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Immunohistochemical localization of BDNF-, TrkB- and TrkA-like proteins in the teleost lateral line system

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Abstract

The lateral line system, formed of both superficial (pit organs) and canal neuromasts, is one of the major mechanosensory systems in fish. It has always been assumed that this system depends on neurotrophins and their cognate Trk receptors for development and maintenance, as has been shown in other mechanosensitive systems of vertebrates. However, until now this issue has not been specifically addressed. In this study we used immunohistochemistry to investigate the occurrence and localization both of neurotrophins (NGF-, BDNF- and NT-3-like) and of Trk-like proteins (TrkA-, TrkB-, TrkC-like) in alevins of *Salmo salar* and *S. trutta.* All cells in the pit organs of *S. salar* displayed strong immunoreactivity for TrkB-like and BDNF-like, whereas they were restricted to the hair cells in *S. trutta*. The hair, supporting and mantle cells of *S. salar*, and the mantle cells of *S. trutta*, also expressed TrkA-like immunoreactivity. In the canal neuromasts BDNF-, TrkA- and TrkB-like proteins were present in all cells, without differences between species. NGF-, NT-3- and TrkC-like immunoreactivity were never detected. The present results suggest that mechanoreceptive hair cells, as well as supporting cells, in the lateral line system are under the control of the BDNF–TrkB-like complex, and probably of ligands of TrkA-like receptors.

Key words hair cells; lateral line system; neuromast; neurotrophins; teleosts.

Introduction

The lateral line system (LLS) represents one of the major sensory systems in bony and cartilaginous fish, as well as in aquatic amphibians and the aquatic larvae of terrestrial amphibians. Its localization varies among species, but is usually in the lateral parts of the body and in the head (see Webb, 1989; Rouse & Pickles, 1991). Two main parts can be considered: one situated in the internal cephalic and trunk canals, and the other one in the body surface forming the so-called pit organs (for a review see Coombs et al. 1989).

The sense organs of the LLS are called neuromasts. They consist of sensory hair, supporting and mantle cells. Hair cells bear one kinocilium and up to 40-stereocilia encapsed in a more or less developed gelatinous cap, i.e. the cupula (Cernuda-Cernuda & García-Fernández, 1996; Alexandre & Ghysen, 1999). The neuromasts are supplied by the peripheral processes of the bipolar sensory neurones of two cranial ganglia, located anterior to or behind the ear, which innervates the head and trunk-tail neuromasts, respectively (Alexandre & Ghysen, 1999). Functionally, the neuromasts of the LLS are regarded as mechanoreceptive, detecting water displacement, sounds at frequencies higher than 500 Hz, magnetic fields and the location of obstacles (for references see Engelman et al. 2000; Northcutt et al. 2000; Popper, 2000).

In higher vertebrates, the sensory systems, including some kinds of mechanoreceptors, are under the control of specific growth factors for development and maintenance. In particular, the muscular (Fan et al. 2000), cutaneous (Ichikawa et al. 2000) and cochleo-vestibular mechanoreceptors (Fritzsch et al. 1997; Vega et al. 1999) depend on specific neurotrophin-Trk receptors (Lewin & Barde, 1996; Huang & Reichardt, 2001). Both

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common (Hashimoto & Heinrich, 1997; Hallbook et al. 1998) and specific (Gotz et al. 1994; Lai et al. 1998; Nilsson et al. 1998; Caminos et al. 1999) neurotrophins and their parent Trk-like receptors (Martin et al. 1995, 1998; Caminos et al. 1999) have been demonstrated in fishes (see also Heinrich & Lum, 2000). Nevertheless, the occurrence of neurotrophins or Trk-like proteins in the LLS has never been investigated in depth, although neuromasts express mRNA and immunoreactivity for BDNF in developing zebrafish (Hashimoto & Heinrich, 1997; Lum et al. 2001) and Trk-like proteins seem to be absent in *Dicentrarchus labrax* (Hannestad et al. 2000).

Thus the present study was designed to investigate the immunolocalization of neurotrophin-like (nerve growth factor – NGF-, brain-derived neurotrophic factor – BDNF- and neurotrophin-3 – NT-3-) and Trk-like proteins (TrkA, TrkB an TrkC) in neuromasts of the LLS in alevins of a *Salmo trutta* and *S. salar*. On the other hand, because the neuromast of the LLS structurally resembles the inner ear, and the hair cells in this place display S100 protein immunoreactivity (Saidel et al. 1990; Foster et al. 1993), we investigated in parallel the localization of this protein, as well as the innervation of the neuromast cells.

Materials and methods

Materials

Alevins of a *S. trutta* (*n* = 10) and *S. salar* (*n* = 10), aged 1–2 months, were obtained from the aquarium of the Department of Functional Biology, Section of Genetics (Prof E. García Vázquez) of the University of Oviedo. Specimens were anaesthetized with MS222 (ethyl-m-amino benzoate; 0.4 g L^{-1}), killed and fixed *in toto* in Bouin's fixative for 24 h and then routinely processed for paraffin embedding. The pieces were cut in serial frontal, horizontal and sagital sections 10 μm thick, and collected on gelatine-coated microscope slides.

Immunohistochemistry

The sections were processed for indirect peroxidase immunohistochemistry as described elsewhere (Hannestad et al. 2000), Briefly, deparaffinated and rehydrated sections were rinsed in Tris-HCl buffer (0.05 M, pH 7.5) containing 0.1% bovine serum albumin and 0.2% Triton-X 100. Endogenous peroxidase activity and non-specific binding were blocked (3% H_2O_2 and 25% fetal calf serum, respectively), and sections were incubated overnight with the primary antibodies (see Table 1). Then sections were washed in the same buffer and incubated for 1.5 h at room temperature with peroxidase-labelled sheep antirabbit IgG (Amersham, UK), diluted 1 : 100. The immunoreaction was visualized using 3–3′ diaminobenzidine as a chromogen.

The antibodies against Trk proteins used here were against mammalian Trks, and map within the catalytic domain of the Trk proteins, a region with a highly preserved evolutionary structure (Barde, 1994; Martin et al. 1995, 1998; Hallbook, 1999; Heinrich & Lum, 2000). Moreover, using Western blot analysis in teleosts, these

Table 1 Antibodies used

Antibody	Raised in	Specificity	Dilution	Supplier
anti-TrkA	Rabbit	TrkA (residues 763–777)	1 : 100	Santa Cruz Biotechnology
anti-TrkB	Rabbit	TrkB (residues 794–808)	1 : 100	Santa Cruz Biotechnology
anti-TrkC	Rabbit	TrkC (residues 798–812)	1 : 100	Santa Cruz Biotechnology
anti-NGF	Rabbit	recombinant NGF	1 : 1000	Chemicon, Inc
anti-BDNF	Rabbit	recombinant BDNF	1 : 1000	Chemicon, Inc
anti-NT-3	Rabbit	recombinant NT-3	1 : 500	Chemicon, Inc
anti-S100 protein	Rabbit	α and β subunits of S100 protein	1 : 1000	Dako
anti-NFP (clone 1592)	Mouse	Medium and high NFP subunits	1 : 100	Boehringer-Manhein

BDNF: brain-derived neurotrophic factor; NFP: neurofilament proteins; NGF: nerve growth factor; NT-3: neurotrophin 3.

antibodies recognize proteins whose estimated molecular masses are equivalent to those of the mammalian full-length Trks (De Girolamo et al. 1999, 2000; Lucini et al. 1999b, 2001). On the other hand, the antibodies used to detect neurotrophins were raised against mammalian proteins, which are also highly preserved during evolution (see Götz & Schartl, 1994; Hallbook, 1999; Heinrich & Lum, 2000).

Additional experiments following an identical protocol were carried out to localize S100 protein (Saidel et al. 1990; Foster et al. 1993) and neurofilament proteins (Blank et al. 1997; Hall & Yao, 2000; Table 1).

Double immunohistochemistry

To better analyse the innervation of the LLS, some sections were incubated overnight in a humid chamber at 4 °C with a 1 : 1 mixture of the working solution of the antibodies against S100 protein or neurofilaments (see Table 1). After rinsing in the above-mentioned buffer, the sections were first incubated for 1 h at room temperature with Texas red-labelled sheep antirabbit IgG (Amersham, UK) diluted 1 : 50, then rinsed again and incubated for another hour with FluoroLinkCy2-labelled goat antimouse IgG (Amersham, UK), diluted 1 : 500. The sections were finally studied and photographed using a Bio-Rad MR-600 confocal-laser scanning microscope (Servicio de Proceso de Imagenes, University of Oviedo).

Controls

The specificity of the immunoreactivity developed was tested by substituting a buffer either for the Trk antibodies, or the antirabbit IgG, in repeated trials. To avoid cross-reactivity of each Trk antisera to another, aliquots of each Trk antiserum were absorbed with an excessive amount of heterologous antigen (for details see Hannestad et al. 2000). For the other proteins investigated, controls were performed using a non-immune rabbit serum instead of the primary antibodies. Under these conditions no specific immunoreactivity was observed.

Results

In both species analysed, the neuromasts were found superficially in the pit organs, and deeply in the cephalic and trunk canals. The structure of these sensory systems was almost identical in *Salmo trutta* and *S. salar*, and they were composed of hair cells, supporting cells and mantle cells. As predicted, the hair cells in the neuromasts, independently of location, displayed immunoreactivity for S100 protein (see Figs 1A,D, 2A and 3A), whereas supporting and mantle cells were unreactive.

Pit organs

The patterns of expression and cell localization for both neurotrophin-like and Trk-like receptors in the species examined were similar, although slight differences were observed. In *S. salar* all cells in the neuromast exhibited a strong immunoreactivity for TrkB-like protein (Fig. 1B), whereas in *S. trutta* it was apparently restricted to the hair cells (Fig. 1E). Furthermore, neuromasts also showed TrkA-like protein immunoreactivity localized in all cells in *S. salar* (Fig. 1G), and in the mantle cells in *S. trutta* (Fig. 1H). Regarding the TrkC-like receptor, it was always absent in the neuromast (data not shown), although it was detected in scattered unidentified cells placed on the skin of the head of *S. trutta* (Fig. 1I).

The only neurotrophin localized in the pit organs was BDNF-like protein, and its distribution matched that of TrkB-like receptor. It was observed in all cells of the neuromasts in *S. salar* (Fig. 1C), and in the hair cells of *S. trutta* (Fig. 1F), but the occurrence of BDNF-like protein immunoreactivity in mantle cells cannot be ruled out in this species.

Outside the neuromasts, some basal and suprabasal epidermic cells were immunoreactive for TrkB- and BDNF-like proteins in *S. salar* (Fig. 1B,C), and a fine and diffuse immunoreactivity for BDNF-like protein was observed in all epidermic layers of *S. trutta* (Fig. 1F).

Canal neuromasts

Although the distribution and organization of hair cells into the trunk and cephalic channels differs between *S. salar* and *S. trutta*, the cell localization of Trk-like and neurotrophin-like proteins was identical. The hair and supporting cells of the canal neuromasts displayed TrkA-like (not shown) and TrkB-like (Fig. 2B) receptors, but not TrkC-like (Fig. 2C) receptor immunoreactivity. Furthermore, faint but specific immunoreactivity was also observed in the non-sensory walls of the canals (Fig. 2B).

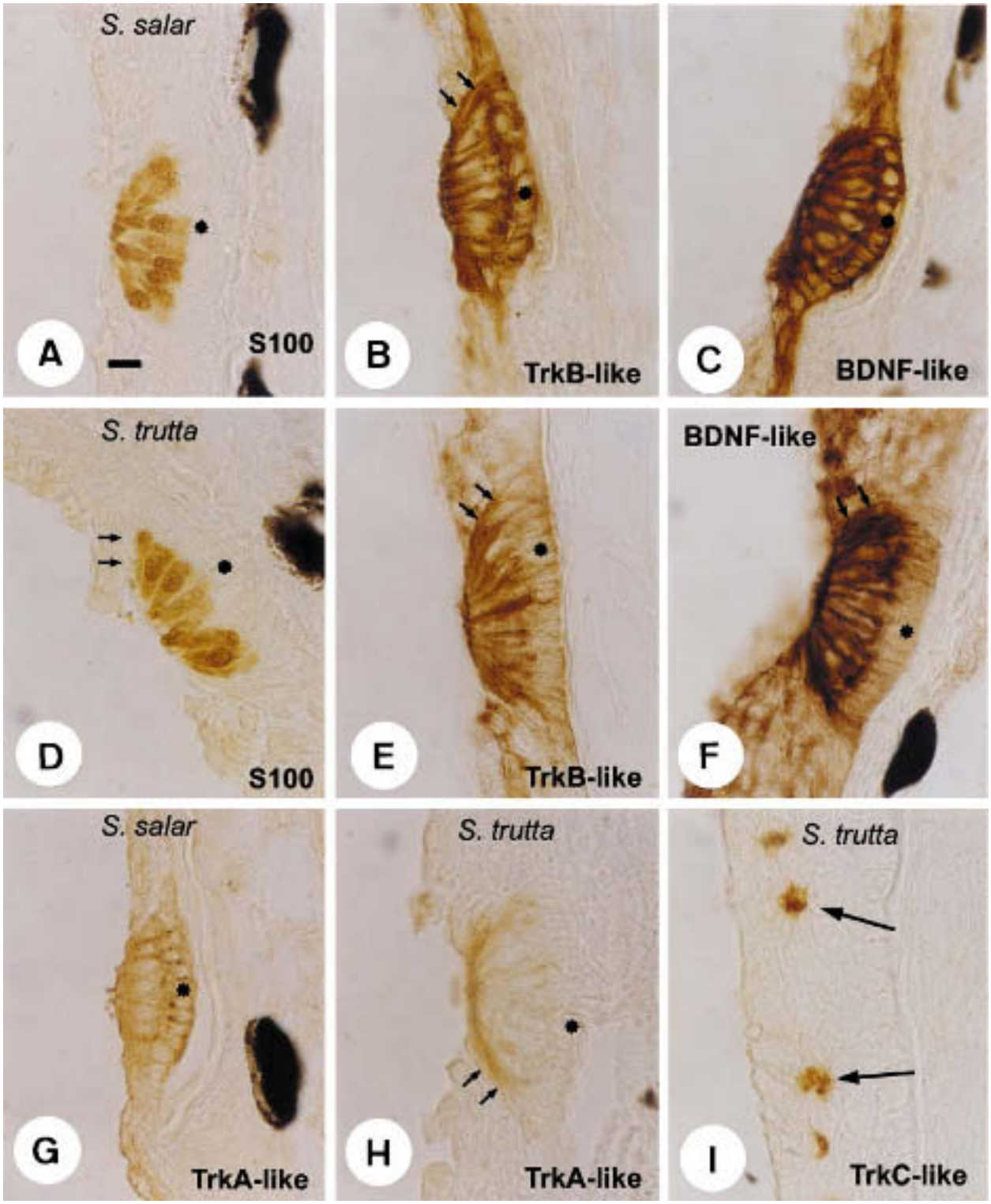

Fig. 1 Immunohistochemical localization of S100 (A,D), TrkB-like (B,E), BDNF-like (C,F) and TrkA-like (G,H) proteins in the trunk pit organs of *Salmo salar* and *S. trutta.* S100 protein was an excellent and selective marker for hair cells. TrkB-like and TrkA-like proteins were detected in all cells of neuromast in *S. salar* whereas in *S. trutta* TrkB-like protein was restricted to hair cells, and TrkA-like protein was found in mantle cells. TrkC-like protein was exclusively detected in unidentified epidermic cells (arrows in I) of *S. trutta*. Small arrows indicate mantle cells. Asterisks show the supporting cells. Scale bar = 10 μm.

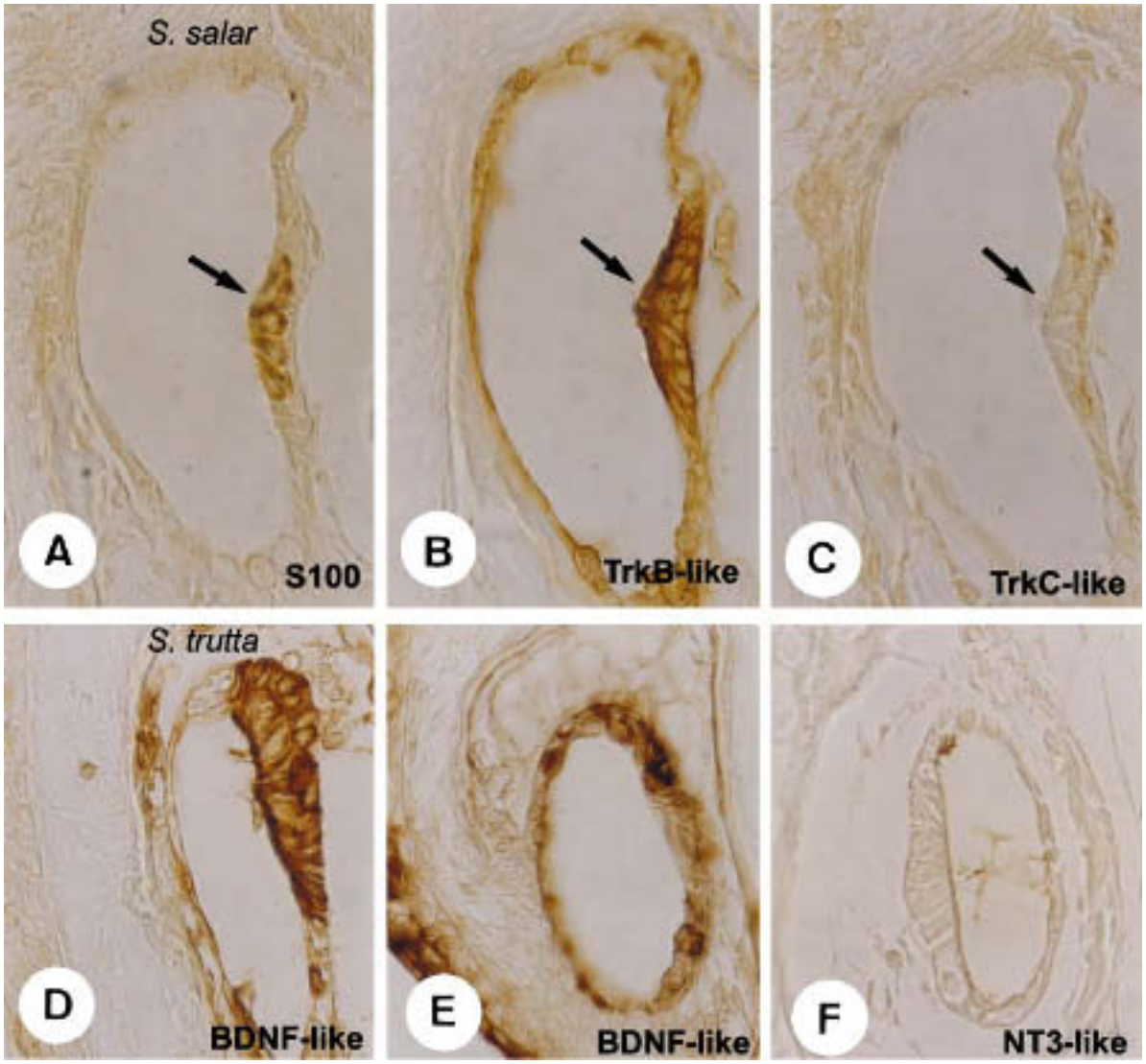

Fig. 2 Immunohistochemical detection of S100 (A), TrkB-like (B), TrkC-like (C), BDNF-like (D,E) and NT-3-like (F) proteins in neuromast of the trunk canals (arrows) of *Salmo salar* and *S. trutta.* S100 protein immunoreactivity was restricted to hair cells, whereas all cells displayed TrkB-like and DBDN-like proteins. TrkC-like and NT-3 proteins were regularly absent. TrkB-like and BDNF-like proteins were also found in non-sensory cells of the canals. Scale bar = 10 μm.

On the other hand, and as for the pit organs, the only neurotrophin detected in the canal neuromasts was BDNF-like protein, which was also present in the non-sensory cells of the walls of canals (Fig. 2D,E); NGF-like (not shown) and NT-3 (Fig. 2F) proteins were regularly absent.

Innervation of the LLS

Neurofilament-immunorective nerve fibres reached neuromasts of the pit organs and canals from large subcutaneous nerves (data not shown). Within the sensory organs, nerve fibres are in contact with the basal pole of hair cells, but also with supporting and mantle cells (Fig. 3). Regarding innervation, no differences were noted between *S. trutta* and *S. salar.*

The results on the distribution of Trk- and neurotrophin-like proteins in pit organs and neuromasts of canals are summarized in Table 2.

Discussion

The sensory systems of vertebrates are under the control of different families of growth factors for development and maintenance, one of the most important being the family of neurotrophins (see

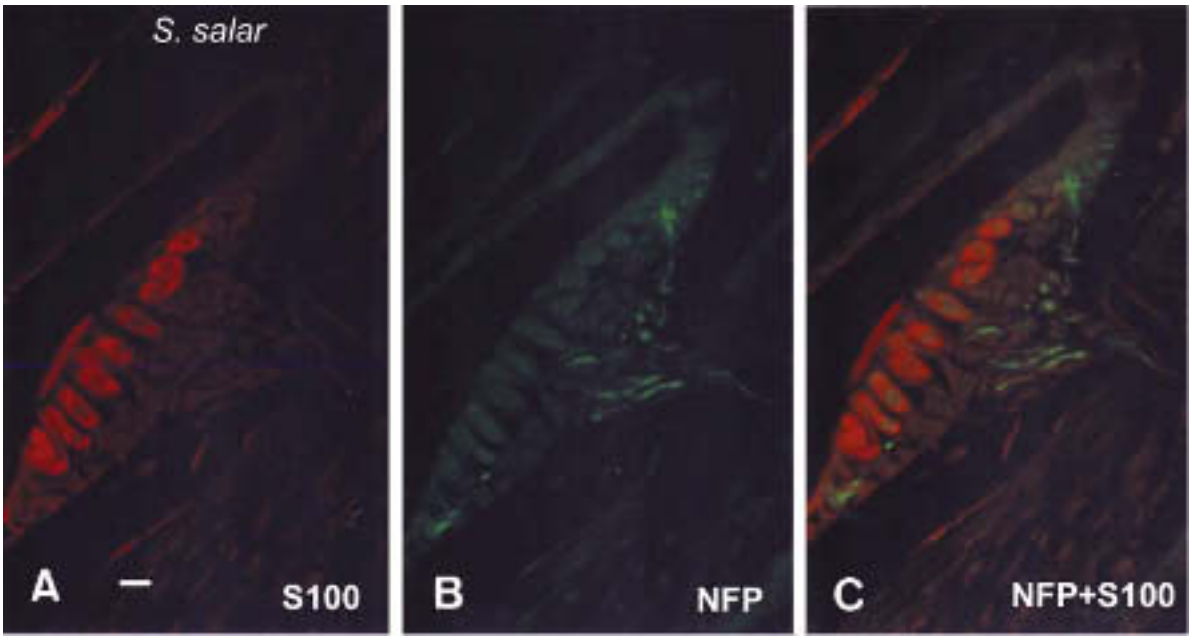

Fig. 3 Single (A,B) and double (C) immunohistochemistry for S100 protein (A) and neurofilament proteins (B) in neuromasts of the trunk canals of *Salmo salar.* Sensory axons innervate hair cells but also supporting cells. Scale bar = 16 μm.

Table 2 Distribution of neurotrophin-like and Trk-like proteins in the lateral line system of alevins of *S. salar* and *S. trutta*

	NGF	BDNF	NT-3	TrkA	TrkB	TrkC
Salmo salar						
Pit organs						
Hair cells	–	+ + +	–	+	+ + +	–
Supporting cells	–	+ + +	–	+ +	+ + +	–
Mantle cells	–	+ + +	–	+ +	+ + +	–
Canal neuromasts						
Hair cells	–	+ + +	–	+ +	+ + +	–
Supporting cells	–	+ +	–	+ +	+ +	–
Mantle cells	–	+ +	–	+ +	+ +	–
Salmo trutta						
Pit organs						
Hair cells	–	+ + +	–	–	+ + +	–
Supporting cells	–	–	–	–	–	–
Mantle cells	–	–	–	+ +	–	–
Canal neuromasts						
Hair cells	–	+ + +	–	+	+ + +	–
Supporting cells	–	+ +	–	+ +	+ +	–
Mantle cells	–	+ +	–	+ +	+ +	–

The intensity of immunostaining in neuromasts was evaluated as strong (+ + +), moderate (+ +), faint (+) or absent (–).

Fariñas, 1999; Huang & Reichardt, 2001). Neurotrophins act on cells through signal-transducing tyrosine-kinase receptors called Trk proteins (Lewin & Barde, 1996). Both neurotrophins and their parent Trk receptors are highly preserved during evolution, and they have been isolated in all vertebrates (see Hallbook, 1999), including fish (Heinrich & Sam, 2000). Trk-like receptors, but not neurotrophin ligands, have been found also in some invertebrates (Van Kestener et al. 1998; Lucini et al. 1999a; see also Jaaro et al. 2001). However, the tissue distribution of neurotrophins and/or Trk receptors in vertebrates other than mammals and birds is poorly known. Recently, Trk-like proteins have been detected in neuronal and non-neuronal cells of fish and reptiles, but attention was not focused on the LLS (De Girolamo et al. 1999, 2000; Lucini et al. 1999b, 2001; Hannestad et al. 2000).

This paper reports for the first time the occurrence and cell distribution of BDNF-like and TrkB-like proteins, and to a lesser extent TrkA-like protein, in mechanoreceptors of LLS in fish. Previous studies have demonstrated the presence of BDNF mRNA in the neuromasts of developing zebrafish (Hashimoto & Heinrich, 1997; Lum et al. 2001), and the absence of Trk-like protein in LLS of *Dicentrarchus labrax* (Hannestad et al. 2000), although we have recently found TrkB-like protein immunoreactivity in hair cells of neuromasts of alevins of this species (Catania et al. unpubl. obs.). Conversely, NGF-like, NT-3-like and TrkC-like proteins were regularly absent from neuromasts although all were detected in other tissues such as the central nervous

system, gills and skin (not shown; see also Hannestad et al. 2000; Lum et al. 2001). BDNF-like protein was strongly expressed in LLS organs (see also Hashimoto & Heinrich, 1997; Lum et al. 2001), mainly in the hair cells but also in the supporting cells in both the pit organs and neuromasts of the canals. Consistently with the expression of BDNF-like protein, the main Trk-like receptor detected in neuromasts was the TrkB-like receptor, regarded as its physiological receptor (Lewin & Barde, 1996). Furthermore, the cellular distribution of TrkB-like receptor basically matched that of BDNF-like protein, thus suggesting autocrinia and/or paracrinia in the neuromasts of LLS.

In addition to TrkB-like protein, TrkA-like receptor was detected in all cells of pit organs in *S. salar*, and apparently restricted to the mantle cells in *S. trutta*. These species-specific differences in the expression of TrkA-like protein were inexistent in the neuromasts of the canals, and could be related to sensory modalities (see Engelmann et al. 2000).

The antibodies against Trk used here map within the catalytic domain of the Trk proteins, a region with a high homology in all vertebrates (Barde, 1994; Hallbook, 1999) being higher than 90% in the zebrafish and rat (Martin et al. 1995, 1998). Furthermore, all the key elements found in mammalian Trks, including those involved in signal transmission, are identical in zebrafish and mammals (Heinrich & Lum, 2000). They recognize full-length Trk proteins in teleosts (De Girolamo et al. 1999, 2000; Lucini et al. 1999b). The neurotrophin sequences are also highly preserved during evolution in vertebrates (see Götz & Schartl, 1994; Hallbook, 1999; Heinrich & Lum, 2000). Nevertheless, only BDNF-like protein was detected in the LLS whereas NGF-like and NT-3-like molecules were always absent. It remains to be established if these negative results are due to poor cross-reactivity of the antibodies employed or these molecules are really absent in the LLS.

It is accepted that LLS neuromasts, especially those of canals, are closely related to the inner ear organ, and represent an adaptation in fishes of the vestibular system. Supporting this view they share a placodal origin, types of sensory cells, presence of supporting cells, localization of the sensory ganglia and central projections (see Northcutt et al. 2000). Interestingly, the sensory vestibular epithelium, especially hair cells, of mammals (Montcouquiol et al. 1998; Popper et al. 1999; Qun et al. 1999), birds (see Vazquez et al. 1994; Pirvola et al. 1997) and amphibians (Don et al. 1997) express BDNF mRNA, as well as TrkB mRNA (Don et al. 1997). As speculated in this paper regarding neuromasts, autocrine/paracrine signalling was also thought to be present in the mammalian inner ear (Knipper et al. 1996; Robinson et al. 1996). The data on the TrkA-like receptor must be further investigated since this receptor is expressed only transiently, and is not necessary for development, in the inner ear of higher vertebrates (see Schimmang et al. 1997; Vega et al. 1999).

Our data regarding the innervation of the LLS are in good agreement with previous studies in other teleost species (for references see Northcutt et al. 2000). Sensory axons contact hair cells, as well as supporting cells and mantle cells (Alexandre & Ghysen, 1999; see also Smith, 2000). Although further studies are necessary, the occurrence of nerve fibres in this latter localization could be explained by the formation of new hair cells in this area (Jorgensen, 1991; Fritzsch et al. 1998; Witte et al. 2001) The relationship between innervation and the pattern of expression of Trk-like or neurotrophin-like proteins remains to be analysed in future studies.

The function of Trk-like and neurotrophin-like proteins in neuromasts, and in fish in general, is essentially unknown, but it could be related to the cell turnover (Balak et al. 1990; Williams & Holder, 2000) and probably to the development or maintenance of synaptogenesis (Montcouquiol et al. 1998).

Finally, it must be noted that our results demonstrate that S100 protein is an excellent marker for hair cells in neuromasts of the LLS, as it occurs in their parent cells in the inner ear (Saidel et al. 1990; Foster et al. 1993).

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