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Neurotrophin Trk Receptors in the Brain of a Teleost Fish, *Nothobranchius furzeri*

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ABSTRACT Trk neurotrophin receptors are transmembrane tyrosine kinase proteins known as TrkA, TrkB, and TrkC. TrkA is the high affinity receptor for nerve growth factor, TrkB is the one for both brain-derived neurotrophic factor and neurotrophin-4, and TrkC is the preferred receptor for neurotrophin-3. In the adult mammalian brain, neurotrophins are important regulators of neuronal function and plasticity. This study is based on *Nothobranchius furzeri,* a teleost fish that is becoming an ideal candidate as animal model for aging studies because its life expectancy in captivity is of just 3 months. In adult *N. furzeri*, all three investigated neurotrophin Trk receptors were immunohistochemically detected in each brain region. TrkA positive neuronal perikarya were localized in the dorsal and ventral areas of the telencephalon and in the cortical nucleus; TrkB immunoreactivity was observed in neuronal perikarya of the dorsal and ventral areas of the telencephalon, the diffuse inferior lobe of the hypothalamus, and Purkinje cells; TrkC positive neuronal perikarya were detected in the most aboral region of the telencephalon, in the magnocellular preoptic nucleus and in few neurons dispersed in the hypothalamus. Numerous positive fibers were widely distributed throughout the brain. Radial glial cells lining the mesencephalic and rhombencephalic ventricles showed immunoreactivity to all three Trks. These findings suggest an involvement of neurotrophins in many aspects of biology of adult *N. furzeri*. *Microsc. Res. Tech. 00:000–000, 2011.* © 2011 Wiley-Liss, Inc.

INTRODUCTION

Neurotrophins are a family of evolutionarily conserved growth factors, which regulate the differentiation, growth, and function of many neuronal populations in the central nervous system (CNS) and peripheral nervous system of vertebrates. Neurotrophins bind with low affinity to p75NTR, a member of the TNF receptor family, and with high affinity to Trk neurotrophin receptors, transmembrane tyrosine kinase proteins known as TrkA, TrkB, and TrkC. Each of Trk receptors interacts specifically, but not exclusively, with different members of the neurotrophin family. TrkA is the high affinity receptor for nerve growth factor (NGF), TrkB binds both to brain-derived neurotrophic factor (BDNF), and neurotrophin-4 (NT-4), and TrkC is the preferred receptor for neurotrophin-3 (NT-3). Both TrkA and TrkB can also recognize with low affinity NT-3. Trks receptors mediate the trophic properties of all neurotrophins. The role of p75NTR is controversial, because it may contribute to the formation of high-affinity receptors, but it can mediate apoptosis in developing neurons. Indeed, in the absence of Trk receptors, p75 binds to proneurotrophins, the secreted immature forms not cleaved by furin proteases (for a review see Schecterson and Bothwell, 2010).

In the adult brain of mammals, neurotrophins control synaptic function and plasticity, sustain neuronal cell survival, morphology, and differentiation (for a review see Skaper, 2008). Moreover, many studies have shown that BDNF, NGF, and NT-3 are also implicated in the mechanisms regulating adult mammalian hippocampal neurogenesis (for a review see Lee and Son, 2009) and a vast amount of evidence indicates that alterations in levels of neurotrophic factors or their receptors can lead to neuronal death and contribute to aging as well as to the pathogenesis of diseases (for a review see Lanni et al., 2010)

Adult neurogenesis appears in vertebrate brains (Kaslin et al., 2008), but qualitative differences exist in neurogenic potential between mammals and teleosts. Studies in teleost fish have indicated that defined neurogenic sites are present over the entire rostrocaudal axis of the nervous system, and are largely associated with the ventricular system (for a review see Zupanc, 2008).

Among teleost fish, *Nothobranchius furzeri* is an emerging animal model for aging studies because its life expectancy in captivity is of a few months, which represents the shortest documented captive lifespan for a vertebrate (Terzibasi et al., 2007). In addition, lifespan can be modulated by nongenetic intervention (Terzibasi, 2009; Valenzano and Cellerino, 2006; Valenzano et al., 2006) and large genetic differences in lifespan exist between different laboratory strains (Terzibasi et al., 2008). Thus, the aim of this study is to investigate the distribution of Trk neurotrophin receptors in the adult brain of *N. furzeri*, as a preliminary study for

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TABLE 1. Primary antisera

Antisera	Antigen	Source	Specificity	Dilution	Host
TrkA	Human COO-domain 763-777	S. Cruz Biotechnology	No cross reaction with TrkB and TrkC	1/100	Rabbit
TrkB	Human COO-domain 794-808	S. Cruz Biotechnology	No cross reaction with TrkA and TrkC	1/100	Rabbit
TrkC	Human COO-domain 798-812	S. Cruz Biotechnology	No cross reaction with TrkA and TrkB	1/100	Rabbit

future investigations regarding the involvement of neurotrophins in neurogenesis and aging.

MATERIALS AND METHODS

N. furzeri develops from a larva to a sexually mature adult in 3–4 weeks, and its maximum lifespan is 13 weeks. To address this preliminary study on adults, brains were taken from 5–6 week old animals obtained from the animal colony of Leibniz Institute for Age Research, Fritz Lipmann Institute (Jena, Germany). The experimental protocols were conducted within the European Communities Council Directive of November 24, 1986 (86/609/EEC) and were approved by the local authority in the State of Thuringia (Veterinär- und Lebensmittelüberwachungsamt). *N. furzeri* were euthanized using 0.1% ethyl 3-aminobenzoate and methanesulfonate (Sigma Chemicals, St Louis, MO).

Six adult *N. furzeri* heads were fixed by immersion in Bouin's fluid for 24 h at room temperature (RT), dehydrated in ethanol series and embedded in paraffin wax. Transverse, sagittal and horizontal 5–7 μm thick sections were cut. Microtomical sections were serially stained by luxol fast blue, cresyl violet, and immunocytochemistry. Immunocytochemical stainings were performed by means of EnVision + system-horseradish antiperoxidase (HRP) (cod. K4002, Dako, Santa Barbara, CA, peroxidase-antiperoxidase (PAP) method (Sternberger, 1986), and avidin biotin complex (ABC) method.

After dewaxing in xylene, sections were treated with 3% H_2O_2 (20 min), washed with phosphate buffered saline solution (PBS) pH 7.4 and incubated in a humid chamber for 24 h at 4°C with each of primary antibody diluted with PBS containing 0.2% TritonX-100, 0.1% bovine serum albumin, and 4% normal goat serum (NGS) (cod. S1000, VECTOR Lab, Burlingame, CA). Primary antibodies are shown in Table 1. After incubation, the sections were washed in PBS and treated for the different methods as follows. EnVision method: the sections were incubated with EnVision for 30 min at RT; PAP method: the sections were incubated with antiserum raised in goat against rabbit IgG (GAR, 1:50; cod. Z0421, Dako) for 30 min at RT, then washed in PBS, and incubated with rabbit PAP complex (1:100; cod. Z0113, Dako) for 30 min at RT; ABC method: the sections were incubated with biotinylated antigoat IgG rabbit serum (1:200, cod. BA-5000 VECTOR) for 30 min., and then in a solution of avidin and biotin-peroxidase complex (cod. PK-6100, VECTOR) for 30 min. After washing, the immunoreactive sites obtained by all three methods were visualized using a fresh solution of 10 μg of 3,3′-diaminobenzidine tetrahydrochloride (DAB) (cod. D5905, Sigma-Aldrich) in 15 ml of a 0.5 M Tris buffer, pH 7.6, containing 1.5 ml of 0.03% H_2O_2. Immunocytochemical stainings were photographed using a Leica microscope DM RA2 (Leica Camera AG, Solms, Germany) attached to a Leica DC300 F camera for light microscopy and stored in a Leica IM 1,000 archive.

To compare results obtained in animals treated as described before, two adult *N. furzeri* heads were fixed in paraformaldehyde for 24 h at 4°C, then cryoprotected in 30% sucrose in PBS and embedded in cryomounting. Serial transverse cryostat 10 μm thick sections were cut and conserved at −20°C. Slides were thawed and washed in PBS, then they were placed in target retrieval solution (Citric buffer pH 7.4) brought to boil using the microwave, and then gently boiled for 10 min at 10% power, then left in the solution to cool for 30 min. Sections were washed with PBS and incubated for 1 h at RT in a solution of PBS with 20% NGS, 1% BSA, and 0.1% Triton X-100. Then they were incubated in a humid chamber for 24 h at 4°C with each of primary antibody diluted with PBS containing 0.2% TritonX-100, 0.1% bovine serum albumin, and 4% normal goat serum. Incubation with primary antibody was carried out overnight at 4°C. After incubation, the sections were washed in PBS with 20% NGS, 1% BSA, and 0.1% Triton X-100 and incubated with EnVision for 30 min at RT. After washing, the immunoreactive sites were visualized using a fresh solution of 10 μg of DAB in 15 ml of a 0.5 M Tris buffer, pH 7.6, containing 1.5 ml of 0.03% H_2O_2.

Each immunocytochemical staining was performed either on brain and retina slides of *N. furzeri*, the latter considered as positive control. In the retina, the presence of Trk receptors was previously described by both immunocytochemistry and in situ hybridization (Caminos et al., 1999). The specificity of immunocytochemical stainings was tested by successively substituting the primary antisera or the EnVision, PAP, or ABC with PBS or normal serum, in repeated trials. Adsorption controls were performed by using each antibody preadsorbed with an excessive amount of its homologous (25 μg/ml) and heterologous (50 μg/ml) antigens (sc-118 P for TrkA, sc-12 P for TrkB, sc-117 P for TrkC, Santa Cruz Biotechnology, Santa Cruz, CA).

To further analyze the specificity of the antisera employed against Trk receptors, we have tested the antibody specificity by means of dot-blotting analysis. The dot blotting was performed using the method described by Lucini et al. (2004). Briefly, strips of nitrocellulose were cut, and 2 μl drops of synthetic blocking peptides (see controls) at various concentrations (25–156 μg/ml) were spotted on the strips, and allowed to dry at RT. The strips were fixed with Bouin's fluid for 1 h at RT. The procedure involved the following steps: (1) wash in PBS and 1% Triton X (PBS-T) for 25-min; (2) block in 5% bovine serum albumin for 1 h; (3) wash in PBS-T for 5-min; (4) incubation with primary antibody (TrK A, TrK B, and TrK C diluted 1/600) at 48°C overnight; (5) wash in PBS-T for 30-min; (6) incubation with GAR 1/100 for 30-min; (7) wash in PBS-T for 30-min; (8) incubation with PAP 1/200 for 30-min; (9) wash in PBS-T for 30-min; and (10) incubation with DAB for 10−45 min.

TABLE 2. Localization of Trk IR

		TrkA	TrkB	TrkC
Telencephalon	Olfactory bulbs	Fibers	Fibers	Fibers
	Telencephalic lobes	Neurons-fibers	Neurons-fibers	Neurons-fibers
Diencephalon	Preoptic zone	Fibers		
	Magnocellular preoptic nucleus		Fibers	Neurons-fibers
	Posterior parvocellular preoptic nucleus		Fibers	Fibers
	Optic tract			Fibers
	Cortical nucleus	Neurons		
	Dorsal hypothalamus			Neurons
	Diffuse inferior hypothalamic lobe		Neurons	
	Posterior recess nucleus	Fibers		
Mesencephalon	Ventricle	Radial glial cells	Radial glial cells	Radial glial cells
	Longitudinal tori	Fibers		Fibers
	Optic tectum	Fibers	Fibers	Fibers
	Glomerular nucleus	Fibers		Fibers
Rhombencephalon	Cerebellum	Fibers	Purkinje cells	Fibers
	Ventricle	Radial glial cells	Radial glial cells	Radial glial cells
	Medulla oblongata		Fibers	Fibers
	Acoustic n. r.			Fibers
	Glosso-pharyngeal n. r.	Fibers		
	Vagal n.r.	Fibers		

n.r. = nerve root.

RESULTS

The immunoreactivity to Trks was observed in the major regions of *N. furzeri* brain. It has been documented in numerous cells that due to their morphology and localization were almost all classified as neurons and radial glial cells. The results are summarized in Table 2.

The description of the *N. furzeri* brain is based on the atlas of Peter et al. (1975) and Anken and Rahmann (1994). They concern two species *Fundulus heteroclitus* and *Xiphophorus helleri,* phylogenetically very close to *N. furzeri*. All three species belong to the order Cyprinodontiformes.

In the telencephalon, TrkA IR was observed in fibers scattered overall the olfactory bulbs and grouped in the glomerular layer (Fig. 1A). Numerous fibers and rare TrkA positive neurons were also distributed in both the dorsal (Figs. 1B and 1C) and ventral areas of the telencephalic lobes. In the diencephalon, numerous TrkA positive fibers were seen in the preoptic zone and in the posterior recess nucleus of the hypothalamus. Some TrkA positive neurons were observed in the cortical nucleus. In the mesecephalon, TrkA IR was localized in radial glial cells, lining the margin of tegmentum projecting toward the mesencephalic ventricle, and in the periventricular gray zone of the optic tectum (Figs. 1D and 1E). Numerous TrkA positive fibers were seen in the longitudinal tori, in the optic tectum and in the glomerular nucleus of the tegmentum (Figs. 1D–1F). In the rhomboencephalon, TrkA positive fibers were preferentially grouped in the molecular layer of cerebellum corpus, and in the lateral and medial granular eminences. Numerous TrkA positive fibers were observed in the glosso-pharyngeal and vagal nerve roots of the medulla oblongata. Positive radial glial cells were observed along the rhombencephalic ventricle.

In the telencephalon, TrkB IR was observed in fibers of the olfactory bulbs and in glomeruli, in scattered fibers and in few neurons of the dorsal and ventral areas of the telencephalic lobes. These neurons were preferentially distributed in the most aboral regions of the dorsal telencephalic area (Figs. 2A and 2B). In the diencephalon, some TrkB positive fibers were seen in the magnocellular and in the posterior parvocellular preoptic nucleus. Scattered intensely positive neurons were also detected in the diffuse nucleus of the inferior lobe of the hypothalamus. In the mesencephalon, positive radial glial cells were lining the mesencephalic ventricle. In the rhomboencephalon, numerous immunoreactive Purkinje cells, surrounded by positive nerve endings basket-like, were detected (Figs. 2C and 2D) in the cerebellum. Very few positive fibers were dispersed in the medulla oblongata. TrkB positive radial glial cells were lining ventrolaterally the rhombencephalic ventricle.

In the telencephalon, TrkC IR was present in intensely stained fibers and glomeruli of the olfactory bulbs. Numerous TrkC positive fibers were seen in the dorsal and ventral areas of the telencephalic lobes, showing a dorsal-ventral and lateral-medial decreasing pattern. Some small round positive neurons were recognized (Figs. 3A and 3B) in the most aboral region of the dorsal telencephalic area. In the diencephalon, positive fibers, single, or grouped, belonging to the optic tract were observed. Some positive neurons were localized in the magnocellular preoptic nucleus. Numerous and deeply stained fibers were seen in the magnocellular preoptic and posterior parvocellular preoptic nuclei (Figs. 3C and 3D). Rare neurons were seen dispersed in the hypothalamus. In the mesencephalon, TrkC immunoreactive fibers were observed in the longitudinal tori and in the optic tectum particularly localized in the most external layer of the superficial white and gray zone. TrkC IR was detected in fibers extending in the glomerular nucleus (Figs. 3E and 3F) of the tegmentum. Numerous radial cells lining the mesencephalic ventricle were TrkC positive (Fig. 3G). In the rhombencephalon some scattered positive fibers were observed in the cerebellum. Positive fibers were decurring in the medial longitudinal fascicle of the medulla oblongata. Radial glial cells, along the rhombencephalic ventricle, were TrkC positive. TrkC positive fibers were seen in the acoustic nerve (Fig. 3H).

Controls for all Trk antisera showed no reaction. In addition, the substitution of primary antibody with antibodies adsorbed by the correlated antigens did not modify

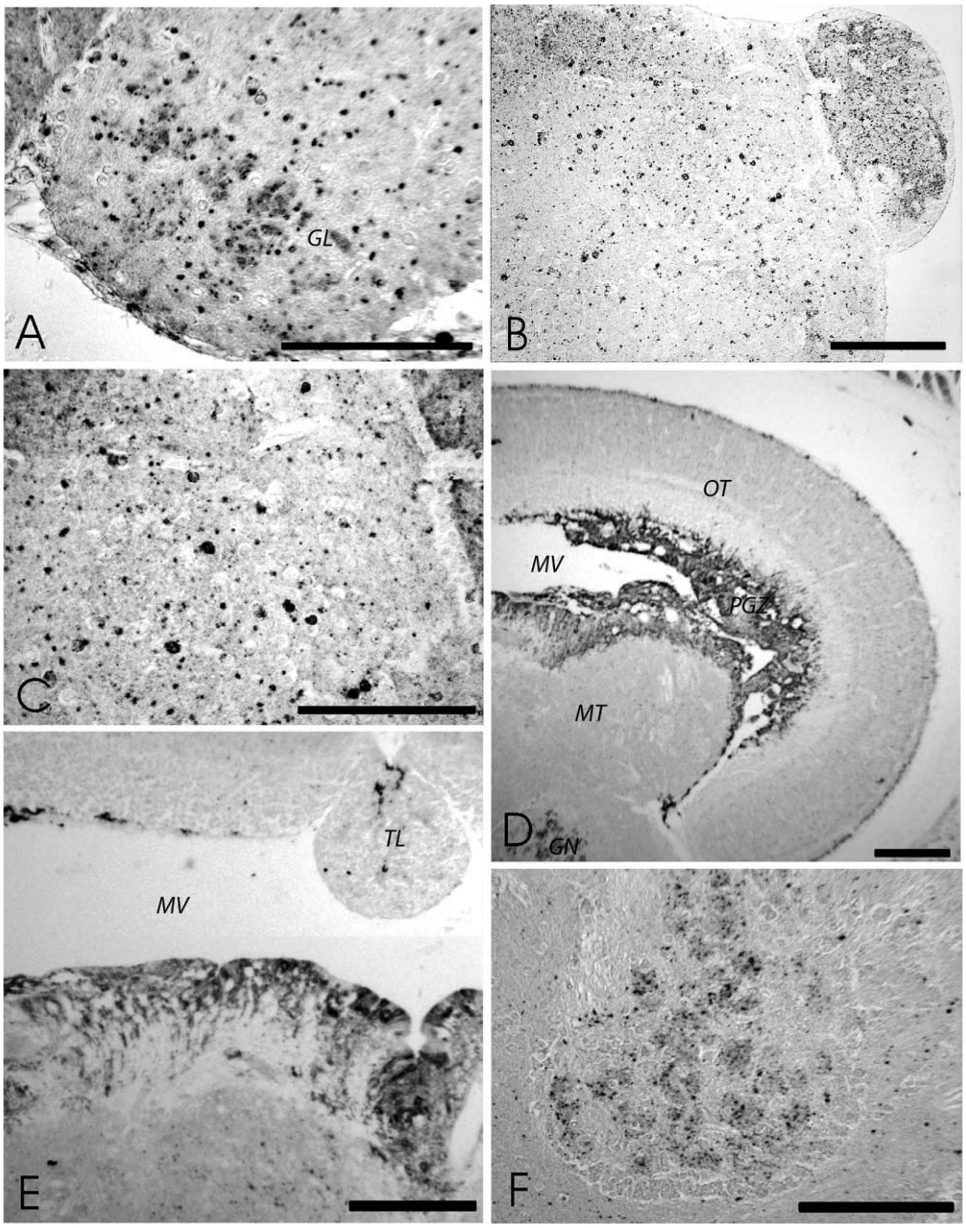

Fig. 1. Trk A IR in transversal sections of the brain of *N. furzeri*. **A**: Olfactory bulbs with dispersed and grouped positive fibers. **B** and **C**: Dorsal telencephalic area at low (B) and high (C) magnification with positive fibers and rare neurons. **D**–**F**: Mesencephalon: optic tectum showing intensely stained periventricular gray zone (D), tegmentum lined by positive radial glial cells at low (D) and high magnification (E) and glomerular nucleus with antero-posterior decurring fibers at low (D) and high magnification (F). GL, glomerular layer; OT, optic tectum; MV, mesencephalic ventricle; PGZ, periventricular gray zone; MT, mesencephalic tegmentum; GN, glomerular nucleus; TL, longitudinal tori. Scale bars: D = 300 mμ; E = 200 mμ; B, C, and F = 150 mμ; A = 100 mμ.

the reaction. The dot-blot technique showed that each antiserum recognized its related antigen (Fig. 4). Immunoreactions carried out on paraffin and cryostatic sections showed overlapping pattern of distribution.

DISCUSSION

Teleostean and mammalian neurotrophins have similar primary and three-dimensional structures (for a review see Heinrich and Lum, 2000). Trk receptors have remained unchanged in number in all tetrapods, while in the teleost fish lineage a specific gene duplication have occurred (for a review see Benito-Gutiérrez et al., 2006). In zebrafish (Martin et al., 1995), there are five genes encoding for Trk receptors, one of the A class, and two of each B and C classes. It implies that one of the duplicated TrkA genes was lost early in the fish lineage. The antisera employed in this study against TrkA, TrkB, and TrkC react against the tyrosine kinase catalytic domain of the specific Trk mammalian protein. Because the five Trk proteins identified in zebrafish are structurally homologous to the only three known mammalian Trk proteins, especially in

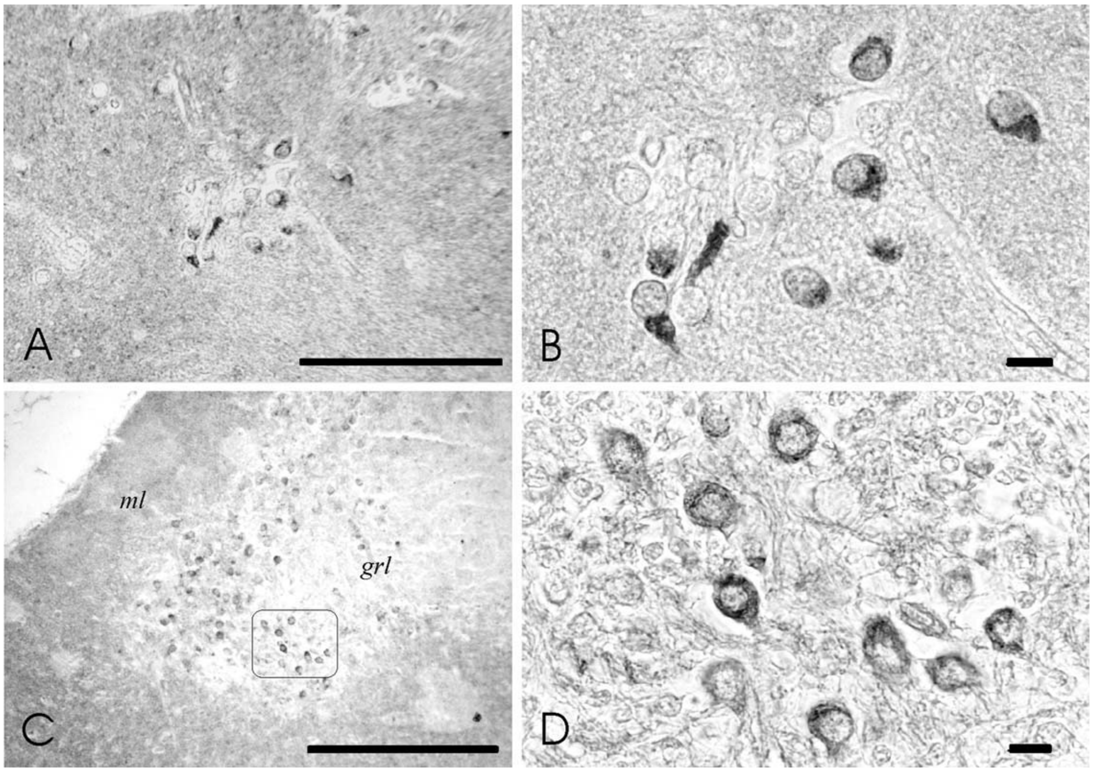

Fig. 2. Trk B IR in transversal sections of the brain of *N. furzeri*. **A** and **B**: Dorsal telencephalic area with positive fibers and neurons at low (A) and high magnification (B); **C** and **D**: Posterior part of corpus cerebelli with numerous positive Purkinje cells at low (C) and high magnification (D). ml, molecular layer; grl, granular layer. Scale bars: C = 150 mμ; A = 100 mμ; B and D = 10 mμ.

the intracellular kinase regions, it is reasonable to assume that the Trk proteins in *N. furzeri* are equivalent to functional isoforms of mammalian proteins.

Although all three Trk receptors were present in the major regions of *N. furzeri* CNS, each of them presented a specific distribution pattern as shown in Table 2: (a) TrkA IR in neurons of the cortical nucleus and in fibers extending in the preoptic zone, posterior recess nucleus, glosso-pharyngeal, and vagal nerve root; (b) TrkB IR in neurons of the diffuse inferior lobe and in Purkinje cells; (c) TrkC in neurons dispersed in the hypothalamus and in fibers belonging to the optic tract and acoustic nerve. These findings further confirm, besides our control results, the absence of any cross-reactivity among the antisera employed. However, some structure, such olfactory bulb fibers and radial glial cells around ventricles, displayed IR to all three receptors and thus a higher grade of redundancy. Also in mouse CNS redundant survival pathways were suggested by analyses of the various neurotrophin and Trk receptor knock-outs, in which mutation of a neurotrophin or Trk gene in no case resulted in the complete disappearance of a distinct subpopulation of central neurons (Huang and Reichardt, 2001).

In the brain, the presence of Trk receptors was previously described in the zebrafish few days after hatching by in situ hybridization (Martin et al., 1995), in *Dicentrarchus labrax* alevins by immunocytochemistry (Hannestad et al., 2000) and TrkB in the eel by RT-PCR analysis (Dalton et al., 2009). While these studies confirmed the presence of Trk receptors in the CNS of teleost fish, they were not devoted to neuroanatomical description of Trks in the brain. Therefore, this study is the first comprehensive characterization of Trks protein distribution in an adult teleost fish brain and the first description regarding the presence of Trk receptors in adult *N. furzeri*.

In general, comparing our results with those reported in mammals, in *N. furzeri* Trk positive perikarya seem to be fewer and less widespread, though a statistical analysis is beyond the scope of this article. These findings could not relate to a scarce sensibility of the antisera, neither the employed method of revelation, because positive controls always showed an intense reaction. Immunoreactions against Trk receptors performed on *N. furzeri* brain, by PAP and ABC method revealed few and very poorly stained neurons (data not shown), while EnVision technique, performed on both paraffin and cryostatic sections, showed some clearly positive neurons and overall numerous fibers. These findings are due to the higher sensitivity of EnVision method (Sabattini et al., 1998; Vosse et al., 2007), which permits to reveal lower Trk concentrations. However, many positive fibers, mainly TrkA positive, were seen throughout the *N. furzeri* brain. This pattern of distribution could be due to an active retrograde transport of signaling endosomes carrying NGF bound to activated TrkA (Campenot, 2009).

In the olfactory bulbs, all Trks IR were well documented in rat and cat (Deckner et al., 1993; Yan et al., 1997), similarly to data obtained in our species. TrkA has been described in cholinergic neurons in the rat basal

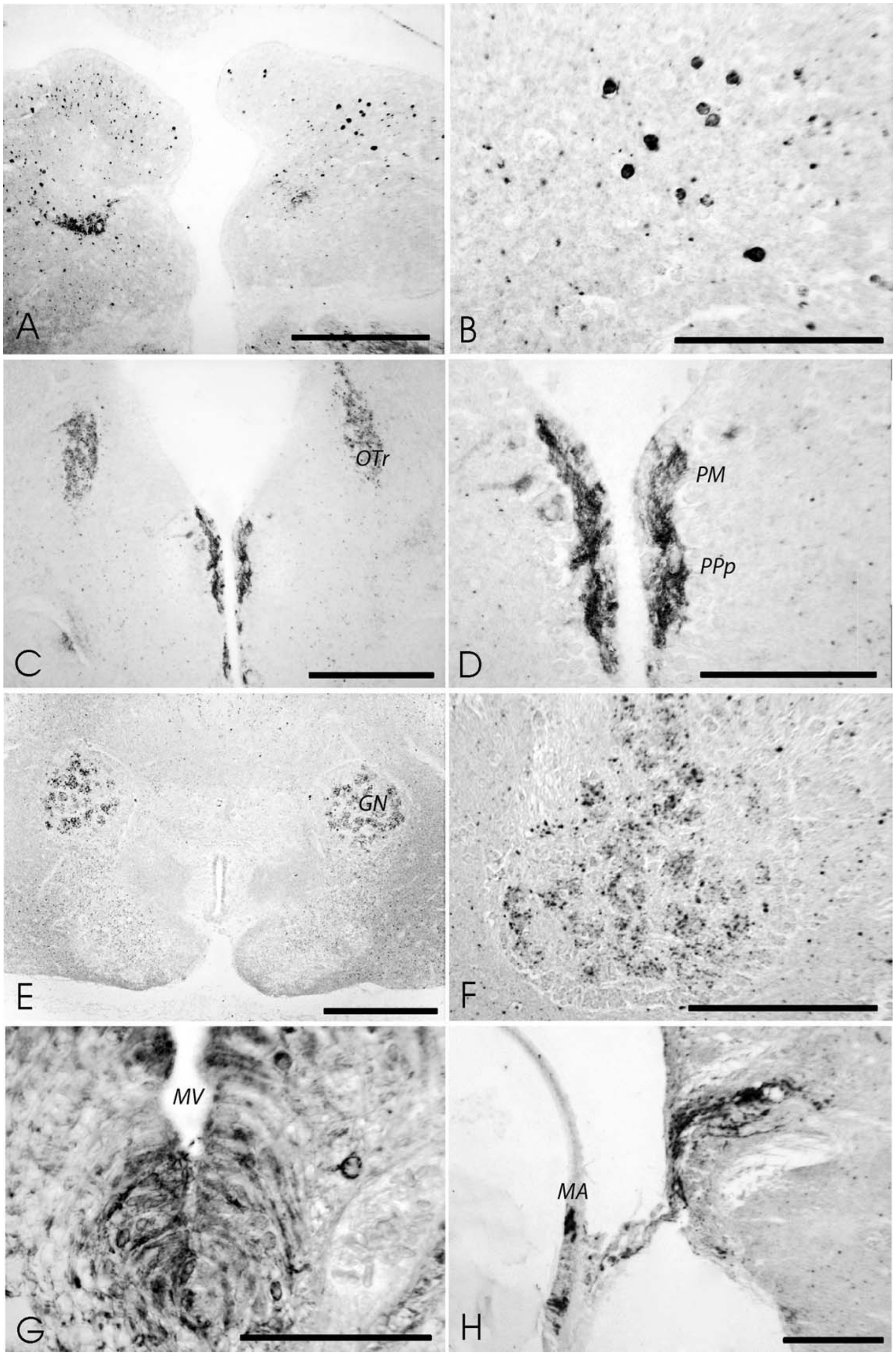

Fig. 3. Trk C IR in transversal sections of the brain of *N. furzeri*. **A**: Aboral region of the dorsal telencephalic area with positive fibers and neurons at low (A) and high magnification (**B**). **C** and **D**: Diencephalon showing positive fibers in the optic tract, fibers, and cells in the magnocellular preoptic nucleus and posterior parvocellular preopticus nucleus at low (C) and high magnification (D). **E** and **F**: Positive fibers in the glomerular nucleus at low (E) and high magnification (F). **G**: Positive radial glial cells along the mesencephalic ventricle. **H**: Acoustic nerve showing intensely stained fibers. OTr, optic tract; PM, magnocellular preoptic nucleus; PPp, posterior parvocellular preoptic nucleus; GN, glomerular nucleus; MV, mesencephalic ventricle; MA, macula. Scale bars: A, C, and E = 250 mμ; D and F = 150 mμ; B, G, and H = 100 mμ.

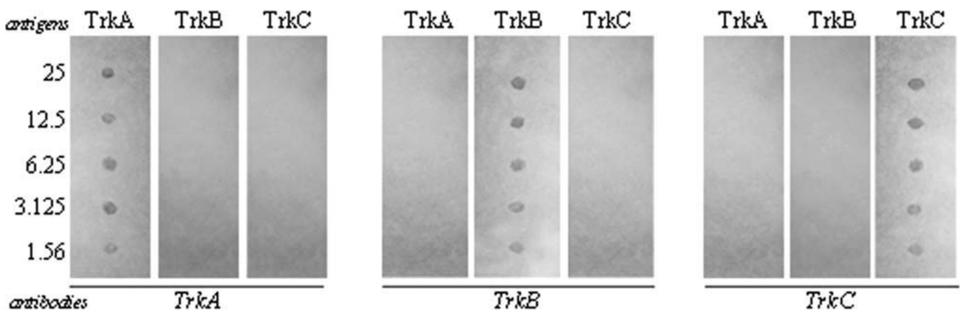

Fig. 4. Dot-blot analysis shows the reactivities of TrK A, TrK B, and TrK C polyclonal antibodies diluted to 1/600, and incubated with homologous and heterologous peptides to various concentrations (from 25 to 1.56 μg/ml).

forebrain and neostriatum (Holtzman et al., 1995), as well as TrkA and TrkB in the rat and guinea pig hippocampus (Cellerino, 1996; Dieni and Rees, 2002; Yan et al., 1997). However, the topology of fish telencephalon is so highly distorted for a developmental process called eversion, hence, it is difficult to correlate teleostean with corresponding mammalian regions, though efforts, sometime contrasting, have been done (Mueller and Wullimann, 2009; Nieuwenhuys, 2009; Yamamoto et al., 2007).

In the periventricular region of the diencephalon, numerous intensely stained TrkB and TrkC fibers were seen, also extending through cells of the magnocellular and posterior parvocellular preoptic nuclei. This area was previously described in zebrafish as neurogenic (Grandel et al., 2006). In *N. furzeri*, some cells of this zone displayed IR to the proliferating cell nuclear antigen (PCNA), a marker of G1 phase of the cell-cycle and generally maintained throughout division (unpublished data). Thus, it seems intriguing to speculate about a possible involvement of neurotrophins in adult diencephalic neurogenesis

Neurotrophins regulate the development and plasticity, the maintenance, and/or the modulation and regeneration of the mammalian visual centers (Avwenagha et al., 2006; Cellerino and Maffei, 1996; von Bartheld, 1998). In *N. furzeri*, the presence of TrkA and TrkC IR in neurons of the cortical nuclei and in fibers inside the glomerular nucleus *and* the optic tract and tectum also suggest in this species an involvement of fish neurotrophins in the visual pathway.

Purkinje cells of *N. furzeri* intensely expressed TrkB and some fibers running in the molecular layer were positive to TrkA and TrkC. These results are in accord with those obtained in rat (Yan et al., 1997), and partially with those of Quartu et al. (2003), Dieni and Rees (2002), which report in human and guinea pig cerebellum neurotrophin Trk receptors extensively expressed in different cell populations and fibers.

Finally, in the rhombencephalon of *N. furzeri* only Trks positive fibers were seen, whereas in rat Trks expression was reported in bulbospinal neurons (King et al., 1999; Yan et al., 1997).

Around mesencephalic and rhombencephalic ventricles, numerous non neuronal cells displayed IR to Trks. Almost the majority of them showed the morphology of radial glial cells, which in fish directly lined ventricles, as no ependymal layer is present (Grupp et al., 2010; Marz et al., 2010). Radial glial cells persist within the adult CNS of teleosts and seem to be involved in the generation and guidance of new neurons (Strobl-Mazzulla et al., 2010; Zupanc and Clint, 2003).

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