Immunohistochemical detection of tyrosine kinase B (TrkB) in the enteric nervous system of the small intestine in pigeon *(Columba livia)*

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The presence and cell localization of TrkB, the main receptor for the neurotrophins (NTs), was investigated immunohistochemically in the small intestine of adult pigeons, with special reference to the enteric nervous system (ENS). Several neuronal (neurofilament proteins and PGP 9.5) and glial cell (S100 protein) markers were studied in parallel. TrkB immunoreactivity (TrkB-IR) was found to be restricted to immunohistochemically-identified glial cells present in the enteric plexuses, and to Schwann cells forming the perivascular plexus. Also, TrkB-IR was detected in enterochromaffin cells and in unidentified dendritic cells within the gut-associated lymphoid tissue. The present results demonstrate that as for mammals, TrkB in the ENS is restricted to the glial cells. The possible function of the TrkB ligands, however, remains to be established.

Key words: Enteric nervous system, neurotrophins, TrkB, glial cells, pigeon

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The neurotrophins (NTs), acting through their parent signal transducing receptors (Lewin and Barde, 1996; Huang and Reichardt, 2003), participate in the development and maintenance of sensory and sympathetic neurons derived from the neural crest (see review by Fariñas, 1999). Conversely, it remains still unclear whether or not NTs are also involved in the differentiation of the neural crest cellular progeny which form the enteric nervous system (ENS; see reviews by Gershon, 1997, 1998). However, evidence suggesting such participation does exist, especially for NT-3 (Gershon *et al.*, 1993; Chalazonitis, 2004; Chalazonitis *et al.*, 1994, 1995; see also Gershon, 1997, 1998).

The developing ENS express *trk*A and *trk*C transcripts (Lamballe *et al.*, 1991, 1994; Parada *et al.*, 1992; Ip *et al.*, 1993; Gershon *et al.*, 1993; Tessarollo *et al.*, 1993; Shelton *et al*, 1995; Sternini *et al.*, 1996), and TrkA-, TrkB- and TrkC-IR have been detected in the mammalian adult ENS (Kobayashi *et al.*, 1994 a, b; Hoehner *et al.*, 1996; Shibayama and Koizumi, 1996; Esteban *et al.*, 1998). But surprisingly, the ENS develops normally in mice deficient for NTs and their receptors (see Fariñas, 1999).

Most data regarding the occurrence and distribution of NTs in the ENS have been obtained from mammals, and recent information is emerging from lower vertebrates (de Girolamo *et al.*, 1999; Lucini *et al.*, 1999, 2001; Maruccio *et al.*, 2004). However, the occurrence and distribution of Trks in the avian ENS has not been studied. To help fill this gap in knowledge, we analyzed the localization of TrkB immunoreactivity in the ENS of the adult pigeon. We used this animal model because the antibody used against TrkB successfully reacts in pigeon tissues (Ciriaco *et al.*, 1996, 1997; Hannestad *et al.*, 1998). TrkB is the main receptor for brain-derived neurotrophic factor (BDNF) and NT-4/5, and also for NT-3 in certain contexts (Klein *et al.*, 1991, 1992). TrkB was found immunohistochemically in the enteric glia of different

adult mammals, including man (Sternini *et al.*, 1996; Esteban *et al.*, 1998), and BDNF, the main ligand for TrkB, is released from gut epithelia and regulates visceral innervation (Lommatzsch *et al.*, 1999)

Materials and Methods

Animals and treatment of the tissues

Adult male King pigeons (*Columba livia*, f. domestica, Morini's strain) aged 90 days (n = 5) were used. Samples 1 cm long of the different segments of small intestine were obtained after sacrifice of the animals under deep anesthesia (chloral hydrate, 350 mg/kg, i.p.), and following the criteria established for Animal Rights. The pieces were collected within 5 min after death, cleaned in 0.9% cold saline, placed in Bouin's fixative for 24 h, and routinely embedded in paraffin. Then, 10 μm thick serial sections perpendicular to the long axis of the gut were obtained, mounted on gelatin-coated microscope slides, and processed for indirect peroxidase immunohistochemistry. To ascertain structural details, selected slides were counterstained with hematoxylin-eosin.

Immunohistochemistry

After rehydratation, sections were rinsed in 0.05 M HCl Tris buffer (pH 7.5) containing 0.1% bovine serum albumin and 0.1% Triton X-100. The endogenous peroxidase activity and non-specific binding were blocked with 3% H_2O_2 and 25% fetal calf serum, respectively. Thereafter, the sections were incubated overnight in a humid chamber at 4°C with a rabbit polyclonal antibody directed against the 794-808 residues of mouse TrkB at a dilution of 1 μg/mL (Santa Cruz Biotechnology, Santa Cruz, CA, USA). This antibody maps within the carboxyl terminal domain, and has been used previously in paraffin-embedded tissues of pigeon (Ciriaco *et al.*, 1996, 1997; Hannestad *et al.*, 1998). After incubation with the primary antibody, sections were washed in the same buffer described above, and incubated with peroxidase-labeled sheep anti-rabbit IgG (Amersham, Buckinganshire, UK), diluted 1:100, for 1 hour at room temperature. The immunoreaction was visualized using diaminobenzidine tetrahydrochloride (Sigma, Saint Louis, MO, USA) as a chromogen.

To identify cells displaying TrkB-IR, neuronal and glial markers were studied in parallel in serial sections. The antibodies used in this part of the study were: a) a rabbit polyclonal antibody against S-100 protein (Dako, Copenhagen, Denmark, diluted 1:1000); b) a mouse monoclonal antibody against phosphorylated 200 kDa neurofilament (clone RT-97, Boehringer Mannheim, diluted to 1 μg/mL); c) a rabbit polyclonal antibody against PGP 9.5 (Biogenesis, Poole, England, UK; diluted 1:1000).

The specificity of the immunoreactivity was tested in representative sections processed in the same way using specifically pre-adsorbed sera (5 μg of the antigen in 1 mL of the primary antibody working solution). Cross pre-adsorption among primary antibodies was also carried out (for details see Ciriaco *et al.*, 1996). Under these conditions, no specific immunostaining was observed.

Results

Specific TrkB immunoreactivity was detected in all gut segments examined. It was found in both nervous and non-nervous cells, especially in the enterochromaffin cells but also in unidentified dendritic cells localized within the gut-associated lymphoid tissue (data not shown). No positive immunoreactivity was detected in the control sections processed as described above. The following description applies exclusively to the ENS.

The distribution of TrkB immunoreactivity in the ENS was identical in all the segments of the gut analyzed; and labeled elements were found in the myenteric and submucous plexuses, in the nerve bundles connecting them, in the perivascular plexuses and in isolated nerve fibers running through the intestinal villosities (Figure 1 and 2). In the enteric microganglia, TrkB immunoreactivity did not label the neuron cell bodies, but the interneuronal spaces occupied by the enteric glia and enteric nerve fibres (Figure 1B and 1D; Figure 2). The pattern of immunostaining, as observed in comparing serial sections did not follow that of neuronal markers (i.e. neurofilaments or PGP 9.5; data not shown), whereas it was almost identical to that obtained for S100 protein (Figure 1A and 1C). On the basis of this distribution, cells expressing TrkB immunoreactivity can be identified as enteric glial cells. On the other hand, the occurrence of TrkB immunoreactivity was also evident in the nerve fibers forming the perivascular plexuses (Figure 2B) which connect the enteric microganglia (Figure 1 and 2). Presumably, the immunolabeled cells correspond to Schwann cells.

Discussion

The present study was designed to investigate the presence and cell localization of TrkB, the physiologic

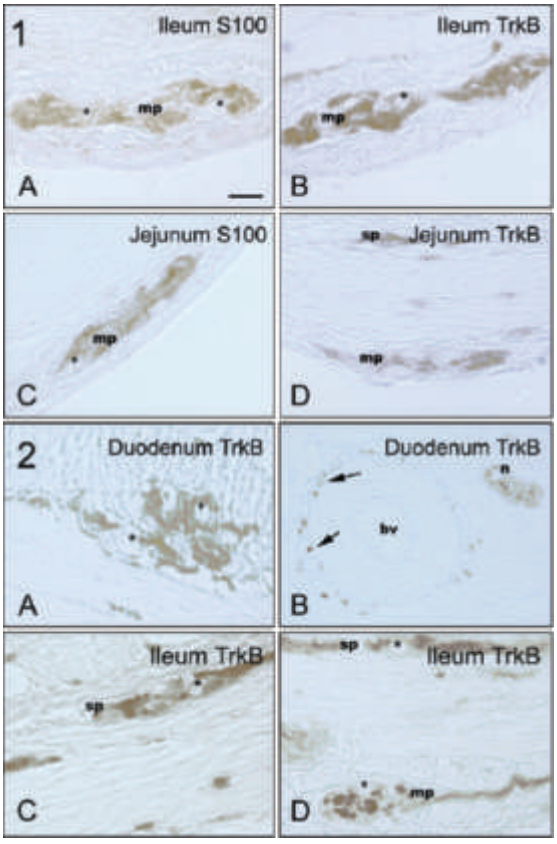

Figure 1. Immunohistochemical detection of S100 protein (A, C) used as a marker for glial cells, and TrkB (B, D) in serial sections of the ileum and jejunum of adult pigeon. Neurons (asterisks) are devoid of immunoreaction for both antigens, whereas it is present in the glial cells of both the myenteric (mp) and submucous (sp) plexuses. Scale bar = 50 m.

Figure 2. TrkB immunoreactivity labels enteric glial cells (A, C, D) of the myenteric (A, mp) and submucous (sp) plexuses, the neurons in the microganglia (asterisks) being unreactive. TrkB immunoreactivity is also present in plexuses (arrows in B) surrounding enteric blood vessels (bv), as well as in intramural nerves (n in B). Scale bar = 50 μm (as in figure 1).

ligand for BDNF and NT-4/5 in the small intestine of the pigeon, with special reference to the ENS. In complete agreement with previous reports in mammals (Esteban *et al.*, 1995; Garcia-Suarez *et al.*, 1997), aves (Hannestad *et al.*, 1998), and lower vertebrates (Lucini *et al.*, 1999; Maruccio *et al.*, 2004), the enterochromaffin cells were TrkB immunoreactive. Also, as for mammals (Levanti *et al.*, 1997) and birds (Hannestad *et al.*, 1998), dendritic cells within the intestinal lymphoid tissues were TrkB immunoreactive.

Regarding the ENS, this paper describes for the first time the presence of TrkB in cells of the myenteric and submucous plexuses, and in other enteric nerves. These cells were identified on the basis of their morphology and immunohistochemical properties as enteric glial cells and Schwann cells. Although pioneer studies failed to demonstrate trkB in the murine ENS (Klein *et al.*, 1990), it was later detected in both developing and adult mammals, including man (Shelton *et al.*, 1995; Hoehner *et al.*, 1996; Sternini *et al.*, 1996). Basically, the present results are in agreement with those obtained in mammals (Esteban *et al.*, 1998), and the main discrepancies occurs with respect to man, since some authors observed a total absence of TrkB immunoreactivity (Shibayama and Koizumi, 1996), while others detected TrkB in both neurons and glial cells (Hoehner *et al.* 1996).

The occurrence of TrkB immunoreactivity in enteric glial cells was surprising because only truncated forms of this protein were detected in other non-myelinating peripheral glial cells and Schwann cells (Wetmore and Olson, 1995; Zhou *et al.*, 1996). Nevertheless, and because the antibody we used maps within the intracytoplasmic domain of TrkB, it can be assumed that the TrkB we have found corresponds to the full-length protein.

At least in mammals, NT-3 seems to be the only member of the family of neurotrophins involved in the development, differentiation and maintenance of the enteric neurons and glial cells both i*n vitro* (Chalazonitis, 2004) and *in vivo* (Palm *et al.*, 1996), presumably in a developmentally regulated manner with other growth factors (Chalazonitis *et al*, 2004). Consistently, the enteric neurons express mRNA for *trk*C (Chalazonitis *et al.*, 1994; Lamballe *et al.*, 1994) and TrkC immunoreactivity (Esteban *et al.*, 1998). Whether or not NT-3 plays similar roles in the avian ENS remains to be demonstrate, but since TrkC is absent from the ENS in birds (Germanà *et al.*, umpublished) NT-3 might signal via TrkB (Klein *et al.*, 1991; Davies *et al.*, 1995).

The functional significance, if any, of TrkB in these cells remains to be established, especially re-examining the ENS of knockout mice lacking NT receptor genes (see Klein, 1994; Snider, 1994, Fariñas, 1999), in which no apparent variations were observed. In addition, more studies are necessary to elucidate this since it is now clear that NTs control the biology of glial cells (Althaus *et al.*, 2000), and it has been recently observed that NT-3 promotes the differentiation of some kinds of glial cells (Bianco *et al.*, 2004). However, the differentiating role of NT as well as the effect of its physiological ligands remains to be demonstrated in the enteric glia.

References

Althaus HH, Richter-Landsberg C. Glial cells as targets and producers of neurotrophins. Int Rev Cytol 2000; 197:203-77.

Bianco JI, Perry C, Harkin DG, Marckay-Sim A, Feron F. Neurotrophin 3 promotes purification and proliferation of olfactory ensheathing cells from human nose. Glia 2004; 45:111-23.

Chalazonitis A, D'Autreaux F, Guha U, Pham TD, Faure C, Chen JJ, et al. Bone morphogenetic protein-2 and -4 limit the number of enteric neurons but promote development of a TrkC-expressing neurotrophin-3-dependent subset. J Neurosci 2004; 24:4266-82.

Chalazonitis A. Neurotrophin-3 in the development of the enteric nervous system. Prog Brain Res 2004; 46:243-63.

Chalazonitis A, Rothman TP, Chen JX, Lamballe F, Barbacid M, Gershon MD. Neurotrophin-3 induces neural crest-derived cells from fetal rat gut to develop in vitro as neurons or glia. J Neurosci 1994; 14:6571-84.

Chalazonitis A, Rothman TP, Gershon MD. Ciliary neurotrophic factor (CNTF) and neurotrophin-3 (NT-3) potentiate one another in promoting enteric neuronal development. Abstr Soc Neurosci 1995; 21: 1545.

Ciriaco E, Dall'Aglio C, Hannestad J, Huerta JJ, Laurà R, Germanà G, et al. Localization of Trk neurotrophin receptor-like proteins in avian primary lymphoid organs (thymus and bursa of Fabricius). J Neuroimmunol 1996; 69:73-83.

Ciriaco E, Garcia-Suarez O, Ricci A, Abbate F, Piedimonte G, Vega JA. Trk-like proteins during the post-hatching growth of the avian bursa of Fabricius. Vet Immunol Immunopathol 1997; 55:313-20.

De Giroloma P, Lucini C, Vega JA, Andreozzi G, Coppola L, Castaldo L. Co-localization of Trk neurotrophin receptors and regulatory peptides in the endocrine cells of the teleostean stomach. Anat Rec 1999; 256:219-26.

Davies AM, Minichello L, Klein R. Developmental changes in NT3 signalling via trkA and trkB in embryonic neurons. EMBO J 1995; 14: 4482-89.

Esteban I, Hannestad J, Levanti B, Del Valle ME, Naves FJ, Vega JA. Neurotrophin receptor proteins immunoreactivity in human gastrointestinal endocrine cells. Brain Res Bull 1995; 38:539-43.

Esteban I, Levanti B, García-Suárez O, Germanà G, Ciriaco E, Naves FJ, et al. A neuronal sub-population in the mamalian enteric nervous system TrkA and TrkC neurotrophin receptor-like proteins. Anat Rec 1998; 251:360-70

Fariñas I. Neurotrophin actions during the development of the peripheral nervous system. Microsc Res Tech 1999; 45:233-42.

Garcia-Suarez O, Hannestad J, Esteban I, Martinez del Valle M, Naves FJ, Vega JA. Neurotrophin receptor-like protein immunoreactivity in human lymphnodes. Anat Rec 1997; 249:226-32.

Gershon MD. Genes and lineages in the formation of the enteric nervous system. Curr Opin Neurobiol 1997; 7:101-9.

Gershon MD. Genes, lineages, and tissue interactions in the development of the enteric nervous system. Am J Physiol 1998; 275:G869-73.

Gershon MD, Chalazonitis A, Rothman TP. From neural crest to bowell: development of the enteric enteric nervous system. J Neurobiol 1993; 24: 199-14.

Hannestad J, Germanà A, Catania S, Laurà R, Ciriaco E, Vega JA. Neurotrophins and their receptors in the pigeon caecal tonsil. An immunohistochemical study. Vet Immunol Immunopathol 1998; 61: 359-367.

Hoehner JC, Wester T, Pahlman S, Olsen L. Localization of neurotrophins and their high-affinity receptors during human enteric nervous system development. Gastroenterology 1996; 110:756-67.

Huang EJ, Reichardt LF. Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 2003; 72:609-42.

Ip NY, Stitt TN, Tapley P, Klein R, Glass DJ, Fandl J, et al. Yancopoulos GD. Similarities and differences in the way neurotrophins interact with the Trk receptors in neuronal and non neuronal cells. Neuron 1993; 10:137-49.

Klein R, Lamballe, F, Bryant S, Barbacid M. The trkB tyrosine protein kinase is receptor for neurotrophin-4. Neuron 1992; 8:947-56.

Klein R, Martin-Zanca D, Barbacid M, Parada LF. Expression of the tyrosine kinase receptor gene trkB is confined to the murine embryonic and adult nervous system. Development 1990; 109:845-50.

Klein R, Nanduri V, Jing SA, Lamballe F, Tapley P, Bryant S, et al. The trkB protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell 1991; 66: 395-03.

Klein R. Role of neurotrophins in mouse neuronal development. FASEB J 1994; 8:738-44.

Kobayashi H, O'Briain DS, Puri P. Nerve growth factor receptor immunostaining suggests an extrinsic origin for hypertrophic nerves in Hirschprung's disease. Gut 1994a; 35:1605-7.

Kobayashi H, Hirakawa H, O'Briain DS, Puri P. Nerve growth factor receptor staining of suction biopsies in the diagnosis of Hirschprung's disease. J Pediatr Surg 1994b; 29:1224-7.

Lamballe F, Klein R, Barbacid M. trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell 1991; 66: 967-79.

Lamballe F, Smeyne R, Barbacid M. Developmental expression of trkC, the neurotrophin-3 receptor, in the mammalian nervous system. J Neurosci 1994; 14:14-28.

Levanti B, Hannestad J, Esteban I, Ciriaco E, Germanà G, Vega JA. Neurotrophin receptor-like proteins in Peyer's patches. Anat Rec 1997; 249:365-72.

Lewin GR, Barde Y-A. Physiology of the neurotrophins. Annu Rev Neurosci 1996; 19:289-17.

Lommatzsch M, Braun A, Mannsfedt A, Botchkarev VA, Botchkareva NV, Paus R, et al. Abundant production of brain-derived neurotrophic factor by adult visceral epithelia. Implications for paracrine and target-derived Neurotrophic functions. Am J Pathol 1999; 155: 1183-93.

Lucini C, De Girolamo P, Lamanna C, Botte V, Vega JA, Castaldo L. TrkA and TrkC neurotrophin receptor-like proteins in the lizard gut. Cell Tissue Res 2001; 303:345-50.

Lucini C, De Girolamo P, Maruccio L, Lamanna C, Castaldo L, Vega JA. Trk neurotrophin receptor-like immunoreactivity in the gut of teleost species. Cell Tissue Res 1999; 296:323-30.

Maruccio L, Castaldo L, de Girolamo P, Lucini C. Neurotrophin and Trk receptor-like immunoreactivity in the frog gastrointestinal tract. Histol Histopathol 2004; 19:349-56.

Pham T, Wade A, Chalazonitis A, Skirboll SL, Bothwell M and Gershon MD. Increased number of myenteric neurons arise in transgenic mice that overexpress neurotrophin-3 (NT-3) directed to the enteric nervous system (ENS) by the dopamine β-hydroxylase promoter. Neurosci Abst 1996; 12:999.

Parada LF, Tsoulfas P, Tessarollo L, Blair J, Reid SW, Soppet D. The Trk family of tyrosine kinase: receptors for NGF-related neurotrophins. Cold Spring Harb Symp Quant Biol 1992; 57:43-51.

Shelton DL, Sutherland J, Gripp J, Camerato T, Armanini MP, Phillips HS, et al. Human trks: molecular cloning, tissue distribution, and expression of extracellular domain immunoadhesins. J Neurosci 1995; 15:477-91.

Shibayama E, Koizumi H. Cellular localization of the Trk neurotrophin receptor family in human non-neuronal tissues. Am J Pathol 1996; 148:1807-18.

Snider WD. Functions of the neurotrophins during nervous system development: with the knockouts are teaching us. Cell 1994; 77: 627-38.

Sternini C, Su D, Arakawa, J, De Giorgio R, Rickman DW, Davis BM, et al. Cellular localization of pan-trk immunoreactivity and trkc mRNA in the enteric nervous system. J Comp Neurol 1996; 368: 597-07.

Tessarollo L, Tsoulfas P, Martin-Zanca D, Gilbert DJ, Jenkins NA, Copeland NG, et al. trkC, a receptor for neurotrophin-3, is widely expressed in the developing nervous and non-neuronal tissues. Development 1993; 118:463-75.

Wetmore C, Olson L. Neuronal and nonneuronal expression of neurotrophins and their receptors in sensory and sympathetic ganglia suggest new intercellular trophic interactions. J Comp Neurol 1995; 353:143-59.

Zhou X-F, Rush RA, McLachlan EM. Differential expression of the p75 nerve growth factor receptor in glia and neurons of the rat dorsal root ganglia after peripheral nerve transection. J Neursoci 1996; 16:2901-11.