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Original Article

Catalytic and non-catalytic forms of the neurotrophin receptor xTrkB mRNA are expressed in a pseudo-segmental manner within the early *Xenopus* central nervous system

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ABSTRACT The induction of anterior-posterior and medio-lateral patterning within the *Xenopus* neural plate leads to the rapid establishment of a functional nervous system. Here we describe two *Xenopus* TrkB neurotrophin receptor genes which are expressed in discrete sets of neuroblasts during this developmental process. The xTrkB mRNAs encode both catalytic and non-catalytic receptors and exhibit membrane-spanning-domain proximal splicing. Expression begins at neural tube closure within the trigeminal ganglion and within the Rohon-Beard neurons of the dorsal spinal cord, providing an excellent dorsal marker of early neural tube patterning. Expression occurs later in the facial ganglia and possibly within the Kolmer-Agduhr neurons. The predominant xTrkB transcripts within the trigeminal and Rohon-Beard neurons and the exclusive early transcripts of the facial ganglia encode C-terminally truncated non-catalytic receptors. Such Trk mRNAs have previously been observed in rodents. However, our observations suggest that they may play a specific role during early development. Anterior-posterior segmentation of the neural tube occurs rostrally within the prospective brain, but previous studies have suggested that segmentation does not extend caudally into the spinal cord. We show that the xTrkB positive Rohon-Beard neurons of the spinal cord do in fact display clear segmental groupings soon after neural tube closure. This is consistent with a role for segmentation in the anterior-posterior patterning of the trunk central nervous system.

KEY WORDS: *Xenopus, development, neurobiology, neurotrophin, TrkB*

Introduction

The early stages of *Xenopus* development provide an ideal model system in which to study development of the vertebrate central and peripheral nervous systems. Soon after neurulation a relatively small number of neuron types develop in the CNS and well before hatching, these neurons have formed relatively simple but functional networks (see Roberts, 1989, 1990). Detailed studies of these networks have provided very plausible explanations for the behavior of the early *Xenopus* larvae in response to external stimuli such as touch, pressure and light. Several genes have been implicated in the definition of the extent and polarity of the neuroectoderm, e.g. *noggin* (Lamb *et al.*, 1993), *follistatin* (Hemmati-Brivanlou *et al.*, 1994), *wnt-1* (Noordermeer *et al.*, 1989; Hidalgo and Ingham, 1990; McMahon and Bradley, 1990) and *s-chordin* and *BMP-4* (Sasai *et al.*, 1995; Schmidt *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995; Xu *et al.*, 1995), and in regulating neuroblast commitment e.g. *xDelta*, *Xotch* (Chitnis *et al.*, 1995) and *NeuroD* (Lee *et al.*, 1995). *Dorsalin* or *BMP4/7* and *hedgehog* have also been shown to regulate the dorsal-ventral patterning of the neural tube (see Kessler and Melton, 1994; Liem *et al.*, 1995). These studies provide major advances in our understanding of the developing nervous system and describe the choice of motoneuron versus sensory neuron fate. They do not, however, describe the developmental cues which determine the several different neuronal fates occurring in the early neural tube. As a step in identifying these cues, we have cloned and studied the expression of the *Xenopus* homolog of TrkB. This neurotrophin receptor provides an excellent marker of certain early neuronal fates.

The high affinity ($gp^{140-145}$) nerve growth factor receptors (NGFRs) form a subfamily of tyrosine kinase membrane receptors encoded by the Trk group of cellular oncogenes, TrkA, -B and -C (Barbacid, 1993, 1995; Glass and Yancopoulos, 1993). These receptors mediate the predominant responses to the neurotrophins NGF, BDNF and NT-3 and -4. *In vitro*, TrkA mediates the cellular

Abbreviations used in this paper: BMP, bone maturation protein; NGF, nerve growth factor; BDNF, brain derived neurotrophic factor; NT-3, neurotrophic factor 3; NT-4, neurotrophic factor 4; RT-PCR, reverse transcription-polymerase chain reaction; a.a., amino acid.

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responses to NGF (Hempstead *et al.*, 1991; Kaplan *et al.*, 1991; Klein *et al.*, 1991a; Loeb *et al.*, 1991), while TrkB has highest affinity for BDNF and NT-4 (Klein *et al.*, 1991b; Ip *et al.*, 1993) and TrkC has highest affinity for and mediates the cellular responses to NT-3 (Lamballe *et al.*, 1991; Ip *et al.*, 1993; Chalazonitis *et al.*, 1994). Cultured mature dorsal root and embryonic cortical neurons require BDNF (or NT-3) for their survival (Barbacid, 1993; Ghosh *et al.*, 1994), the former relying on a TrkB/BDNF autocrine loop (Acheson *et al.*, 1995). In mouse, inactivation of TrkB is lethal, leading to severe loss of sensory neurons from both the central (CNS) and peripheral nervous systems (PNS) (Klein *et al.*, 1993). Inactivation of TrkA or C causes a less severe phenotype which affects predominantly the PNS (Klein *et al.*, 1994; Smeyne *et al.*, 1994).

In mammals, birds and fish, expression of the Trk receptors occurs in overlapping patterns throughout the embryonic and adult nervous system (Heuer *et al.*, 1990; Klein *et al.*, 1990; Martin-Zanca *et al.*, 1990; Tessarollo *et al.*, 1993; Yao *et al.*, 1994; Martin *et al.*, 1995; Shelton *et al.*, 1995). Within given cells, the Trks are expressed in a non-exclusive manner, expression of TrkB usually being accompanied by TrkA or TrkC expression, but rarely or never by both (McMahon *et al.*, 1994). Neuroblast commitment and the differentiation of given neurons are dependent on local auto- and paracrine-derived, as well as target-derived, neurotrophins (e.g. see Johnson *et al.*, 1986; Kalcheim *et al.*, 1989; Sieber-Blum, 1991; Miller *et al.*, 1994). Thus, the development and plasticity of sensory neurons is the result of a complex balance involving more than one ligand-receptor combination and both autocrine and paracrine loops.

Here we present a temporal and spatial study of the onset of TrkB expression in *Xenopus* development. The data show an overt segmentation of the early dsc and suggest non-catalytic forms of TrkB may play a distinct role in early neuroblast differentiation.

Results

Five cDNA clones (Fig. 1a) were isolated using a 75bp probe previously obtained by RT-PCR (9732; see Materials and Methods). Their sequences revealed two highly related xTrk genes, α and β, and a splicing variant of the α gene (Fig. 1b). The α cDNAs encoded proteins of 821 or 811 amino acids (aa) containing an obvious hydrophobic putative membrane spanning domain and a C-terminal kinase domain. Although the coding region of the β cDNAs was incomplete, the deduced peptide sequence varied only 5% from that of the α gene. The only possibly surprising difference between the α and β genes was the deletion of serine-525 just N-terminal of the kinase domain in β. This residue is otherwise conserved between species (see Figs. 1b and 3).

Interspecies comparisons clearly showed that both the α and β genes encoded the *Xenopus* TrkB homologs (xTrkB) (Fig. 2). Alignment of the xTrkBα1 sequence with other TrkBs (Fig. 3) revealed a very high degree of homology throughout the cytoplasmic domains. The extracellular domains were distinctly less well conserved. Though no 3D structure exists for the ligand specific extracellular domain of the Trk receptors, certain polypeptide motifs have been identified by primary sequence comparisons. Following the N-terminal signal peptide, several cysteine rich (cysteine cluster) and leucine rich (LRM) motifs and two immunoglobulin C2 homology (Ig) domains are found (Fig. 3 and Schneider and Schweiger, 1991). Recently, it was shown that the

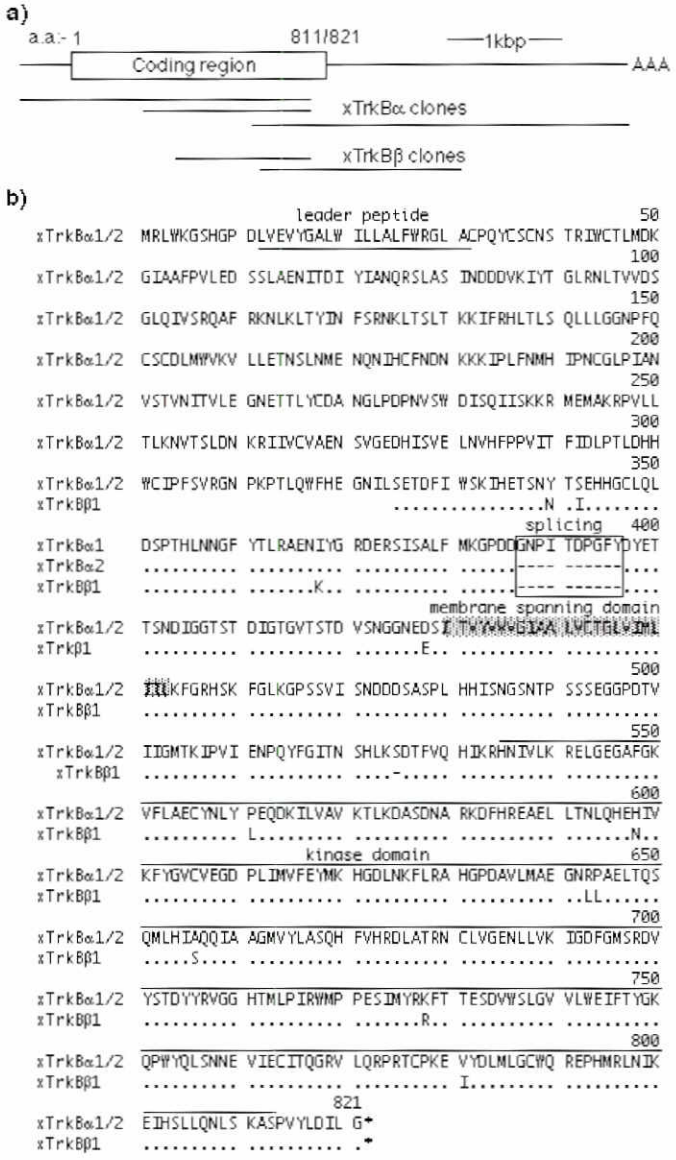

Fig. 1. Two different genes for the *Xenopus* TrkB homolog. (a) *The organization of the xTrkB mRNA and the 5 cDNA clones from which this general organization was deduced.* **(b)** *The deduced amino acid sequences of xTrkBα and -β of 821/811 and 820 residues respectively. The putative leader and membrane spanning peptides are indicated as is the differential splicing of xTrkα mRNA leading to two variant receptors, xTrkBα1 and -α2, of 821 and 811 aa, respectively. Genbank accession numbers U39670-39672.*

C-terminal of the two Ig domains is predominantly responsible for the ligand specificities of the Trk receptors (Urfer *et al.*, 1995). Consistent with this, Figure 3 shows that of the two Ig domains, it is the C-terminal, specificity determining one, which is the more conserved across species.

Differential splicing in the extracellular domain

Two splice alternatives of the extracellular domain of xTrkBα were isolated during this study (Fig. 1b). The splicing event removes 10 a.a. from a short peptide segment between the membrane spanning domain and the more C-terminal of the two Ig-

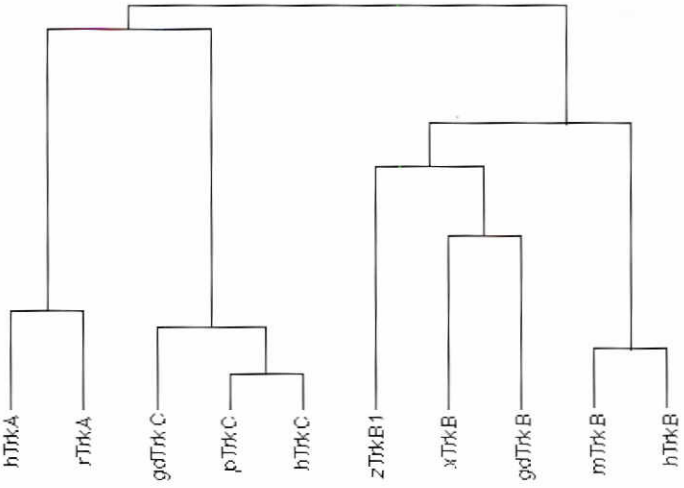

Fig. 2. Homologies among the Trk family. *The deduced protein sequences were aligned using the program PILEUP (Devereux et al., 1984). The sequences were derived from Klein et al. (1989), Lamballe et al. (1991), Meakin et al. (1992), Dechant et al. (1993), Garner and Large (1993), Martin et al. (1995) and Shelton et al. (1995).*

domains (Figs. 1 and 3). Although similar differential splicing has been observed for mammalian TrkA and C receptors (Barker *et al.*, 1993; Shelton *et al.*, 1995), the only other TrkB for which membrane spanning domain proximal splicing has previously been observed is that of zebrafish (Martin *et al.*, 1995 and Fig. 3). The proximity of the spliced region to the ligand specificity determining Ig-domain (McDonald and Chao, 1995; Urfer *et al.*, 1995), suggests that this splicing could modulate receptor function *in vivo*. Analogous splicing of the TrkA receptor has, in fact, been shown to confer an enhanced response to NTF-3 (Clary and Reichardt, 1994).

Two major forms of xTrkB mRNA are expressed from stage 21

Two major forms of xTrkB mRNA, a 6.0 kb corresponding to the full length cDNA and a 2.9 kb truncated form, were detected as early as stage 21 (Fig. 4b). The 2.9 kb mRNA species hybridized with the extracellular domain (JA) probe, but not with the kinase domain (Xb) probe nor with two probes from the immediate 3' noncoding region (AH & H). Thus the 2.9 kb mRNA encoded a kinase-negative or non-catalytic form of xTrkB (xTrkB-tk$^-$) (Fig. 4a). Such TrkB mRNAs are known to exist in mammals (Klein *et al.*, 1991b; Middlemas *et al.*, 1991; Shelton *et al.*, 1995) and probably occur in zebrafish (Martin *et al.*, 1995). Between the 6 and 2.9 kb xTrkB mRNAs, a broad band of hybridization was also noted in the region of the 28S rRNA (Fig. 4b). The intensity of this hybridization was not constant but followed that of the 2.9 kb mRNA form, indicating that it was not simply due to cross-hybridization with the 28S rRNA. These RNAs hybridized with both the extracellular JA and the kinase (Xb) probes, but did not hybridize with the immediate 3'-noncoding AH probe. The exact nature of these transcripts must therefore await a more detailed analysis. However, the situation in *Xenopus* is very reminiscent of that in mammals and zebrafish, where several different TrkB messages, some of around 4 kb, have been observed.

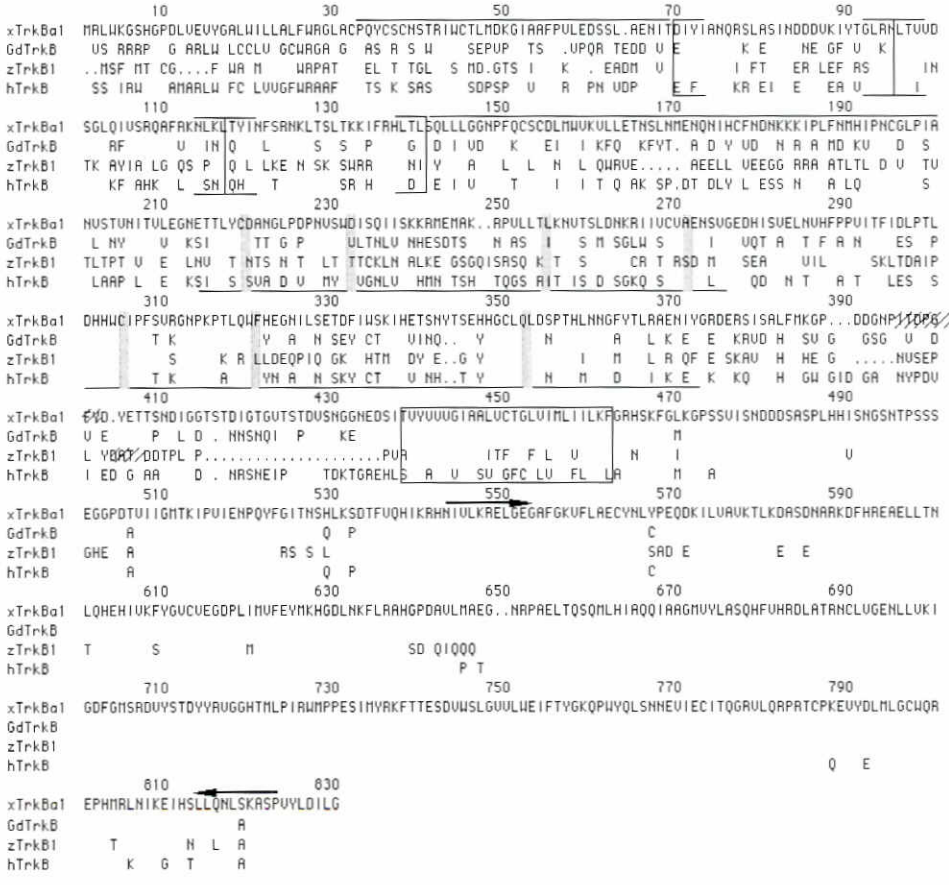

Fig. 3. Alignment of the deduced TrkB protein sequences. *Four representative TrkB homologs, Xenopus xTrkBα1, chicken GdTrkB (Dechant et al., 1993), zebrafish zTrkB1 (Martin et al., 1995), and human hTrkB (Shelton et al., 1995), are shown and the major structural features highlighted (see Schneider and Schweiger, 1991). The cysteine clusters are overlined, the leucine rich motifs (LRM) are bracketed, the immunoglobulin C2 (Ig-C2) homologies are underlined and the putative membrane spanning domain is boxed. The kinase domain is indicated by arrows. The more conserved residues within the Ig-C2 domains are shown shaded and the membrane-spanning-domain-adjacent splicing is shown hatched.*

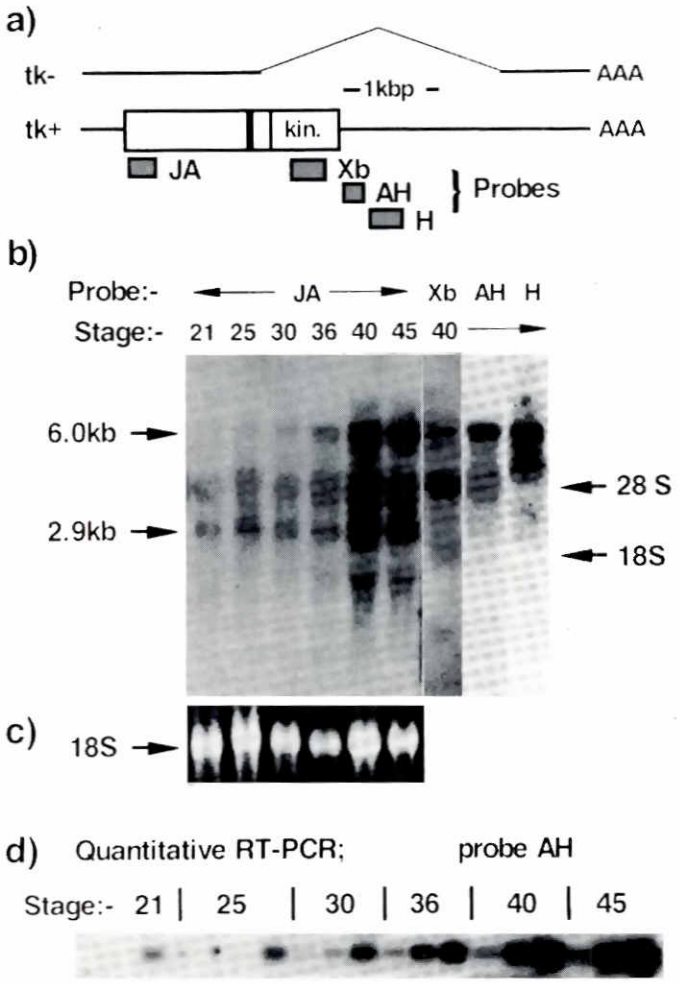

Fig. 4. Expression of two major mRNA forms from stage 21. (a) *The positions of the various hybridization probes and the approximate extent of the differential splicing leading to a 2.9 kb mRNA coding a non-catalytic truncated receptor.* **(b)** *Northern analysis of staged total Xenopus RNA with the four different xTrkB probes.* **(c)** *18S RNA on the original gel as revealed by EtBr staining.* **(d)** *Semi-quantitative RT-PCR using two oligonucleotides taken from the sequences immediately flanking the AH probe. A cDNA reaction was prepared for each RNA using the 3' oligonucleotide. Each cDNA preparation was then divided in aliquots of 1 part, 2 parts and 4 parts and all aliquots subjected to 15 cycles of PCR in parallel. After separating the PCR products by agarose gel, they were blotted and the resultant membrane was hybridized with the AH probe.*

Semi-quantitative RT-PCR showed that the full length xTrkB-tk$^+$ message increased in concentration gradually and by about 4-fold, from stage 21 to stage 36, then by about a further 2-fold to stage 40/45, i.e. about 8-fold in all from stages 21 to 45 (Fig. 4d). Northern analysis showed that the 2.9 kb xTrkB-tk$^-$ mRNA predominated from stages 21 through 30, while at later stages the tk$^-$ and tk$^+$ mRNA forms were equally expressed. The earliest neurons begin to differentiate at neural tube closure, stage 20 or 21 and by stage 33/34 they form part of a functional network which remains essentially unchanged until stage 36/37 (Hughes, 1957; Nieuwkoop and Faber, 1967; Hartenstein, 1993). The xTrkB-tk$^-$ mRNA therefore predominates during the differentiation of these neurons and expression of the tk$^+$ form becomes significant only as the first neurons become fully differentiated. The large increase in mRNA concentration seen at, and subsequent to, stage 40 corresponds with the differentiation of the many retinal neurons (Cohen-Cory and Fraser, 1994).

xTrkB expression first occurs in the trigeminal and early dorsal spinal cord

In situ whole-mount hybridization revealed that the xTrkB message is first expressed at stage 21/22 within the trigeminal ganglia (tg) and the dorsal spinal cord (dsc) (Fig. 5). (As with both Northern and RT-PCR analyses, attempts to detect expression at earlier stages were consistently negative). The dorsal tip of the developing trigeminal ganglion (tg) hybridized strongly at stage 21-23 and weaker hybridization was noted throughout the ophthalmic and maxillary/mandibulary branches of the tg (Figs. 5a,b and 6a). At the same stages, expression was also evident in two rows of dsc cells (Fig. 5a,b and c). Hybridization in the dsc became more extensive by stage 24, spreading caudally (Fig. 6d). By stage 33/34 xTrkB mRNA was found throughout the dsc with the exception of the tail bud (Fig. 5e and f). It was also present in the hindbrain as far rostral as rhombomere 6 or 7 (see Fig. 6 and below). xTrkB expression in the tg did not become more extensive at the later stages, but it did become more intense.

xTrkB positive cranial neurons at stage 33/34

The major cranial nerves of the stage 33/34 embryo are summarized in Figure 6a. The major xTrkB positive cranial ganglion at this stage could be unequivocally identified from its positioning in both lateral views and in transverse sections as the tg (Fig. 6b and d). The ophthalmic and maxillary/mandibular branches, which encompass the eye cup, respectively, dorso-anteriorly and ventro-posteriorly, were very well defined in lateral views and in transverse section hybridization occurred at mid eye cup. At earlier stages (Fig. 5a to d), it was also noted that, as expected for the tg, xTrkB hybridization first occurred in two anterior cranial branches which subsequently moved caudally.

The tg contains at least two sensory neuron types, the pressure receptors in the head skin and cement gland which inhibit swimming and the touch receptors in the head skin which excite swimming (Roberts and Blight, 1975; Hayes and Roberts, 1983). Single somata could be identified in the tg by the exclusion of xTrkB staining from their nuclei (Fig. 6c) and the density of staining suggested that most tg neurons expressed xTrkB. xTrkB hybridization was also systematically observed in one isolated cell (in optical sections actually one cell on either side of the midline), lying within the anterior hindbrain, dorsal of the entry of the trigeminal nerve and within or close to rhombomere 2 (as determined by parallel whole-mount Krox20 hybridizations to identify rhombomeres 3 and 5; Papalopulu *et al.*, 1991). The position of this cell corresponds to one of the two central trigeminal somata which lie either side of the midline. Subsequent to stage 30, hybridization also occurred at the dorso-anterior boundary of the otic vesicle and extended a short way antero-ventrally parallel to the maxillary/mandibulary branch of the tg (Fig. 6b and c). This hybridization identified the developing VIIth or facial ganglion which traverses the region (Fig. 6a). Other sensory cranial nerves present at stages 30 onwards such as the tanabin positive glossopharyngeal ganglion (gg), the vagus ganglion (vg) and the lateral line (LL) ganglia

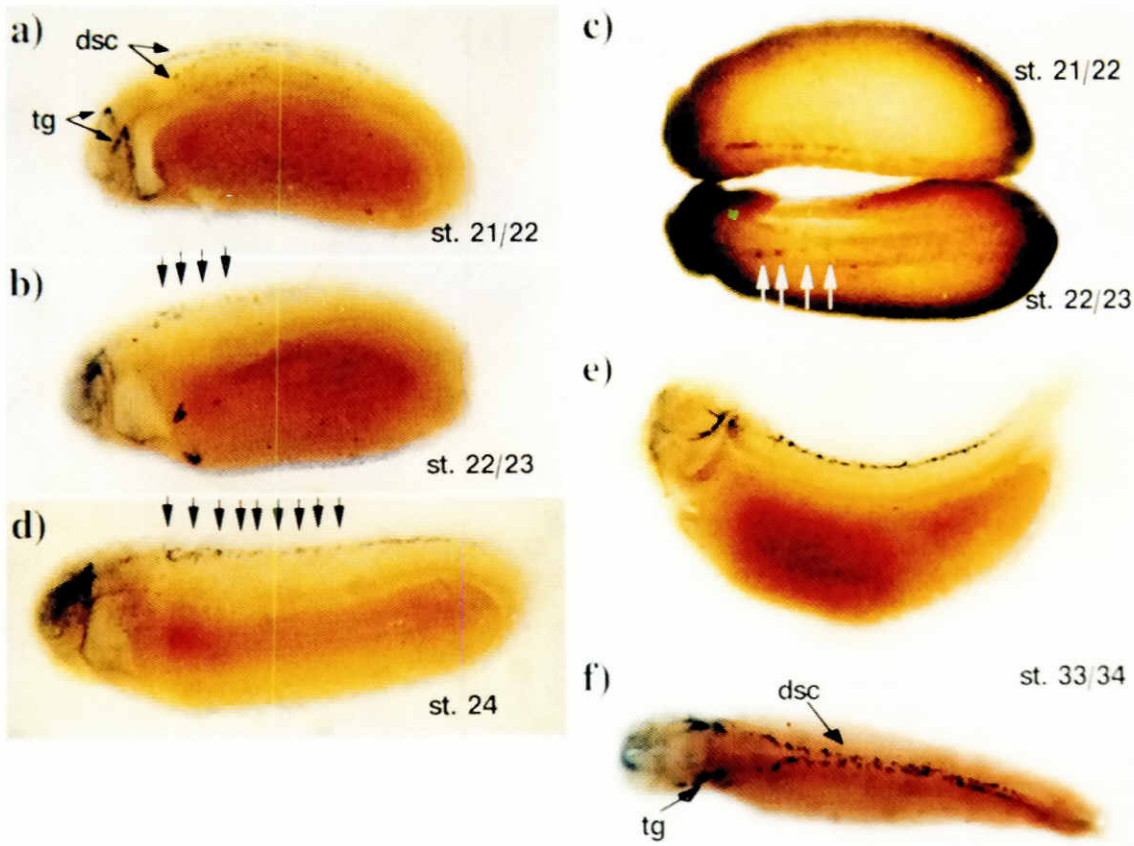

Fig. 5. Spatial expression of xTrkB in *Xenopus laevis* embryos. (a,b,d, and e) *Lateral views of cleared embryos at stages 21/22, 22/23, 24 and 33/34, respectively.* **(c)** *Dorsal view of uncleared stage 21/22 and 22/23 embryos.* **(f)** *Dorsal view of cleared stage 33/34 embryo. The early dorsal spinal cord (dsc) and trigeminal ganglia (tg) are indicated. Shaded arrows indicate regularity in the patterns of expression within the dsc at the earlier stages.*

(Hemmati-Brivanlou *et al.*, 1992), as well as the vestibular nerve or the Mauthner cells (found at the same level of the vestibular nerve and implicated in the startle response of amphibians and fish), did not hybridize. (Staining within the otic vesicle itself was very variable, compare Figs. 6 and 8, and was probably due to probe captured non-specifically in the vesicle). It was concluded that, to stage 33/34, the major cranial neurons expressing xTrkB lie within the tg and to a lesser extent the fg and hence all have their origins in the neural crest (see Gilbert, 1994).

The xTrkB positive cells of the dsc correspond to the Rohon-Beard cells

By stage 33/34, eight different types of neurons are present in the *Xenopus* spinal cord (Fig. 7a). The trunk skin is innervated by transient neurons which initiate swimming in response to touch. These neurons are called Rohon-Beard (R-B) cells and have characteristically large somata. Each R-B cell has a peripheral process which penetrates between the myotomes to innervate the skin as well as ascending and descending axons. Unlike the tg cells, the R-B cells originate directly from the neural ectoderm. Whole-mount *in situ* hybridization resolved the somata of the individual spinal cord neurons and in many cases it was possible to discern that the hybridization was cytoplasmic (Fig. 7b and inserts). The large xTrkB positive dsc cells appeared in lateral and dorsal views (see Figs. 5, 7b and c and 8) to lie near the dorsal surface of the neural tube. It was therefore likely that they corresponded to R-B cells. To verify their transverse position, whole-mount hybridized embryos were imbedded in wax and sections taken throughout the trunk region (e.g. Fig. 7c and d). Strongest xTrkB hybridization occurred within the superficial dorsal cells of the neural tube, identifying these cells predominantly or exclusively as R-B neurons. Some hybridization was also seen at a more lateral position (Fig. 7d), possibly corresponding to the position of dorsolateral interneurons. However, the number of xTrkB positive cells per somite segment, estimated here as 4 to 6 per ganglion at mid-trunk (Fig. 7f) was very close to the 6 to 8 R-B cells per ganglion determined using an anti-acetyl-tubulin antibody (Hartenstein, 1993). Thus, the xTrkB positive dsc cells were probably all R-B cells. Rare staining was also noted in cells attached to the dorsal surface of, but not within, the neural tube (data not shown). These cells probably represented examples of extramedulary neurons, most probably a rare subclass of R-B cells whose somata have migrated out of the neural tube (Roberts, 1989).

In most whole-mount samples it was possible to discern weak but clear xTrkB hybridization within ventral neural tube cells (Fig. 7b). It was unlikely that this was due to cross-hybridization with xTrkA or -C mRNAs, since hybridization occurred with both the extracellular (JA) and the 3'-noncoding (H) probes (see also Figures 4a and 8). The ventral xTrkB positive cells most probably represent either motoneurons or Kolmer-Agduhr (K-A) sensory neurons (see Discussion).

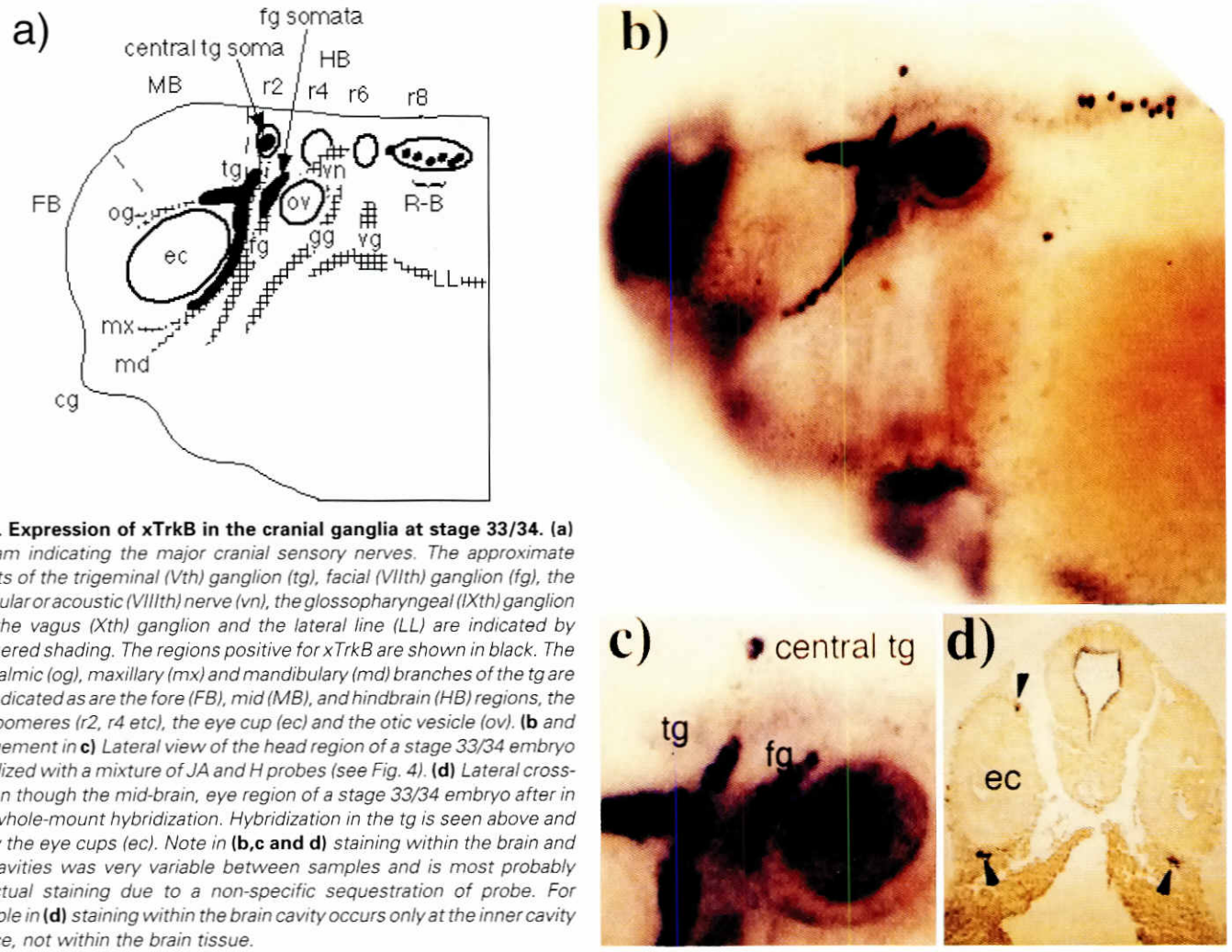

Fig. 6. Expression of xTrkB in the cranial ganglia at stage 33/34. (a) *Diagram indicating the major cranial sensory nerves. The approximate extents of the trigeminal (Vth) ganglion (tg), facial (VIIth) ganglion (fg), the vestibular or acoustic (VIIIth) nerve (vn), the glossopharyngeal (IXth) ganglion (gg), the vagus (Xth) ganglion and the lateral line (LL) are indicated by chequered shading. The regions positive for xTrkB are shown in black. The ophthalmic (og), maxillary (mx) and mandibulary (md) branches of the tg are also indicated as are the fore (FB), mid (MB), and hindbrain (HB) regions, the rhombomeres (r2, r4 etc), the eye cup (ec) and the otic vesicle (ov).* **(b** *and enlargement in* **c)** *Lateral view of the head region of a stage 33/34 embryo hybridized with a mixture of JA and H probes (see Fig. 4).* **(d)** *Lateral cross-section though the mid-brain, eye region of a stage 33/34 embryo after in situ, whole-mount hybridization. Hybridization in the tg is seen above and below the eye cups (ec). Note in* **(b,c and d)** *staining within the brain and otic cavities was very variable between samples and is most probably artifactual staining due to a non-specific sequestration of probe. For example in* **(d)** *staining within the brain cavity occurs only at the inner cavity surface, not within the brain tissue.*

Expression of xTrkB-tk$^+$ and -tk$^-$ mRNAs most probably occurs in the same tg and R-B cells

Little or nothing is known of the function of the non-catalytic or tk$^-$ forms of TrkB. Northern analysis (Fig. 4b) showed that the tk$^-$ mRNA predominated during neuronal differentiation. This suggested that either non-catalytic receptors play a special role during differentiation or their expression is neuron specific. We therefore determined the spatial distributions of the tk$^+$ and tk$^-$ mRNAs. Whole-mount hybridization performed with the JA probe detected both the tk$^+$ and tk$^-$ messages while the 3'-noncoding region probe H detected only the tk$^+$ message (see Fig. 4). Since the tk$^-$ mRNA predominated from stage 21 through stage 30, mixed probe hybridization in the tg and dsc at these stages (Fig. 5a-d) suggested that tk$^-$ mRNA may be present in one or both of these ganglia. Throughout these stages the separate JA and H probes were also shown to hybridize to both the tg and the R-B cells confirming that the tk$^+$ and tk$^-$ mRNAs most probably existed in both cell types (data not shown). At stage 33/34 little difference in the tissue distribution of xTrkB hybridization was detected with the two probes, each probe staining the tg, the R-B cells and weakly some ventral spinal cord (Kolmer-Agduhr) cells (Fig. 8). The number of xTrkB-positive R-B cells per somite segment was found to be essentially the same whether the JA, H or a combination of these two probes was used (e.g. compare Figs. 5e,f and 8). Hence, at both early and late stages of R-B and tg neuroblast differentiation it is possible that the same cell types express tk$^+$ and tk$^-$ mRNAs.

In contrast to the probable overlap of tk$^+$ and tk$^-$ mRNAs in the tg and dsc, hybridization within the developing facial ganglion (fg) at stage 33/34 (Figs. 6 and 8) showed a very distinct dependence on the probe used. Fg hybridization was first noted around stage 30 (data not shown), corresponding roughly with neuroblast commitment in this ganglion, and was seen only with the JA probe (compare Fig. 8a and b). Thus, at least until stage 33/34 the developing fg exclusively expresses the tk$^-$ mRNA form.

Segmentation of the dsc

An important question in studies of spinal cord development is whether or not neuron positioning reflects the segmentation of the embryonic trunk. If so, is neuroblast commitment regulated by molecular cues similar to those which lead to the rostro-caudal segmentation of the embryo, as is the case in insects (Bate, 1976; Hartenstein and Campos-Ortega, 1984)? Data supporting the idea of a segmental arrangement of primary neurons has been obtained in zebrafish (Myers, 1985; Metcalfe *et al.*, 1986; Bernhardt *et al.*,

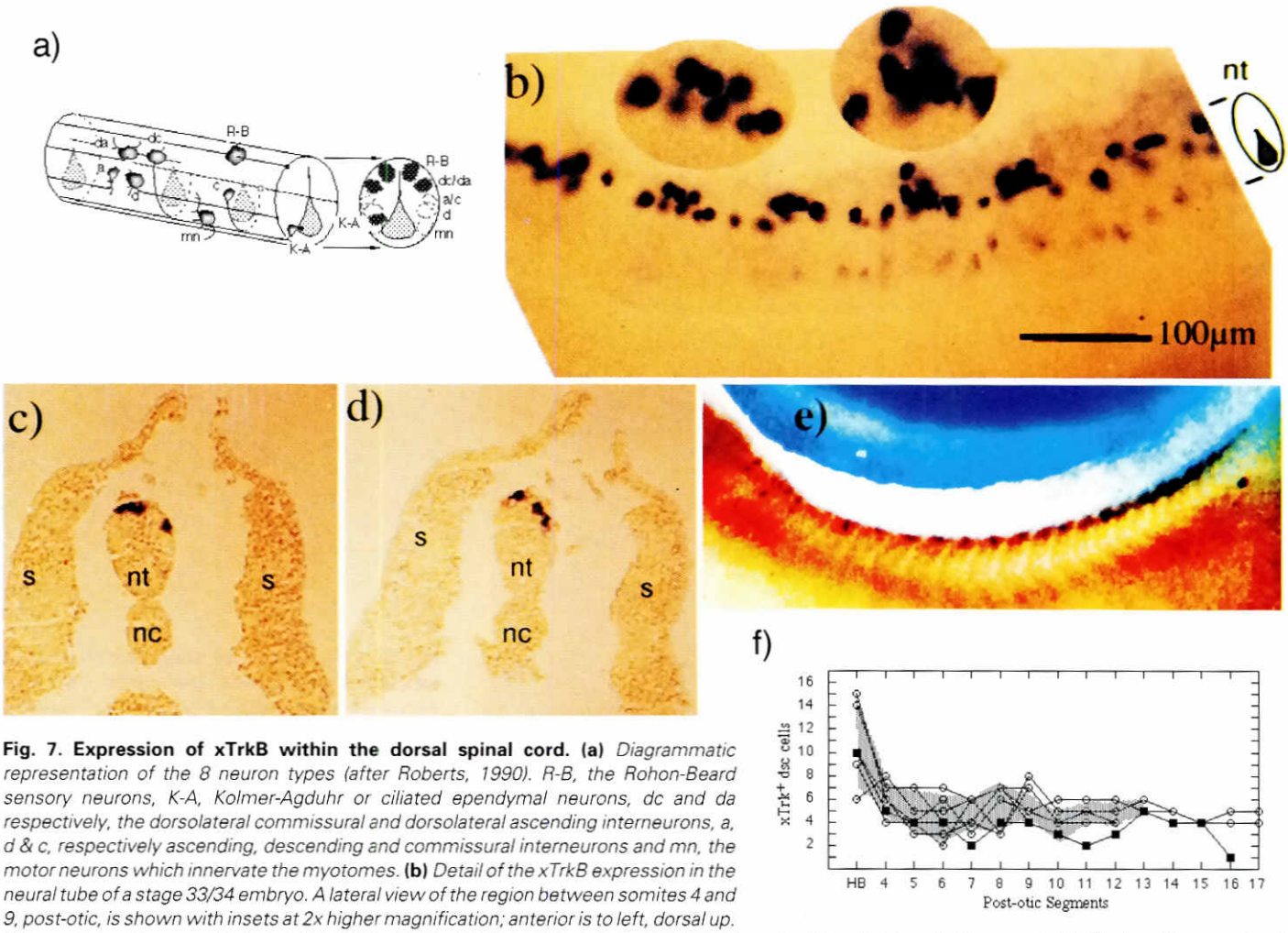

Fig. 7. Expression of xTrkB within the dorsal spinal cord. (a) *Diagrammatic representation of the 8 neuron types (after Roberts, 1990). R-B, the Rohon-Beard sensory neurons, K-A, Kolmer-Agduhr or ciliated ependymal neurons, dc and da respectively, the dorsolateral commissural and dorsolateral ascending interneurons, a, d & c, respectively ascending, descending and commissural interneurons and mn, the motor neurons which innervate the myotomes.* **(b)** *Detail of the xTrkB expression in the neural tube of a stage 33/34 embryo. A lateral view of the region between somites 4 and 9, post-otic, is shown with insets at 2x higher magnification; anterior is to left, dorsal up. The position of the neural tube, nt, is indicated.* **(c and d)** *Transverse cross-sections taken at mid-trunk after whole-mount hybridization. The notochord, nc, neural tube, nt, and somites, s, are indicated.* **(e)** *Lateral view of an uncleared embryo showing the relative positions of somite segments and TrkB positive cells, anterior is to left, dorsal up.* **(f)** *Analysis of the numbers of xTrkB-positive dsc cells within different segments. Numbers are given per segment, per side and somites are identified by their number post-otic. HB refers to the hindbrain region. Open circles indicate data from 4 embryos (6 counted ganglia) at stages between 30 and 34 while solid squares give data from a single stage 24 embryo. The shaded area indicates the standard deviation of the stage 30 to 34 data.*

1990). However, work in other vertebrates suggests that no overt segmentation of neurons occurs in the embryonic spinal cord (Keynes and Lumsden, 1990; Lim *et al.*, 1991; Hartenstein, 1993). To some extent our data was in agreement with these latter studies in that it showed R-B cell positioning within each segment to be highly variable. At the same time the data showed a very obvious tendency for R-B cells to form segmental groupings (see Figs. 5e and f, 7b and 8). These segmental groupings of R-B cells corresponded to the segmentation of the adjacent somites (Fig. 7e).

The anterior 8-9 somites of the *Xenopus* embryo are first segregated at stage 21/22 while the anterior 15 are segregated by stage 24. At first sight, xTrkB-positive cells appeared randomly positioned at these stages (Fig. 5a to c). However, with closer inspection of these and other images, it became evident that some preference for R-B cell positioning probably existed even at very early developmental stages. For example, in Figure 5b and d, groupings of xTrkB positive neuroblasts are just apparent in the rostral and mid-trunk regions. This patterning is more obvious in the dorsal view of an uncleared stage 22/23 embryo shown in Figure 5c. In the original sample staining could be seen to correspond with the somite repeat. Hence, though the distribution of xTrk positive cells is more variable in the early dsc than it is at later stages, some underlying patterning probably already exists.

Discussion

As a step towards understanding early neuroblast differentiation and CNS patterning in the *Xenopus* system, we have cloned and studied the temporal and spatial expression of the *Xenopus* neurotrophin receptor TrkB (xTrkB). Two xTrkB genes, -α and -β, encoding polypeptides differing by only 5%, were isolated and their transcripts were shown to be subject to at least two different splicing events. The first event spliced a 30 nucleotide putative

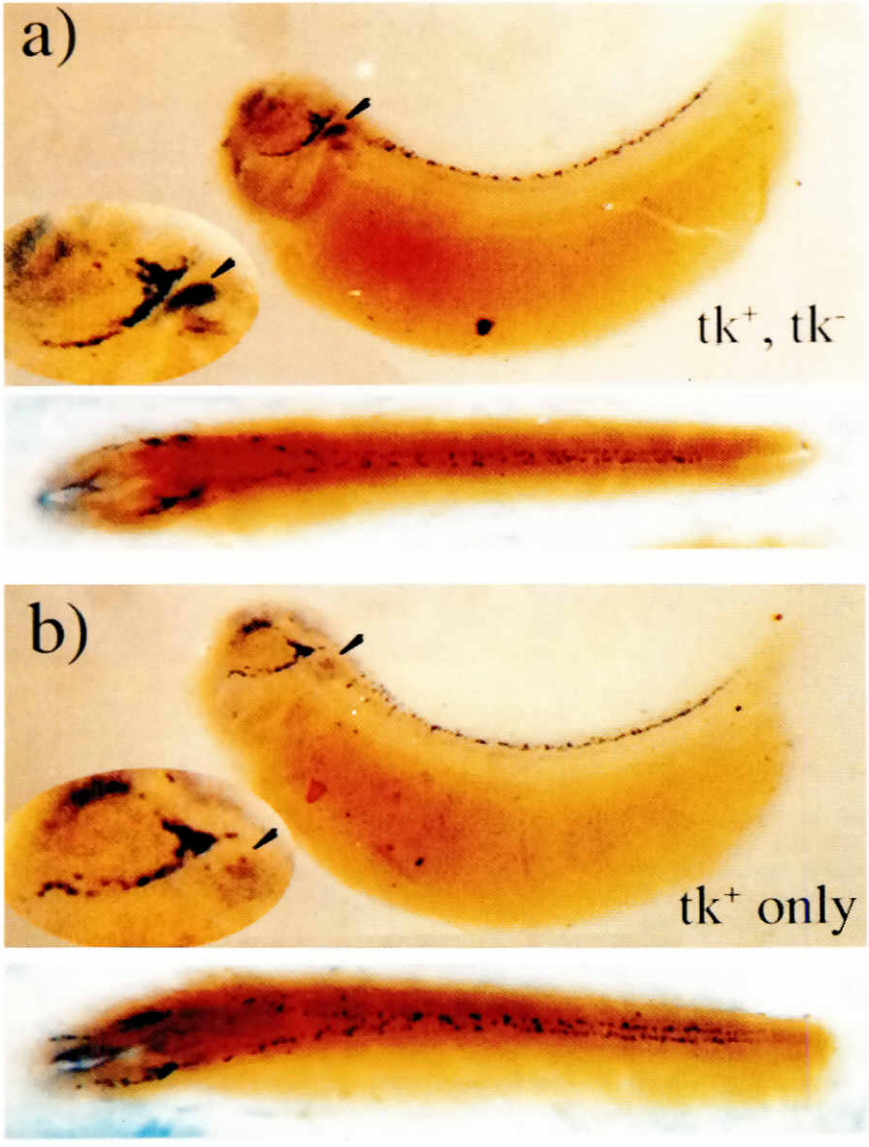

Fig. 8. Comparison of stage 33/34 embryos hybridized with probes *detecting* **(a)** *the catalytic and non-catalytic (JA, tk^+, tk^-) and* **(b)** *only the catalytic (H, tk^+) forms of the xTrkB mRNA. Both lateral and dorsal views are shown for each embryo. The inset shows a higher magnification of the trigeminal and facial ganglion regions and the arrow indicates in* **(a)** *tk^- mRNA within the facial ganglion and in* **(b)** *the presumed region of the facial ganglion in a tk^+ stained embryo. Differences in staining of the anterior cranial regions and otic vesicle between whole-mount samples were observed (e.g. compare Fig. 8a and b with 6b). These differences did not correspond to discrete cellular staining nor did they correlate with given probes and were most probably due to non-specific staining in cranial cavities.*

membrane spanning domain adjacent segment of possible importance in ligand specificity (Clary and Reichardt, 1994; McDonald and Chao, 1995; Urfer *et al.*, 1995), while the second produced an mRNA encoding a non-catalytic (tk^-) receptor. Expression of the xTrkB mRNAs began at stage 21 in the tg and the R-B cells of the dsc ganglia and corresponded with both neural tube closure and early neuroblast differentiation (Nieuwkoop and Faber, 1967; Hemmati-Brivanlou *et al.*, 1992). Expression was therefore observed in cells of both neural tube and neural crest origin, but was limited to a small subset of embryonic sensory neurons.

Somewhat surprisingly, we found that the noncatalytic, tk^-, xTrkB mRNA predominated during the early stages of neuronal differentiation. Between stages 30 and 33/34 the developing fg exclusively expressed the tk^- mRNA. Thus, predominant or exclusive expression of a xTrkB-tk^- mRNA may correlate with neuroblast differentiation. To our knowledge, such a correlation has not previously been made. It is, however, consistent with the observation of a potential tk^- TrkB mRNA in early mouse embryos (Klein *et al.*, 1989). Expression of tk^- TrkB has also been correlated with axonal sprouting in the adult hippocampus (Beck *et al.*, 1993). Tk^- receptors may function to concentrate, sequester or limit the distribution of growth factors. The exclusive or predominant expression of a tk^- receptor might then aid in the differentiation of discrete neuron groupings such as those of the *Xenopus* R-B cells. By analogy with the *Drosophila* Trk homolog, it is possible that tk^- receptors could also function as cell adhesion molecules and hence play a role in axon guidance (Pulido *et al.*, 1992). The availability of the xTrkB gene will allow us to test some of these possibilities *in vivo*.

Strong expression of xTrkB in the R-B cells provides an excellent marker of dorsal-ventral patterning of the neural tube. However, in most whole-mount samples a weak but clear xTrkB hybridization was also observed within ventral neural tube cells, (e.g. see Figs. 7b and 8). Since TrkB expression during early development is generally associated with sensory neurons, it is possible that the xTrkB positive ventral spinal cord cells correspond to Kolmer-Agduhr (K-A) neurons. These are the only ventral sensory neurons in the embryonic neural tube (see Fig. 7a). Further, the density of the xTrkB positive cells roughly corresponded to that expected for

K-A neurons, i.e. 2-5 cells per segment per side (Roberts, 1989). The K-A neurons or ciliated ependymal cells have sensory cilia which penetrate the neural canal, their function is, however, presently unknown. It is also possible that the weak ventral xTrkB expression may be occurring within motoneurons, in which case it could be associated with early programmed cell death (McKay *et al.*, 1996).

Segmentation of the developing CNS is a possible means of achieving correct neuron positioning and neuroblast induction is clearly controlled in a segmental manner in insects (Bate, 1976; Hartenstein and Campos-Ortega, 1984). Antibody staining and HRP backfilling studies in zebrafish have suggested a segmental pattern of spinal cord motor neurons and reticulospinal hindbrain neurons (Myers, 1985; Bernhardt *et al.*, 1990). However, most other studies of the vertebrate embryonic CNS have failed to reveal an obvious segmental positioning of neurons, (see Keynes and Lumsden, 1990; Lim *et al.*, 1991). In particular, anti-acetyl-tubulin and HRP-backfilling studies have suggested that segmentation of the spinal CNS may not occur in *Xenopus* (Hartenstein, 1993). In contrast, whole-mount xTrkB hybridization revealed clear segmental groupings of dorsal spinal cord (dsc) neurons. Our data do agree with the previous work in that the exact positioning of specific xTrkB positive R-B neurons varied between segments, between ganglia and also between individuals. However, this variability did not mask overt groupings of R-B neurons. The segmental groupings most probably appear concurrently with the formation of the somites, very early during neuroblast differentiation. Thus, our data leave open the possibility that the positioning of dsc neurons is subject to segmental cues similar to those which induce segmentation of the somites themselves.

Materials and Methods

Embryos were produced by *in vitro* fertilization (Newport and Kirschner, 1982) and were staged according to Nieuwkoop and Faber (1967).

Cloning and sequencing

Two initial TrkB cDNAs were isolated from a *Xenopus laevis* brain derived λgt10 cDNA bank prepared by K. Richter and kindly provided by T. Sargent. In order to obtain complete cDNAs, a further λgt10 cDNA bank was established using a degenerate primer to kinase domain IX (Hanks *et al.*, 1988; Hanks and Wuinn, 1991; Islam *et al.*, 1994). Screening of both cDNA banks was performed in 50% formamide, 5xSSC, 5xDenhardt's, 100 $\mu g/ml^{-1}$ of torula RNA (Sigma) at 42°C using riboprobes transcribed from the 75bp RT-PCR clone Xltk5 (Islam *et al.*, 1994). After hybridization washing consisted of two washes in 6xSSC and two washes in 2xSSC at 42°C for 15 min each. The filters were then treated with RNase A, 20 $\mu g/ml^{-1}$, and RNase T1, 10 u/ml^{-1}, in 2xSSC at 37°C for 30 min before further washing in 0.5xSSC, 0.1% SDS at 55°C for 30 min. After subcloning, the cDNA sequences were established using a combination of Exo III deletion (Pharmacia) and specific priming.

mRNA expression analyses

RNA was isolated from staged embryos using guanidinium isothiocyanide and LiCl precipitation (Cathala *et al.*, 1983). Northern blots were prepared from formaldehyde gel separations (Brown, 1987) and hybridization was performed using antisense riboprobes. Hybridization was performed in 5xSSC, pH 6.0, 50% formamide, 5xDenhardt's, 100 $\mu g/ml^{-1}$ torula RNA (Sigma) at 60°C. The blots were then washed twice in 6xSSC and twice in 2xSSC at 60°C, 15 min each and finally once or twice in 0.2xSSC for 30 min at 65°C (probe JA) or at 70°C (probes AH & H). Semi-quantitative RT-PCR was carried out, using the 5' primer; 5'TTGATATCCTTGGCTAA and the 3' primer 5'ACAAGAGAGTCATGGCA, homologous to the xTrkB 3' non-coding region sequences flanking probe AH. 10 μg of total RNA from the various embryo stages was reverse transcribed using 37 ng of 3' primer. The cDNA was recovered in 36 μl of 10 mM Tris, 1 mM EDTA, pH 8.3 (TE). Aliquots of 1, 2 and 14 μl were then PCR amplified, using 50 pMoles each of the 5' and 3' primers in a 100 μl reaction, through 15 cycles of 1 min at 94.5°C, 30 sec at 45°C and 30 sec at 72°C. One tenth of each reaction was electrophoresed on 1.5% agarose, transferred to Hybond N (Amersham) and the resulting blots hybridized with probe AH and washed as for cDNA bank screening (see above). Probe JA was derived from xTrkBα and probes AH and H from xTrkBβ. The equivalent AH and H sequences differed by only 1.5 and 7%, respectively, between the two xTrkB "pseudo-alleles".

In situ hybridization

Whole-mount *in situ* hybridization was carried out on albino *X. laevis* (Nasco) as described (Hemmati-Brivanlou *et al.*, 1990; Jowett and Lettice, 1994) with some modifications to avoid the need for manual removal of the vitelline membrane from pre-hatching stage embryos (Islam and Moss, 1996). Sectioning was carried out after whole-mount hybridization by standard paraffin embedding.

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