Histol Histopathol (2004) 19: 349-356

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Neurotrophin and Trk receptor-like immunoreactivity in the frog gastrointestinal tract

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Summary. Nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are members of the neurotrophin family, which is involved in the differentiation, growth, repair, plasticity and maintenance of many neuronal populations. They act through three tyrosin-kinase (Trk) specific receptors: NGF bind to TrkA, BDNF to TrkB and NT3 to TrkC.

Despite increasing evidence regarding the presence of neurotrophin and their receptors in many vertebrate species, in amphibians there are very few data concerning them. Thus, the aim of this study was to extend the investigation to the presence of both neurotrophins and their Trk receptors in the gut of an anuran amphibian, *Rana temporaria*.

In the frog gut NT-3- like immunoreactivity (IR) was observed in both the nervous system and endocrine cells of the stomach and intestine, while NGF-like IR was observed only in the enteric nervous system, and BDNF-like IR in the intestinal endocrine cells. TrkA- and TrkB-like IR was detected in both neurons and endocrine cells of the intestine, while TrkC-like IR was observed only in intestinal neurons. No Trk IR was detected in the stomach.

The occurrence of the IR to neurotrophins and their receptors in the gut of the frog further confirms the well-conserved presence of this family of growth factors and Trk receptors during the evolution of vertebrates and suggests their complex involvement in the biology of the gastrointestinal neuro-endocrine system.

Key words: NGF, BDNF, NT-3, Neurotrophin receptors, Amphibia

Introduction

Neurotrophins are a family of structurally and functionally related growth factors that in all vertebrates include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) (Barbacid, 1995). A fourth neurotrophin, NT4/5 has only been described in mammals and reptiles (for a review see Ibañez, 1996). In fish also two neurotrophins were other described: NT6 and NT7 (for a review see Heinrich and Lum, 2000). Mammalian neurotrophins bind to both low affinity and high affinity membrane receptors (Meakin and Shooter, 1992; Bothwell, 1995). The p75 LNGRF receptor is the low-affinity receptor (Chao and Hempstead, 1995), and its role is still controversial (Barrett, 2000). Tyrosine kinase proteins are the high affinity signal-transducing receptors (Trks). TrkA, TrkB, and TrkC are the preferred receptors for NGF, BDNF and NT4/5 and NT-3, respectively. NT-3, however, can also bind with low affinity to TrkA and TrkB receptors, and NT4/5 to TrkA (Lewin and Barde, 1996). Trks receptors mediate the action of neurotrophins in the regulation of growth, repair, plasticity, differentiation and maintenance of many neuronal populations (Fariñas, 1999). However, increasing evidence also suggests the involvement of these factors and receptors also in non-neuronal cells (for a review see Tessarollo, 1998).

The involvement of neurotrophins in gut physiology of mammals has been suggested by the occurrence of neurotrophin receptor mRNA (Lamballe et al., 1991, 1994; Parada et al., 1992; Gershon et al., 1993; Ip et al., 1993; Tessarollo et al., 1993; Sternini et al., 1996) or proteins (Chesa et al., 1988, Ernfors et al., 1991; Kobayashi et al., 1994a,b; Hoehner et al., 1996a,b; Shibayama and Koizumi, 1996; Esteban et al., 1998) in the intestinal neuro-endocrine system, by experimental studies in vitro (Saffrey and Burnstock, 1984; Belai et al., 1992; Mulholland et al., 1994; Saffrey et al., 2000) and by clinical trials (Coulie et al., 2000). During development, neurotrophins resulted important for the migration and differentiation of neuronal precursor cells. Successively, they seemed to be involved in regulating neuropeptide synthesis and synaptic functions throughout life (for a review see Von Boyen et al., 2002). All neurotrophins and their receptors appear well conserved during evolution (Hallböök, 1999).

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Neurotrophin receptors have been reported by immunocytochemical studies in the enteric neuroendocrine system of pigeon (Hannestad et al., 1998), lizard (Lucini et al., 2001), xenopus (Holmberg et al., 2001) and goldfish, bass, gilt-head and carp (de Girolamo et al., 1999; Lucini et al., 1999; Hannestad et al., 2000). On the contrary, fewer studies have been devoted to the localization of neurotrophins in the gut (Hoehner et al., 1996a,b; Lommatzsch et al., 1999; Lucini et al., 2002). Thus, in order to continue the investigation regarding neurotrophin and their receptors in vertebrate species, we chose to study the gastrointestinal tract of an amphibian species, *Rana temporaria*.

Materials and methods

Animals

Five adult *Rana temporaria* of both sexes were bought from a local hatchery. Samples were taken from the stomach, small and large intestine.

Tissue preparations

Animals were anaesthetized with 0.1% ethyl 3-aminobenzoate, methane sulfonate (Sigma, St. Louis, MO, USA) and killed by decapitation.

The samples were taken quickly, stretched and fixed by immersion in Bouin's fixative (12-24h) at room temperature (RT). After washing, the samples were processed for paraffin embedding *in vacuo*, and were cut 5-7 μm transversally. Then they were immunohistochemically stained. Some of them, after dewaxing, were also subjected to microwave oven treatment (0.001% sodium citrate buffer, pH 6.0, for 10 minutes at 750W) (Reynolds et al., 1994) to unmask antigens.

Immunohistochemistry

Simple immunohistochemical staining was performed using the peroxidase anti-peroxidase (PAP) method according to Sternberger (1986). Endogenous peroxidase activity was blocked by treating sections with 3% H_2O_2 for 20 minutes at room temperature (RT) and then rinsing for 15 minutes in PBS (pH 7.4) containing 0.2% Triton X-100 and 0.1% bovine serum albumin. Background staining was prevented by incubating the sections with normal goat serum (S-1000, Vector, Burlingame, CA, USA) diluted 1/5 for 30 minutes at RT. Then the sections were incubated overnight in a humid chamber at 4 °C with one of the polyclonal primary antibodies raised in rabbit against NGF, BDNF, NT3, TrkA, TrkB and TrkC (see Table 1).

After incubation with primary antibodies, sections were washed in PBS and incubated with goat-anti rabbit IgG (1:50) for 30 minutes at RT (AI-1000 Vector, Burlingame, CA, USA). Subsequently sections were rinsed in PBS for 15 minutes and incubated with PAP complex (1:100; A200/V, UCB, Braine-l'Allend. Belgium) for 30 minutes at RT. Each incubation was performed in a humid chamber. The sections were rinsed again, and the peroxidase reaction was visualized using a freshly prepared solution of 3.3'-diaminobenzidine tetrahydrochloride (DAB; Sigma; 10 mg in 15 ml of 0.5 Tris buffer pH 7.6) containing 1.5 ml hydrogen peroxide (final concentration 0.03%). All dilutions were made using PBS. Finally, the sections were dehydrated by passing them through an ethanol series and they were cleared in xylene, mounted, observed and photographed

Table 1. Antisera employed in the study.

ANTISERA RAISED TO	CODE	SOURCE	SPECIFICITY	DILUTION
NGF	sc-548	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	Slightly cross-reacts with BDNF	1/500 (s.i.) 1/50 (d.i.)
BDNF	sc-546	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	Does not cross react with any heterologous antigens	1/500 (s.i.) 1/50 (d.i.)
NT3	sc-547	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	Slightly cross-reacts with BDNF	1/500 (s.i.) 1/50 (d.i.)
TrkA	sc-118	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	Does not cross react with any heterologous antigens	1/200 (s.i.) 1/20 (d.i.)
TrkB	sc-12	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	Does not cross react with any heterologous antigens	1/200 (s.i.) 1/20 (d.i.)
TrkC	sc-117	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	Does not cross react with any heterologous antigens	1/200 (s.i.) 1/20 (d.i.)
Chromogranin A	PH 176	The binding site, Birmingham, UK	Does not cross react with any heterologous antigens	1/40 (d.i.)
ChromograninB	PH 191	The binding site Birmingham, UK	Does not cross react with any heterologous antigens	1/40 (d.i.)

s.i.: simple immunohistochemistry; d.i.: double immunohistochemistry.

using a Leica DMRA2 microscope.

Double immunohistochemical staining

The sections were dewaxed and rinsed in PBS for 5 min. Background staining was prevented by incubating the sections with 1:5 normal donkey serum (017-000-121; Jackson, West Grove, PA, USA) for 30 min at RT. After drawing off the excess sera, consecutive sections were incubated for 24 h at RT with each rabbit antibody against NTs and Trks and with sheep antibody against Chromogranin A or B (Table 1), in repeated trials.

After rinsing in PBS, the sections were incubated for 1 h at RT with a mixture containing TRITC-conjugated donkey anti-rabbit IgG serum (711-025-152, Jackson, West Grove, PA, USA; diluted 1/100) and FITC-conjugated donkey anti-sheep IgG serum (713-095-147, Jackson, West Grove, PA, USA; diluted 1/100). All secondary antibodies were immunoabsorbed for multiple labeling.

Finally the sections were rinsed in PBS and mounted under coverslips with glycerol in PBS (Sigma 1000-4). Immunofluorescence was observed with a Leica DMRA2 microscope.

Specificity of the immunoreactivity

The specificity of the immunoreactivity (IR) was tested by successively substituting a buffer either for neurotrophins and their receptor antibodies, or the anti-rabbit IgG, or the PAP complex, in repeated trials.

Adsorption controls and dot blotting tested the cross-reactivity of the primary antibodies. They were performed by using the following antigens: NGF (sc-549 P), BDNF (sc 546P), NT3 (sc547P), NT4/5 (sc545P), TrkA (sc-118P), TrkB (sc-12P), TrkC (sc-117P), (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

For adsorption controls, sections of the studied specimen were treated with antibodies that had been pre-adsorbed with excessive amounts of their homologous (25 μg/ml) and heterologous (50 μg/ml) antigen.

For dot blotting analysis, strips of nitrocellulose were cut and 2 μl drops of antigens at varying concentrations (25 μg, 12.5 μg, 6.25 μg, 3.125 μg) were spotted on the strips and allowed to dry at RT. The strips were then fixed with Bouin's fluid for 1hr at RT. The procedure involved the following steps: 1) a 25 min wash in PBS and 1% Triton X (PBS-T); 2) blocking with normal goat serum (1/5) for 1hr at RT ; 3) a 5 min PBS-T wash; 4) incubation with primary antibody (NGF, BDNF, NT-3, diluted 1/500 and TrkA, TrkB, TrkC diluted 1/200), overnight at 4 °C; 5) a 30 min wash in PBS-T; 6) a 30 min incubation with GAR 1/100; 7) a 30 min wash in PBS-T; 8) a 30 min incubation with PAP 1/200; 9) a 30 min wash in PBS-T; and 10) a 10-45 min incubation with DAB.

Results

The structure of the gastroenteric apparatus of the frog consisted of: a) a mucosae (composed of high columnar epithelial cells and containing mucous cells); b) a lamina propria (formed of loose connective tissue). In particular, the lamina propria of the gastric region contained tubular glands which opened into crypts of the mucosal folds; c) a thick muscularis mucosae; d) a tunica muscolaris (formed by an inner circular and outer longitudinal muscle layers); and e) a thin tunica serosa. In the intestine, two zones, anterior and posterior, were identified on the basis of the height in the mucousal folds (that decreased cranio-caudally), and of the mucous cell number (that increase cranio-caudally).

The enteric nervous system was composed by neurons mostly grouped between the longitudinal and circular muscle layer (myenteric plexus) and between the circular muscle and the muscolaris mucosae (mucousal plexus).

In the text, for the sake of simplicity, neurotrophin-like IR, and Trk-like IR are referred as neurotrophin- and Trk-IR. The results are summarized in Table 2.

Neurotrophin immunoreactivity

NGF- IR was observed in neurons of the myenteric plexus of the small and large intestine (Fig. 1a).

BDNF-IR was observed only in cells of the small and large intestinal epithelium. They were numerous, ovoidal or elongated in shape with IR distributed in all the perinuclear area, in the thin supranuclear cytoplasm and in swelling basal processes (Fig. 1b). They cranio-caudally decreased in number.

NT-3-IR was observed throughout the whole gut, in

Table 2. Presence or absence of the neurotrophins and their Trk receptors in the gastrointestinal tract of frog.

	NGF		BDNF		NT3		TrkA		TrkB		TrkC	
	e.c.	n.c.	e.c.	n.c.	e.c.	n.c.	e.c.	n.c.	e.c.	n.c.	e.c.	n.c.
Stomach	-	-	-	-	+	+	-	-	-	-	-	-
Small intestine	-	+	+	-	+	+	+	+	+	+	-	+
Large intestine	-	+	+	-	+	+	+	-	+	+	-	+

e.c.: endocrine cells; n.c.: nerve cells ; +: presence; -: absence

both the nervous system and in cells of the epithelium. Regarding the enteric nervous system, mainly the myenteric plexus showed positive neurons (Fig. 1c). NT-3-positive cells in the stomach epithelium were distributed in the pits of the gastric glands. They were small in size, roundish or ovoidal in shape, mainly closed in type with the IR in all the perinuclear cytoplasm (Fig. 1d). In the intestine NT3 positive cells were ovoidal or elongated in shape, mainly "open" in type with the IR distributed in both supranuclear and basal cytoplasmic region.

Trk receptor immunoreactivity

TrkA-IR neurons were seen almost exclusively in the myenteric plexuses of the small intestine (Fig. 2a). TrkA-immunopositive cells were scattered throughout the epithelium of the intestine. They were elongated in shape with the IR in both supranuclear and basal cytoplasmic area (Fig. 2b).

TrkB-IR was observed both in the enteric nervous system and in cells along the epithelium of the intestine. The TrkB-positive neurons were observed above all in the myenteric plexus and sometime in the circular muscle (Fig. 2c). TrkB-positive cells in the epithelium of intestine were ovoidal or elongated in shape, mainly "open" in type with the IR distributed in both supranuclear and basal cytoplasmic region (Fig. 2d). They progressively decreased cranio-caudally in number. TrkC- IR was observed in nerve cells mostly belonging to the myenteric plexus of the small and large intestine (Fig.2e).

Double immunostaining revealed that the NTs- Trks-immunoreactive cells in the epithelium also displayed IR for chromogranin A and B.

Controls

No aspecific staining was obtained by the PAP method. In fact, replacement of primary, secondary and PAP antiserum with PBS and normal serum gave negative staining. In the absorption controls the incubation of NT and Trk antisera with their homologous antigens did not show any IR. The incubation of NGF and BDNF antisera with the correlated antigens did not modify the normal pattern of

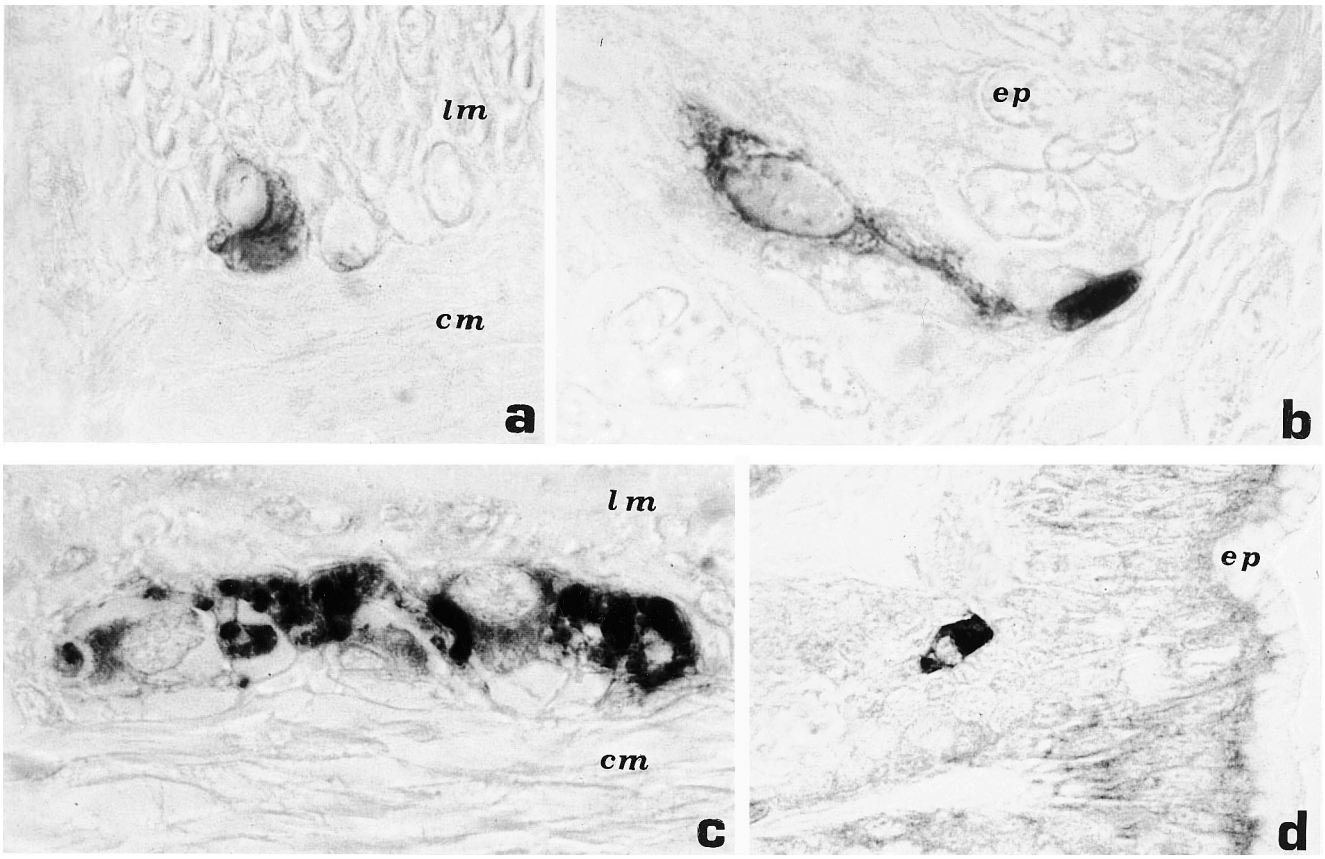
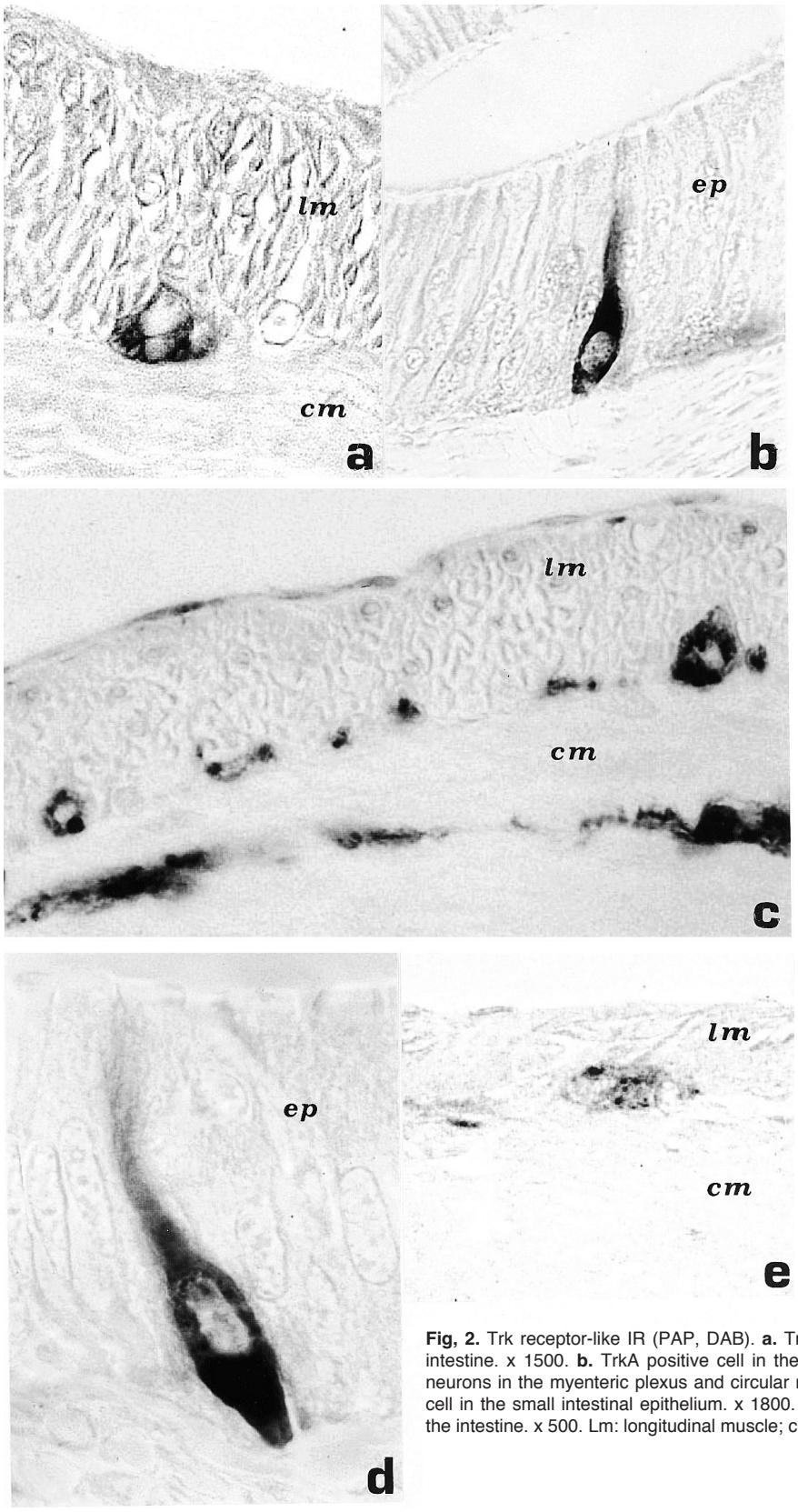


Fig. 1. Neurotrophin-like IR (PAP, DAB). **a.** NGF positive neuron in the large intestine, x 1800; **b.** BDNF positive cell in the small intestinal epithelium, x 1800; **c.** NT3 positive neurons of the myenteric plexus of the stomach, x 1800; **d.** NT3 positive cell in the stomach epithelium. x 700. Lm: longitudinal muscle; cm: circular muscle; ep: epithelium.



Fig, 2. Trk receptor-like IR (PAP, DAB). **a.** TrkA positive neuron in the myenteric plexus of small intestine. x 1500. **b.** TrkA positive cell in the small intestinal epithelium. x 700. **c.** TrkB positive neurons in the myenteric plexus and circular muscle of the large intestine. x 500. **d.** TrkB positive cell in the small intestinal epithelium. x 1800. **e.** TrkC positive neurons of the myenteric plexus of the intestine. x 500. Lm: longitudinal muscle; cm: circular muscle; ep: epithelium.

immunostaining, while the incubation of NT3 antiserum with BDNF slightly reduced the IR in the intestine.

In the dot blotting technique, NT and Trk antisera recognized their homologous antigens. BDNF, TrkA, TrkB and TrkC antisera did not stain the heterologous antigens, while NGF and NT3 antisera reacted very slightly with the BDNF protein (data not shown).

Sections of frog brain, used as positive controls, showed NTs- and Trks-positive neurons (data not shown).

Discussion

In this study we describe the occurrence and localization of neurotrophins- and their Trk specific receptors-IRs in the gut of an amphibian species, *Rana temporaria*, by employing antisera against mammalian neurotrophins and Trk proteins. These growth factors and their receptors appear well conserved during vertebrate evolution (Gotz and Schartl, 1994) because they probably are co-evoluted (Hallböök, 1999). Thus, it is reasonable to assume that the IR to neurotrophins and Trk receptors in *Rana temporaria* is due to the presence of isoforms of mammalian proteins. However we cannot exclude the occurrence of unknown amphibian neurotrophin and/or receptors, neither the presence of subgroups of these molecules, as found in fish for TrkB and TrkC (for a review see Heinrich and Lum, 2000).

Neurotrophins and their Trk receptor-IR was localized in both the enteric nervous system and/or in cells lining the gut epithelium. These epithelial cells also generally showed IR to chromogranin, a common marker of endocrine cells (Facer et al., 1983; Buffa et al., 1988). Thus we could retain the cells showing neurotrophin or Trk-IR in the epithelium as "endocrine cells", which presumably synthesize, store and secrete amines and/or peptides (Solcia et al., 1981).

Among neurotrophins, NT3 is the most widely diffused, being localized throughout the whole gastrointestinal tract and both in endocrine and nervous cells. On the contrary, NGF and BDNF IR were, respectively, observed only in neurons and endocrine cells of the small and large intestine. By dot blot and immunocytochemical controls, NT3 antiserum was seen to cross-react slightly with BDNF, while BDNF did not cross-react with any other tested neurotrophins. In the small and large intestine both NT-3 and BDNF IR was seen in endocrine cells. Thus, because of the data reported above, NT-3 positive endocrine cells in the intestine could be retained as BDNF immunoreactive cells. By dot blot control, NGF antiserum was seen to cross-react slightly with BDNF. However, because NGF-IR was detected exclusively in neurons and BDNF-IR in endocrine cells, it could be ipothesizable that in histological sections the NGF antiserum did not cross react with BDNF.

To our knowledge, there are no previous data regarding the distribution of neurotrophins in amphibian gut.

Trk receptor IR was detected only in the intestine. TrkA and TrkB IR were seen in both nervous and endocrine cells, while TrkC IR was limited to a neuronal localization. Our results are partially in agreement with those reported by Holmberg et al. (2001) in X*enopus laevis* gut where TrkB and TrkC were found in the nervous system of the gastrointestinal tract. The discrepancies could be ascribed to species-specific characteristics or to different specimen processing. The antisera employed in this study against Trk receptors are directed to the intracytoplasmic tyrosine kinase catalytic domain. Thus, the localization of Trk IR in both neurons and endocrine cells of frog gut seem to indicate the presence of functional mammalian Trk neurotrophin isoforms which mediate the action of Trk-ligands in this cell population.

Neurotrophins are known as typical target-secreted trophic factor acting on many neuronal population (Lewin and Barde, 1996). Consistently, the presence of IR to Trk receptors in enteric frog neurons suggests a possible influence of neurotrophins. It would be intriguing to consider endocrine cells showing neurotrophin IR in the gut as target cells of Trk positive neurons. Thus, the localization of neurotrophin IR in the neurons could be due to retrograde transport of Trk receptors and neurotrophin binding complex (Di Stefano et al., 1992; Li et al., 2001). On the other hand, interaction between neurons and endocrine cells is well know. Hormones, released by gut endocrine cells, excite primary afferent neurons (for a review see Furness et al., 1999) and experimental gut denervation induces entero-endocrine cell hyperplasia (Santos et al., 2000). However, another mode of neurotrophin action cannot be excluded. The presence of neurotrophins in the neuronal population has also been recently reported in both the central and peripheral nervous system and supposed to be due to an autocrine and/or paracrine mechanism (Gill and Windenbank, 1998; Pitts and Miller, 2000; Schutte et al., 2000). Presynaptic neurons were seen to release neurotrophins in order to stimulate postsynaptic neurons (Nawa and Takey, 2001; Heerssen and Segal, 2002).

The presence of IR to Trk receptors in endocrine cells could further confirm the possible influence of neurotrophins on non-neuronal tissues (Tessarollo, 1998). Particularly, in the gut endocrine cells Trk-IR has been reported in many vertebrate species (Esteban et al., 1995; Shibayama and Koizumi, 1996; Hannestad et al., 1998; de Girolamo et al., 1999; Lucini et al., 2001) and experimental studies suggest an involvement of neurotrophins on rat endocrine cells (Mahr et al., 1998). Furthermore, the localization of neurotrophins, besides their receptors, in frog endocrine cells, could suggest a paracrine/autocrine mechanism of neurotrophin action in these cell populations, as previously suggested in endocrine pancreatic cells (Kanaka-Gantenbein et al., 1995; Rosenbaum et al., 1998; Lucini et al., 2003).

In conclusion, this study firstly reports the presence of neurotrophin IR and also extends the knowledge

concerning the presence of Trk IR in amphibian gut. The results seem to suggest a complex mode of action of neurotrophins and their involvement in entero-neuroendocrine interaction.

Acknowledgements. We thank Mrs. Annamaria Zollo for her excellent technical assistance.

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Accepted October 24, 2003