nuclei (*pn*), spinal nerves (*sn*), thalamus (*thal*), and tongue (*ton*). Scale bars: *A–C*, 1350 μ m; *D*, 200 μ m.

gion, which gives rise to projections throughout the facial region, and the otic ganglion, which provides innervation to the auditory system (Fig. 3*F,G*). Outside the nervous system, expression of *trkC* was limited to regions around the heart, including the aorta and pericardium, as well as the diaphragm and gut (Fig. 3*A,B*). As in previous embryonic stages, specificity of *trkC* expression in the above sections was demonstrated through the use of a sense cRNA probe (Fig. 3*C*).

At the E15.5 stage, there is further differentiation of the CNS, specifically in the regions of the cerebellum and hippocampus. *trkC* hybridization signal is diffusely distributed through most levels of the central neuraxis (Fig. 4*A,B*). Highest levels are observed in the tectum, telencephalon, septal region, mesencephalon, brainstem, and the germinal trigone of the developing cerebellum (Fig. 4*A,B*). In addition, *trkC* hybridization was newly noted in the pontine nuclei and in the developing hippocam-

Figure 5. *trkC* expression in an E17.5 mouse embryo. *A–C*, Bright-field (*A*) and dark-field (*B*, *C*) photomicrographs of a midsagittal section through an E17.5 mouse embryo. Adjacent sections were hybridized with antisense (*B*) and sense (*C*) *trkC* cRNA probes. Labeled are aorta (*a*), adipose tissue (*ad*), cerebellum (*cb*), dorsal root ganglia (*drg*), foot (*f*), olfactory epithelium (*oe*), paw (*p*), submaxillary gland (*smg*), and anterior tier of thalamic nuclei (*thala*). Scale bar, 2350 μ m.

pus (data not shown). In the PNS, *trkC* transcripts were observed in the otic and trigeminal ganglia as described in the E13.5 embryos (data not shown) as well as the spinal nerves exiting between vertebrae and DRGs (Fig. 4*A,B,D*). In the DRGs, *trkC* transcripts exhibited a more discrete localization than was seen at earlier times (compare Figs. 2*H*, 4*D*). At E15.5, the majority of the *trkC*-expressing cells are located in the ganglia's periphery. Outside of the nervous system, several areas were found to express high levels of *trkC* transcripts. In the head, expression was found in the posterior tongue and lingual glands. In the body cavity, hybridization signal was detected in the wall of the aorta and in the diaphragm. The developing kidney and genital ridge also showed high levels of *trkC* expression (Fig. 4*A,B*). Figure 4*C* shows control adjacent sections hybridized with the sense *trkC* riboprobe.

trkC expression during late embryogenesis

In the late-stage E17.5 embryo, the majority of the nervous system has been generated and *trkC* expression is present throughout all levels of the neuraxis (Fig. 5*A,B*). Hybridization signal was detected in the caudatoputamen as well as a fiber pathway located between the caudate and forebrain subventricular zone. In the diencephalon, heterogeneous expression of *trkC* was noted, with high levels of hybridization signal detected specifically in nuclei of the anterior tier of thalamus (Fig. 5*A,B*). Relatively high levels of expression were also noted in the medial septal nucleus and the hypothalamus (data not shown). In the mes- and metencephalon, *trkC* expression was located in the tectum and cerebellum, whereas in the hindbrain, labeling was observed in the medulla and spinal cord (Fig. 5*B*). Specificity of the signal was shown using a corresponding sense cRNA probe (Fig. 5*C*). In the PNS, the majority of the *trkC* hybridization

signal was detected in a subset of neurons located at the periphery of each individual DRG (Fig. 6*A,D*). This pattern differs from that observed at E11.5, when regardless of position in the ganglia, all of the neurons express *trkC*.

In body regions outside of the CNS and PNS, we observed intense *trkC* hybridization in facial structures such as the vibrissae of the snout, which provide tactile sensory information via the maxillary branch of the trigeminal nerve (Hebel and Stromberg, 1986) (Fig. 5*A,B*). In this region, the expression of *trkC* colocalized with neurofibers, as determined by immunostaining of adjacent sections with a monoclonal antibody directed against NFH (data not shown). A similar observation was noted within the tongue and olfactory epithelium (Fig. 5*A,B* and data not shown). In addition, we identified *trkC* transcripts in multilocular adipose tissue (Fig. 5*A,B*), cartilaginous regions of the paw (Fig. 5*A,B*), acini of the submandibular and sublingual glands (Fig. 6*B,E*), and in the aortic wall (Fig. 6*C,F*).

trkC transcripts in the ENS

At E17.5, high levels of *trkC* expression were detected in the mesentery of the small intestine as well as in the external muscle layers of the intestinal wall (Fig. 7*A,B*). The *trkC* hybridization signal observed in the mesentery is most likely derived from fibers originating in the superior mesenteric, inferior mesenteric, and coeliac ganglia of the PNS. In contrast, the expression of *trkC* in the submucosal and myenteric plexuses located within the muscular layers of the gut wall is found in regions corresponding to the ENS (Gershon, 1981). To confirm that *trkC* expression did correlate with nerve fibers, we immunostained adjacent sections with the anti-NFH monoclonal antibody. As shown in Figure 7*C*, the *trkC* transcripts appear to be located in the same regions of the splanchnic viscera as NFH immu-

nostaining. These observations indicate that *trkC* is expressed in the three identified branches of the mammalian nervous system, the CNS, PNS, and ENS.

trkC expression in adult mouse brain

Northern blot analysis of adult mouse tissues revealed *trkC* abundant transcripts in the brain (Lamballe et al., 1991). To determine the specific distribution of these transcripts in the CNS, we performed *in situ* hybridization on frozen coronal sections of adult mouse brain. As shown in Figure 8A, *trkC* is expressed in regions of the forebrain, including the cerebral cortex, hippocampus, thalamus, and hypothalamus. Higher magnifications of these regions are depicted in Figure 9A–D. In the cerebral cortex, which has a six-layer histotypic organization (Boulder Committee, 1970), *trkC* shows intense expression in cortical layers 2, 3, and 6 (Fig. 9A). In the hippocampus, the pyramidal cells of Ammon's horn in CA1, CA2, CA3, and the hilus exhibit very strong *trkC* hybridization. Similarly high levels of *trkC* expression were detected in the granule cells of the dentate gyrus (Fig. 9B). In the diencephalon, a strong hybridization is observed in the centromedian region of the thalamus and in the more superficial habenular nuclei (Fig. 9C). The mediodorsal thalamic nuclei that expressed *trkC* during embryogenesis do not show any *trkC* expression in the adult (Fig. 9C). In the hypothalamus, strong *trkC* expression can be detected in the dorso- and ventromedial nuclei located adjacent to the third ventricle, as well as in the medium eminence (Fig. 9D). These results show that *trkC* expression is maintained in adult brain and is specific for certain populations of neurons.

Discussion

The development of the nervous system requires the coordinated expression of many complex factors. Perhaps foremost of these include the interaction of diffusible factors such as the NGF family of neurotrophins with their cognate signaling receptors. A detailed analysis of the temporal as well as spatial expression of these molecules should provide a better understanding of the basic mechanisms that underlie the organization of the nervous system. In this report, we have investigated the expression of *trkC*, a gene encoding the receptor responsible for mediating the trophic properties of NT-3 (Lamballe et al., 1991).

In the developing mouse embryo, *trkC* expression can first be observed in the forebrain and spinal cord at E9.5. From E10 through birth, there is a dynamic pattern of expression that involves both the CNS and PNS. Moreover, the presence of *trkC* transcripts appears to correlate temporally with axon outgrowth and organogenesis. For example, between E11.5 and E13.5, *trkC* transcripts are observed in three areas of the CNS, the auditory system, the cerebellum, and the tectum. In the developing auditory system, *trkC* expression correlates with the appearance of otic ganglion cell fibers leaving the auditory ganglion to begin their migration toward the otic vesicle (Carney and Silver, 1983). A similar correlation is observed in the intermediate zone of the cerebellum where axons from the nuclear and Purkinje cells first project toward the cerebellar surface (Yamamoto et al., 1986). In the tectum, which includes the superior and inferior colliculi, two gradients of spatial development occur simultaneously. On one hand, initiation of axon outgrowth proceeds in the cortical plate in an outside-in direction, whereas the tectum develops in a rostral-to-caudal direc-

tion. As a result of these developmental gradients, the superior colliculus matures several days before the inferior colliculus (Yamamoto et al., 1986). This pattern of tectal development and axon outgrowth also mirrors *trkC* expression. In fact, by E15.5, when the inferior colliculus has begun axonogenesis (Pickford et al., 1989), *trkC* transcripts can be homogeneously found throughout the whole of the tectum.

In later embryos (E15.5–E17.5), two additional sites within the CNS, the pyramidal cells of Ammon's horn and the germinal trigone of the cerebellum, begin to display *trkC* transcripts. As observed in the younger embryos, expression of *trkC* correlates both spatially and temporally with cytogenetic gradients of cell growth. In the hippocampus, *trkC* is expressed as the pyramidal cells are born and differentiate from the CA1 through the CA3 regions (Bayer and Altman, 1987). In the cerebellum, a second region of proliferation and differentiation forms several days following the generation of Purkinje and nuclear cells from the ventral neuroepithelium of the fourth ventricle (E11.5; see above). This zone, known as the germinal trigone of the cerebellum, is the initial site of granule cell generation (Smeyne, 1989). *trkC* expression appears to be turned on when the granule cell precursors begin their initial movement from this laterally situated neuroepithelial region over the surface of the cerebellar cortex to form the external granular layer. To date, the mechanism by which these cerebellar cells translocate over the surface of the cerebellum remains unknown. It has been speculated that this migration occurs through cell–cell interactions mediated by fibronectin (Hatten et al., 1982). Alternatively, it is possible that such migration is mediated by a gradient of neurotrophic factors. The expression of *trkC* transcripts in these cells, along with the previously reported presence of NT-3 in the cerebellum at this stage (Maisonpierre et al., 1990a), lends supports to this hypothesis.

In the PNS, the pattern of *trkC* expression also mimics the timing of axon outgrowth. At E11.5, *trkC* RNA is already detectable in the DRGs, where axons begin to project to target organs (Rugh, 1990). Interestingly, the pattern of *trkC* expression in the DRGs undergoes dynamic changes through development. At E11.5, DRGs exhibit a *trkC* hybridization signal homogeneously distributed in all of the large DRG neurons. By E15.5 there is a decline in the total number of DRG neurons expressing *trkC*, and by E17.5 *trkC*-expressing cells are distributed solely in the periphery of the ganglia. It is possible that *trkC* expression is shut off in certain DRG neurons as part of a defined developmental program. Alternatively, those *trkC*-positive neurons located in the central part of the DRGs may die, perhaps as a result of decreasing NT-3 concentrations (Schedterson and Bothwell, 1992). Finally, it is possible that the *trkC*-positive ganglion neurons are redistributed to the periphery as part of a developmental reorganization of the DRGs. To date, however, there have been no reports of this type of morphogenetic reorganization within the DRGs.

While the appearance of *trkC* transcripts correlates with the initiation of axon outgrowth, its expression is clearly maintained throughout adulthood when no CNS axon growth is observed. This suggests that *trkC*, and thus its ligand NT-3, may subserve diverse functions at different times in development. One possibility is that during the initial stages of neuronal development, the signal transduction initiated through the interaction of NT-3 and the TrkC receptor may play a role in cellular differentiation and survival while later in development, this complex may be used for cellular maintenance.

Figure 6. High-power photomicrographs of non-CNS regions of an E17.5 mouse embryo. *A–C*, Toluidine blue-stained bright-field photographs showing DRGs (*A*), submaxillary gland (*B*), and heart and aorta (*C*). *D–F*, Adjacent sections to *A–C*, respectively, hybridized for *trkC* expression. Note the distribution of *trkC* transcripts in the periphery of the DRGs and vertebrae (*D*), submaxillary acini (*E*), and aorta (*F*). Scale bars: *A* and *D*, 400 μ m; *B*, *C*, *E*, and *F*, 175 μ m.

The overall pattern of *trkC* expression in the developing CNS corresponds rather well with that of its cognate ligand, NT-3. Whereas the timing for the onset of NT-3 expression has not been determined, E11 and E12 embryos display a dramatic increase in NT-3 transcripts. Detailed examination of NT-3 expression in three CNS structures, including spinal cord, hippocampus, and cerebellum, revealed a striking correlation between high levels of expression of this neurotrophin with active neurogenesis and the initial formation of axons (Maisonpierre et al., 1990a). For instance, whereas the highest levels of NT-3 transcripts appear at E12 and E13 in the spinal cord, they can

be observed between E17 and postnatal day 0 in the hippocampus and during the first 3 weeks after birth in the cerebellum. As described in this report, expression of *trkC* coincides, both temporally and spatially, with these periods of high NT-3 expression.

Unlike *trkC*, NT-3 expression declines after these periods to reach low levels in the adult (Maisonpierre et al., 1990a). The reasons for this differential regulation are not well understood. It is possible that once neurogenesis has occurred, the amount of NT-3 required for homeostasis is not as high as for the proliferative and/or growth stages. Regardless of the relative levels

Figure 7. *trkC* expression in the ENS of an E17.5 animal. *A*, Bright-field photomicrograph of a coronal section through the small intestine showing intestinal villi (*iv*) and outer (*oml*) and inner (*iml*) muscle layers. Arrowheads indicate a minor population of the individual ganglion cells that comprise the myenteric plexus of the ENS. *B*, Dark-field photomicrograph of small intestine in a section representative of that shown in *A*. *trkC* hybridization is seen in the intestinal muscle layers. No expression is seen within the intestinal villi. *C*, Immunocytochemical staining with a monoclonal antibody against NF-H. Axons, visualized using an orange chromogen, are observed surrounding the ganglion cells located within the myenteric plexus. The large orange and blue area seen at the right of this picture is debris. Scale bar, 80 μ m.

Figure 8. *trkC* expression in the adult mouse brain. *A* and *B*, Dark-field photomicrograph of adjacent coronal sections of adult mouse brain taken at the level of the dorsal hippocampus and hybridized with either antisense (*A*) or sense (*B*) *trkC* cRNA probes. Expression of *trkC* is observed in the cerebral cortex (*ctx*) and structures of the limbic system including the hippocampus (*HF*), amygdaloid nucleus (*an*), thalamus (*thal*), and hypothalamus (*hypothal n*). Magnification, 15 $\times$.

of expression, there is a striking correlation between those regions expressing *trkC* and NT-3 transcripts. In the adult brain, the highest levels of NT-3 expression were detected in the cerebellum and hippocampus (Maisonpierre et al., 1990a). In the hippocampus, expression of NT-3 is predominantly found in the vast majority of dentate granule cells as well as in subpopulations of pyramidal neurons located in CA1 and CA2 (Ernfors

et al., 1990b). Low levels of NT-3 mRNA have also been detected in CA3 and CA4 (Winslow et al., 1992). *trkC* expression is observed throughout the entire pyramidal cell layer as well as in the dentate gyrus. Likewise, *trkC* is abundantly expressed in the granular layer of the cerebellum, the other adult structure known to maintain significant levels of NT-3 expression. These observations raise the possibility that hippocampal neurons and

Figure 9. Heterogeneity of *trkC* expression in the adult brain. *A*, In the cerebral cortex, *trkC* hybridization signal is detected primarily in layers 2, 3, and 6. *B*, In the hippocampus, all regions of Ammon's horn, including CA1, CA2, CA3, and the hilus (*H*) express *trkC*. The granule cells located in the dentate gyrus (*DG*) also express *trkC*. *C*, In the diencephalon, several thalamic nuclei express *trkC*, including the mediodorsal nucleus (*md thal nuc*) and the centromedian nucleus (*cm thal nuc*). Expression of *trkC* is also detected in the habenular nucleus (*hab nuc*). *D*, In the region of the hypothalamus, high levels of *trkC* expression are noted in both the dorsomedial (*DM*) and ventromedial (*VM*) nuclei. In addition, *trkC* transcripts are also seen in the median eminence (*ME m*). No expression of *trkC* was detected in the ependymal cells lining the third ventricle (*3V*). Scale bar, 275 μ m.

granular cerebellar neurons may be maintained by a mechanism involving autocrine stimulation of gp145^{*trkC*} receptors.

The pattern of NT-3 mRNA expression has been described in various regions of the PNS as well as in skin, hair follicles (innervated via the trigeminal and DRG), muscle (innervated through the spinal cord motor neurons), and aorta (innervated through the superior cervical ganglia and nodose ganglia) (Schecterson and Bothwell, 1992; Scarisbrick et al., 1993). Comparison of this pattern of expression with that of *trkC* indicates a significant parallelism between *trkC* and NT-3 expression in the embryo. We do not know, however, if within each structure, *trkC* and NT-3 are colocalized in identical regions, since there are no published developmental *in situ* studies for this neurotrophin.

The developmental expression of the other known members of the *trk* gene family has been previously reported (Klein et al., 1990b; Martin-Zanca et al., 1990; Schecterson and Bothwell, 1992). The *trk* proto-oncogene codes for the signaling NGF receptor (Hempstead et al., 1991; Kaplan et al., 1991; Klein et

al., 1991a), while the *trkB* gene encodes the functional receptor for BDNF (Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991) and NT-4 (Berkemeier et al., 1991; Ip et al., 1992; Klein et al., 1992). *trk* gene expression is restricted to sensory cranial and peripheral ganglia (Martin-Zanca et al., 1990; Schecterson and Bothwell, 1992). In contrast, *trkB* is widely expressed in the PNS and CNS (Klein et al., 1989, 1990a,b). Comparison of *trk* and *trkC* expression revealed limited areas of overlap, such as the DRGs and the trigeminal ganglia (Table 1). Whether there is *trk* and *trkC* coexpression in some of the other cranial sensory ganglia known to contain *trk* mRNA remains to be determined.

The expression of *trkB* and *trkC* exhibits significant areas of overlap, although their spatial and temporal distributions differ (Tables 1, 2). For example, these genes are both expressed in the cerebral cortex and in the pyramidal cells of the hippocampus. However, the expression of *trkC* in the dentate gyrus is significantly more pronounced than that of *trkB*. In addition to these similarities, there are a number of structural subregions

Table 1. *trkC* expression in the mouse nervous system: comparison with the *trk* and *trkB*

Structure	<i>trkC</i>	<i>trk</i>	<i>trkB</i>
CNS^a			
Cerebral cortex	+++ ^b	—	+++
Corpus callosum	ND	—	—
Hippocampus			
Ammon's horn	+++	—	+++
Dentate gyrus	+++	—	+
Fimbria	ND	—	—
Habenular nuclei	+++	—	+++
Thalamus			
Mediodorsal	+++	—	ND
Centromedian	+++	—	ND
Parafascicular nuclei	ND	—	—
Hypothalamus			
Dorsomedial	+++	—	ND
Ventromedial	+++	—	ND
Median eminence	+++	—	+++
Striatum	ND	+++	ND
Cerebellum			
Purkinje cells	—	—	+++
Granule cells	+++	—	—
Spinal cord	+++	—	+++
Ventricular system			
Choroid plexus	—	—	+++
Ependyma	—	—	+++
PNS^c			
Cranial ganglia			
Trigeminal	+++	+++	+
Facial	ND	—	+++
Acoustic/otic	+++	—	+++
Superior/jugular	ND	+++	+++
Petrosal/nodose	ND	—	+++
Dorsal root ganglia	+++	+++	+
Autonomic system	++	—	+++
ENS^c			
Enteric ganglia	+++	—	—

Expression of *trk* and *trkB* has been previously reported (Klein et al., 1989, 1990a,b; Martin-Zanca et al., 1990).

^a Tissue distribution determined in adult animals.

^b High (+++), medium (++), or low (+) levels of expression. —, no detectable expression. ND, not determined.

^c Tissue distribution determined in late stage embryos.

in which these neurotrophin receptors are differentially expressed. For instance, in the cerebellum, high levels of *trkB* expression are localized in Purkinje cells whereas *trkC* transcripts are detected in cells of the underlying granule cell layer. Other examples where *trkB*, but not *trkC*, are expressed include the internal granular layer (layer 4) of the cerebral cortex, the choroid plexus, and the ependymal cell layer of the cerebral ventricles. Moreover, *trkC* appears to be the only member of the *trk* gene family to be expressed in the autonomous ENS (Table 1). Finally, *trkC* is abundantly expressed in certain structures outside the nervous system such as the mesenchymally derived cells of arterial walls, acini of the submaxillary and sublingual glands, and dorsal sweat glands (Table 2). To date, neither *trk* nor *trkB* has been found to be expressed in non-nervous tissue.

Table 2. *trkC* expression in tissues outside the nervous system: comparison with *trk* and *trkB*

Structure	<i>trkC</i>	<i>trk</i>	<i>trkB</i>
Craniofacial structures^a			
Retina	ND ^b	—	+++
Vestibular system	ND	—	+
Olfactory epithelium	+++	—	+
Snout	+++	—	+
Submaxillary gland	+++	—	+
Sublingual gland	++	—	ND
Tongue	+++	—	+
Other body structures			
Dorsal sweat glands	+++	—	—
Aorta	+++	—	—
Paws	+++	—	+
Diaphragm	++	—	—
Hair follicles	+++	—	++

Expression of *trk* and *trkB* has been previously reported (Klein et al., 1989, 1990a,b; Martin-Zanca et al., 1990).

^a Tissue distribution determined in late-stage embryos.

^b High (+++), medium (++), or low (+) levels of expression. —, no detectable expression. ND, not determined.

In conclusion, we have shown that *trkC*, a gene encoding the functional receptor for NT-3, is differentially regulated throughout development. Moreover, its expression correlates well with the process of axon outgrowth and the presence of NT-3 transcripts. These observations suggest that NT-3 and its signal-transducing gp145^{trkC} receptors may generate paracrine and/or autocrine loops responsible, at least in part, for the proliferation and differentiation of neuronal precursors as well as for the growth of newly formed neurons. This possibility can be explored using mice with a targeted mutation of the *trkC* locus. Recently, we have disrupted the *trkC* gene in embryonic stem cells (unpublished observations) and achieved germ-line transmission of the targeted allele. The *in situ* expression studies described here, along with the characterization of mice lacking gp145^{trkC} receptors, should help unveil the role of *trkC* in the development and maintenance of the mammalian nervous system.

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