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A COMPREHENSIVE ANALYSIS OF NEUROTROPHINS AND NEUROTROPHIN TYROSINE KINASE RECEPTORS EXPRESSION DURING DEVELOPMENT OF ZEBRAFISH

Running Title: *nt* and *ntrk* expression in zebrafish embryos.

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Abstract

Neurotrophins (NTF) are a family of secreted nerve growth factors with affinity for tyrosine kinase (Ntrk) and p75 receptors. To fully understand the variety of developmental roles played by NTFs, it is critical to know when and where genes encoding individual ligands and receptors are transcribed. Identification of *ntf* and *ntrk* transcripts in zebrafish development remains to be fully characterized for further uncovering the potential function(s) of the NTF signal transduction pathway. Here, we conducted a systematic analysis of the expression profiles of four *ntf* and five *ntrk* genes during zebrafish development using whole-mount *in situ* hybridization. Our study unveils new expression domains in the developing embryo, confirms those previously known, and shows that *ntf* and *ntrk* genes have different degrees of cell- and tissue-type specificity. The unique and overlapping expression patterns here depicted indicate the coordination of the redundant and divergent functions of NTFs and represent valuable tools for deciphering the molecular pathways involved in the specification and function of embryonic cell types.

Key words: neurotrophins, neurotrophin tyrosine kinase receptors, zebrafish, development, phylogeny; RRID:SCR_008439

1 INTRODUCTION

The discovery of the neurotrophin family, structurally similar to nerve growth factors, was a seminal event in the history of research in neurobiology (reviewed in Aloe & Calzà, 2004). At the cellular level, neurotrophins (NTFs) play fundamental roles in several aspects of developing and mature neural phenotypes, including proliferation, survival and synaptic plasticity in both the central and peripheral nervous systems (Chao, 2003; Antal et al., 2010). Because NTFs are thought to be involved in activity-dependent synaptic plasticity, there is great interest in their role in higher-order activities such as learning and memory (Yamada & Nabeshima, 2003; Choi et al., 2010). There is growing evidence that reduced neurotrophic support is a significant factor in the pathogenesis of neurodegenerative diseases (Dawbarn & Allen, 2003; Arancio & Chao, 2007; Frade & Lopez-Sanchez, 2010; Laske et al., 2011; Vilar & Mira, 2016). An implication of NTFs in pathogenesis and treatment of neurological and psychiatric disorders has been proposed (Nagahara & Tuszynski, 2011). Besides their action on neurons, new functions have been assigned to NTFs in several non-neural tissues including heart, lung, muscle, pancreas, adipose tissue, liver, vascular and the haemopoietic-immune system, providing insights into the way NTFs act in the embryo and in the adult (Oberg-Welsh & Welsh 1996; Miralles, Philippe, Czernichow, & Scharfmann, 1998; Teitelman, Guz, Ivkovic, & Ehrlich, 1998; Larrieta, Vital, Mendoza-Rodríguez, Cerbon, & Hiriart, 2006; Durand et al. 2007; Meloni et al., 2010). In particular, several data support the hypothesis that the immune/inflammatory system modulates NTFs through multiple mechanisms and that NTF expression may be involved in the differentiation of lymphoid and myeloid cell types (Calabrese et al., 2014; Minnone, De Benedetti, & Bracci-Laudiero, 2017).

Mature NTF proteins exist as non-covalently associated dimeric polypeptides that are first synthetized as precursors and then cleaved to biologically active proteins (Chao & Botwell, 2002). ProNTFs and mature NTFs can serve both autocrine and paracrine functions. NTFs undergo anterograde trafficking from the cell bodies through long axons (Baquet, Gorski, & Jones, 2004) or, alternatively, target-derived factors may be transported retrogradely to distant cell bodies (Harrington & Ginty, 2013; Ito & Enomoto, 2016). NTFs mediate their action on responsive cells by binding to different receptors: all bind, both as proNTFs and

as mature NTFs, to a common receptor p75, a member of the tumor necrosis factor receptor superfamily, and each mature protein also binds to one or more of the members belonging to neurotrophin tyrosine kinase receptors (Ntrk). NTF/Ntrk binding then exerts its functions by connecting a variety of intracellular signaling cascades, which include MAPK/ERK, PI-3/AKT kinase, and PLCγ pathways (Nakagawara, 2001; Reichardt, 2006; Chalampopoulos et al., 2012).

Important achievements have been made in the understanding of NTF and NTF receptor evolutionary history: a functional NTF/Ntrk system was already present in bilaterian ancestor, predating the split between protostomes and deuterostomes, and secondarily lost in some clades like nematodes, insects and tunicates (Jaaro et al., 2001; Benito-Gutiérrez et al., 2005; Benito-Gutiérrez, Garcia-Fernàndez, & Comella, 2006; Bothwell, 2006; Burke et al., 2006; Lapraz et al., 2006; Wilson, 2009; Lauri, Bertucci, & Arendt, 2016). The presence of one *Ntf* and one *Ntrk* gene in protostomes and in non-vertebrate chordates suggests that much of the complexity of the NTF/Ntrk system has evolved in the course of vertebrate radiation (Hallböök, Lundin, & Kullander, 1998), likely as a result of whole-genome duplications (Ohno, 1993; Dehal & Boore, 2005). The gnathostome genome typically include the nerve growth factors, *NGF*, *BDNF*, *Ntf3,* and *Ntf5*, and the three *Ntrk* receptors, *NtrkA*, *NtrkB*, and *NtrkC* (Cohen, Levi-Montalcini, & Hamburger, 1954; Barde, Edgar, & Thoenen, 1982; Hohn, Leibrock, Bailey, & Barde, 1990; Berkemeier et al., 1991; Benito-Gutiérrez, Garcia-Fernàndez and Comella, 2006; Sossin, 2006), while birds have lost *Ntf5* (Hallböök, Wilson, Thorndyke, & Olinski, 2006). The additional whole-genome duplication which occurred in the common ancestor of teleosts (teleost-specific whole genome duplication, TSGD) (Taylor, Van de Peer, Braasch, & Meyer, 2001; Taylor, Braasch, Frickey, Meyer, & Van de Peer, 2003), was followed by lineage-specific gene losses, thus resulting in the presence of an additional fifth *ntf* family member called *ntf6* or *ntf7*, together with five Ntrk receptor coding genes, *ntrk1*, *ntrk2a*, *ntrk2b*, *ntrk3a* and *ntrk3b* (Heinrich & Lum, 2000).

The zebrafish embryo is an attractive model for developmental biology and genetics of vertebrates for its conserved anatomy in terms of tissue formation and organization, including neuroectodermal derivatives such as brain and spinal cord. Despite zebrafish has undergone evolutionary changes, yet it is likely still bearing traits of the early expansion of the neurotrophin signaling system in vertebrates, thus representing a useful

model for functional approaches and phenotypic characterization. In zebrafish, expression patterns of *bdnf*, *ntrk2b*, *ntrk3a* and *ntrk3b* were previously reported, while those of *ntf4/5* and *ntf6/7* are still unknown (Martin, Marazzi, Sandell, & Heinrich, 1995; Martin, Sandell, & Heinrich, 1998; Williams et al., 2000; Lum, Huynh, & Heinrich, 2001; Thisse & Thisse, 2004, 2005; Knaut et al., 2005; Thisse, Wright, & Thisse, 2008; Tanaka et al., 2011; Pan et al., 2012; Morimura, Nozawa, Tanaka, & Ohshima, 2013; Palanca et al., 2013; De Felice et al., 2014; Dyer, Linker, Graham, & Knight, 2014; Auer et al., 2015). The current study was thus finalized at adding further insights into transcriptional activity of the whole complement of *ntf* and *ntrk* genes during zebrafish embryogenesis. The interpretation of our data is based on the anatomical and topographical distribution of ligand and receptor transcripts by whole-mount *in situ* hybridization method from 12 to 96 hours post fertilization (hpf). Furthermore, increasing availability of key fish genomes opens novel perspectives on the yet obscure evolution of *ntrk* genes. Here, we shed light on the phylogeny of neurotrophin tyrosine kinase receptors, which was deeply influenced by genome duplications and gene losses. Our approach, together with literature and genome browser data, prompted us to use a standardized nomenclature to indicate the genes of the NTF/Ntrk system.

2 MATERIAL AND METHODS

2.1 Ntrk *phylogenetic analysis*

Ntrk predicted amino acid sequences used in our evolutionary reconstruction were retrieved from public databases and aligned using Clustal Omega (Sievers et al., 2012). We included Ntrk proteins from selected species based on their key phylogenetic position and genome features. Ntrk sequences from *Homo sapiens* (Hs_NTRK1: NP_001012331; Hs_NTRK2: ENSP00000365386; Hs_NTRK3: ENSP00000377990) and *Danio rerio* (Dr_Ntrk1: NP_001288285; Dr_Ntrk2a: NP_003199193_2, Dr_Ntrk2b: XP_001184090; Dr_Ntrk3a: XP_005163050; Dr_Ntrk3b: XP_002662799 – here manually implemented) were retrieved from NCBI and Ensembl. The set of proteins was enriched by the search of novel sequences from the West Indian Ocean coelacanth, *Latimeria chalumnae* (Lc_Ntrk1: ENSLACP00000017880; Lc_Ntrk2: ENSLACP00000003030), the Australian ghost shark,

Callorhinchus milii (Cm_Ntrk2: XP_007904999; Cm_Ntrk3: XP_007908653), and the spotted gar, *Lepisosteus oculatus* (Lo_Ntrk1: XP_006643007; Lo_Ntrk2: ENSLOCP00000002703; Lo_Ntrk3: ENSLOCP00000017879). Phylogenetic trees were inferred using MrBayes 3.1.2 generated with default parameters: two independent runs of 1,000,000 generations were performed, each with 4 chains. The single amphioxus Ntrk receptor *Branchiostoma floridae* (AAX94285), representative of the invertebrates' pre-duplicative gene, was used as outgroup (D'Aniello et al., 2008).

2.2 *Embryos*

Adult zebrafish (*Danio rerio*) were maintained according to standard procedures on a 14 h light/10 h dark lighting cycle at 28.5°C as previously described (Westerfield, 2000). Embryos were obtained by natural spawning, and were staged according to hours and days post-fertilization (hpf and dpf, respectively), and to morphological criteria (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). At 22 hpf, the embryo medium was supplemented with 0.003% (v/v) phenylthiourea to inhibit melanin synthesis.

2.3 *Whole-mount in situ hybridization and immunohistochemistry*

For each gene (*ngf, ntf3*, *ntf4/5*, *ntf6/7*, *ntrk1*, *ntrk2a*, *ntrk2b*, *ntrk3a*, and *ntrk3b*) we generated digoxigenin-labeled sense and antisense riboprobes, ranging in length from 267 to 1117 nucleotides, to enable us to gain a view on the expression of nearly all alternative transcripts (Supporting Fig. 1, Supporting Tab. 1). Single and double whole-mount *in situ* hybridization (WISH) analyses were performed as previously described (Westerfield, 2000). Embryos were post-fixed in 4% paraformaldehyde (PFA) in 1X phosphate-buffered saline (PBS) for 20 min and finally stored in 95% glycerol at 4°C until use. Embryos were imaged using a Zeiss Axio Imager M1 microscope equipped with Axiocam digital camera (Zeiss). WISH experiments were conducted in triplicate with consistent results. No signal was observed with the sense riboprobes, indicating the specificity of the antisense riboprobes generated (data not shown). For *ntf3*, after the *in situ* hybridization experiments, 72 hpf embryos were embedded in a mix of BSA and gelatin and sectioned with vibratome at a thickness of 20 µm.

Double-fluorescent WISH was performed following the protocol of Brend and Holley, 2009. Embryos were fixed overnight at 4°C using 4% PFA in PBS, then washed with PBS,

transferred through a series of 25%, 50%, 75% methanol in PBS and incubated in 100% methanol at -20°C. Embryos were rehydrated in 75%, 50%, 25% methanol in PBS-Tween20 (PBST) at RT, washed in PBST, fixed again for 20 min in 4% PFA in PBS at RT and washed 2 times in PBST. Prim-5 stage embryos were permeabilized by treatment with proteinase K (10 µg/ml in PBST) at RT for 5 min. After brief washes in PBST to stop the reaction, embryos were fixed again for 20 min in 4% PFA in PBS at RT. Then they were incubated for 5 min at 65°C in HYB^- buffer (50% deionized formamide, 5x SSC, 0.1% Tween20) and prehybridized at 65°C for at least 1 hour in HYB^+ (Hyb^-, 500 µg/ml yeast tRNA, 50 µg/ml heparin). 1-2 µl of each riboprobe for 100 µl Hyb^+ (digoxygenin- and fluorescein-labeled riboprobes) were added to the embryos and incubated overnight at 65°C. After riboprobe incubation, embryos were washed 3 x 30 min at 65°C in 50% formamide/2x SSC, 3 x 15 minutes at 65°C in 2 x SSC, and 2 x 30 minutes at 65°C in 0.2x SSC. The embryos were blocked for at least 1 hour at RT in 500 µl of a solution of 1x maleic acid buffer plus 2% blocking reagent (Roche). The anti-Fluorescein-POD antibody (Roche), was added at a 1:400 dilution in the blocking solution and incubated overnight at 4°C. After incubation, we washed 4 x 20 minutes in 1x maleic acid buffer and twice for 5 minutes each in PBS, and then incubated 60 minutes in Perkin Elmer amplification diluent buffer (TSA™ Plus Cyanine 3 & Cyanine 5 System Perkin Elmer). For the reaction, we diluted AlexaFluor647 reagent (TSA™ Reagent, Alexa Fluor® 647 Tyramide, Life Technologies) 1:100 in Perkin Elmer amplification diluent buffer. After, we washed for 10 minutes each in 25%, 50%, 75% and 100% methanol in PBS, incubated in a solution of 1% H_20_2 in methanol for 30 minutes to inactivate endogenous peroxidases, and washed again 10 minutes each in 75%, 50%, 25% methanol in PBS, and 3 x 10 minutes in PBS. Embryos were blocked again for at least 1 hour at RT in a solution of 1x maleic acid buffer plus 2% blocking reagent. The anti-DIG POD antibody (Roche) was added at a 1:400 dilution in above blocking solution and embryos were incubated overnight at 4°C. Embryos were washed 4 x 20 minutes in 1x maleic acid buffer, 4 x 10 minutes in PBS and incubated 60 minutes in Perkin Elmer amplification diluent buffer. For the reaction, we diluted Cy3 tyramide reagent 1:100 in amplification buffer, after we washed three times for 10 minutes each and then overnight in PBST. Finally, the embryos were incubated for 10 minutes in 25% and 50% glycerol in PBST and cleared overnight in 75% glycerol at 4°C. Whole-mount fluorescent zebrafish preparations mounted on slides were imaged on a Zeiss LSM

700 laser scanning confocal microscope with 10x or 20x objective with 1x zoom. Excitation and emission wavelengths were set according to the fluorophore used. Pictures were edited with Adobe Photoshop CS4.

3 RESULTS

3.1 *Ntrk phylogenetic reconstruction*

The evolutionary history of NTFs in vertebrates is substantially well defined, due to the presence of a single representative of each NTF family member in all species considered. On the contrary, the evolutionary outline of Ntrk receptors is made more complex by gene redundancy (Hallböök et al., 2006; Sossin, 2006; Lanave, Colangelo, Saccone, & Alberghina, 2007; D'Aniello et al., 2008; Tettamanti et al., 2010). Literature data suggest that the unique invertebrate *Ntrk* represents the pre-duplicative ancestor of the three duplicates found in vertebrates. Therefore, we performed a phylogenetic reconstruction to better define the evolutionary history of Ntrk receptors (Fig. 1). In particular, the phylogenetic relationship between the duplicated *Ntrk2* and *Ntrk3* genes of sarcopterygian lineage (comprising human) and actinopterygians (two ray-finned fish) remains obscure. For this purpose, in the analysis we included the spotted gar *Lepisosteus oculatus* as representative of unduplicated Actinopterygii, which has a single *ntrk* gene for each clade, like tetrapods as human. We also included the coelacanth *Latimeria chalumnae*, and the ghost shark, *Callorhinchus milii*, that possess two *ntrk* genes, according to current available genomic data (Venkatesh et al., 2014). Our analysis points to the three genes present in vertebrates (*Ntrk1*, *Ntrk2*, *Ntrk3*) as descendants of a unique invertebrate ancestor, as a result of the WGDs occurred at the stem of vertebrates (Ohno, 1993; Dehal and Boore, 2005). Moreover, it is parsimonious that teleost *ntrk2a-ntrk2b* and *ntrk3a-ntrk3b* genes are couples of ohnologs arisen by TSGD (Taylor, Van de Peer, Braasch, & Meyer, 2001; Taylor, Braasch, Frickey, Meyer, & Van de Peer, 2003), while a single copy gene of *Ntrk1* in all surveyed gnathostome species would suggest that a second *Ntrk1* paralogue has been lost across evolution. Noteworthy, *ntrk1* and *ntrk3* resulted lost in *C. milii* and *L. chalumnae*, respectively (Fig. 1). It would be important in future to understand the molecular implications of the modification of NTF family in fishes according to their

specific ecological niche.

3.2 *Nomenclature*

There is currently considerable confusion concerning the acronyms indicating neurotrophins and their tyrosine kinase receptors. Whereas BDNF and NGF are known with these names in all vertebrates, other neurotrophin pathway genes are indicated with multiple acronyms in different species. The fact that different NTF names are used in distinct species is misleading for evolutionary and comparative studies that, for instance, aim to find similarity or novelties using a cross-lineage approach. The absence of a univocal nomenclature used by different animal models in research communities is particularly evident in the case of human or mouse in respect to teleost fishes. Phylogenetic reconstructions assessed with limited dataset, or gene name attributions incoherent with the orthology principle, may hinder direct transfer of morphological and functional information from teleost fishes to human. To mention some examples, one gene is named *ntf4* in zebrafish (Auer et al., 2015), *NT-4/5* in mice (English, Cucoranu, Mulligan, Rodriguez, & Sabatier, 2011), *NT4* in rat (Proenca, Song, & Lee, 2016) and *NT5* in human (Berkemeier, Ozçelik, Francke, & Rosenthal, 1992). Therefore, hereon we will indicate this gene as *Ntf4/5* in mammals and *ntf4/5* in fish. Similarly, *NT-6* in platyfish (Gotz et al., 1994) is named *NT-7* in zebrafish (Nilsson, Fainzilber, Falck, & Ibanez, 1998), and hereon we name it *ntf6/7*. Of note, genome databases (*e.g.* MGI, NCBI, HGNC) have recently (November 2017) approved Ntrk1, Ntrk2 and Ntrk3 as new symbols for mammalian TrkA, TrkB and TrkC proteins. Here we refer to the zebrafish genes *bdnf*, *ngf*, *ntf3*, *ntf4/5*, *ntf6/7*, *ntrk1*, *ntrk2a*, *ntrk2b*, *ntrk3a* and *ntrk3b*.

3.3 *Expression patterns*

3.3.1 Ligands

3.3.1.1 *ngf*: As reported previously in the context of a large scale whole-mount *in situ* hybridization screening, zebrafish *ngf* is expressed in the epidermis surrounding anteriorly the neural plate during early somitogenesis (12 hpf) (Thisse & Thisse, 2004; 2005). Most optic tectum cells appeared to be labeled strongly at 24 hpf (Fig. 2a,a'). Positive cells were also observed in the ventral margin of the otic vesicle, in a small cluster of head mesenchymal cells within the pharyngeal arches, in lateral line primordium and

posteriormost somites (Fig. 2a,b). At 36 hpf, *ngf* expression ceased in the optic tectum and caudal somites, and became intense in pharyngeal arches, neurons within the trigeminal ganglion and posterior pharynx (Fig. 2c,d). At 48 hpf, expression persisted in the lower jaw structures, likely including the ventral oral epithelium and paired ceratobranchials, and in the posterior pharynx (Fig. 2e,f). No expression was observed at later stages (data not shown).

3.3.1.2 *ntf3*: The transcriptional anatomy of *ntf3* during zebrafish development is very peculiar in terms of tissue specificity when compared with the other neurotrophin genes. Like *ngf*, *ntf3* expression was activated at the early somitogenesis stage (12 hpf) in the epidermis along the neural plate border, in cardiac progenitor cells and in the lateral line primordium (Fig. 3a). At 22 hpf, *ntf3* positive cells were observed in the linear heart tube, otic vesicle, lateral line primordium, pancreas and posterior somites (Fig. 3b,c). Williams and co-authors (2000) showed that *ntf3* is important for Rohon-Beard (RB) neuron development in zebrafish. During our experiments, no sign of *ntf3* expression was detected in the spinal cord (Fig. 3b,c). At 48 hpf, cells expressing *ntf3* were present in the central retina, pituitary gland and pancreas (Fig. 3d,e,g). No more expression was detected in lateral line and inner ear, while the cardiac signal remained ubiquitous (Fig. 3d,e). At 72 hpf, discrete bilateral groups of neuronal cell types expressing *ntf3* were observed in the brain and cranial nerve region (Fig. 3f). Expression in the retina had spread from central to peripheral photoreceptors (Fig. 3g-j) and to the inner nuclear layer in the temporal retina (Fig. 3h,k). No expression was seen at later stages (data not shown).

3.3.1.3 *ntf4/5*: The only information available in literature concerning the embryonic expression in zebrafish of this neurotrophin coding gene consists on transcriptional levels measured by RT-qPCR at 4 dpf (Auer et al., 2015). In our investigations, the first expression of *ntf4/5* was found in the posterior epiblast during early somitogenesis (12 hpf) (Fig. 4a-c). At 24 hpf, mRNA staining occurred in cranial nerves, lateral line primordium and in the median finfold surrounding the tail (Fig. 4d,e). At 48 hpf, positive cells formed columns that were arranged around the pharyngeal arch region (Fig. 4f,g). This signal was

confined to the anterior most group of pharyngeal cells at 72 hpf (Fig. 4h). Finally, a strong signal labeled the inner wall of the intestine bulb at 96 hpf (Fig. 4i,j).

3.3.1.4 *ntf6/7*: The fish lineage-specific neurotrophin *ntf6/7* was originally identified in the platyfish, *Xiphophorus maculatum* (Gotz et al., 1994). Based on our knowledge of the literature, no whole-mount expression study of *ntf6/7* has so far been conducted in any fish species. Its developmental expression in zebrafish is unique because it was restricted to the otic vesicle (OV). Early transcript was detected at 16 hpf in two clusters of cells adjacent to the anterior and posterior of the inner ear primordium (16 hpf) (Fig. 4k,l). As development proceeds, *ntf6/7* expression was extended in 3-4 small sensory OV patches at 24 and 48 hpf (Fig. 4m-o). Expression was lost at later stages (data not shown).

3.3.2 Receptors

3.3.2.1 *ntrk1*: In spite of teleost-specific third whole genome duplication (Taylor, Van de Peer, Braasch, & Meyer, 2001; Taylor, Braasch, Frickey, Meyer, & Van de Peer, 2003), the zebrafish genome contains only one *ntrk1* gene, which could be explained by a specific gene loss (Heinrich & Lum, 2000). At 24 hpf, the zebrafish *ntrk1* was expressed in two domains of cranial nerve ganglia flanking the hindbrain: a large and a small group anterior and posterior to the otic vesicle, respectively (Fig. 5a,b). The *ntrk1* transcript was detected in RB neurons localized in the dorsal aspect of the spinal cord (Fig. 5c). At 48 and 72 hpf, *ntrk1*-positive cells were similarly distributed as they were seen in two columns projecting toward the eye in the ventral head mesenchyme, and in trigeminal sensory neuron subtypes (Knaut, Blader, Strähle, & Schier, 2005; Tanaka et al., 2011; Pan, Choy, Prober, & Schier, 2012; Palanca et al., 2013) (Fig. 5d-f). Cells expressing *ntrk1* were found at the base of the pectoral fins, suggesting a correspondence to pectoral motor nerve ganglia (Fig. 5d-f). No spinal cord labeling was detected at this stage (data not shown). At 6 dpf, *ntrk1* was expressed in the trigeminal ganglion and in the rostral hindbrain (Martin, Marazzi, Sandell, & Heinrich, 1995).

3.3.2.2 *ntrk2a*: The expression of the *ntrk2a* gene in the trigeminal ganglia and RB neurons has been previously described (Martin, Marazzi, Sandell, & Heinrich, 1995; Palanca et al., 2013). Here, we observed hypoblast labeling throughout the embryo at

early somitogenesis stage (12 hpf) (Fig. 6a). At 24 hpf, *ntrk2a* transcription occurred in discrete groups of neuronal cell types in the posterior telencephalon, hypothalamus, ventral thalamus, ventral tegmentum, ventral hindbrain, cranial ganglia and spinal cord (Fig. 6b-d). Cells expressing *ntrk2a* in the spinal cord are RB neurons localized dorsally, as well as ventral cells that may represent motoneurons or interneurons (Fig. 6d). At 48 hpf, *ntrk2a* mRNA marked the brain, with new signals appearing in trigeminal ganglion and ganglion cell layer (GCL) (Fig. 6e,f). The cerebral signal was still diffuse at the stage of 96 hpf, when strong staining was found in the pharyngeal epithelium (Fig. 6g).

3.3.2.3 *ntrk2b*: At 12 hpf (6 somite stage), *ntrk2b* mRNA was evenly localized in the anterior half of the embryo (Fig. 6h). In agreement with earlier findings (Thisse, Wright, & Thisse, 2008), *ntrk2b* was expressed in posterior telencephalon, ventrorostral thalamus, ventral tegmentum and ventral hindbrain at 24 hpf (Fig. 6i,j). Hybridization staining was spread in the brain at 48 hpf, when a new expression domain appeared in the ganglion cell layer (Fig. 7k,l). The cerebral signal was still diffuse at 96 hpf (Fig. 6m).

3.3.2.4 *ntrk3a*: The available evidence with regard to the expression of *ntrk3a* in the brain of 24 hpf embryo included several subsets of neuroanatomical domains (Martin, Sandell, & Heinrich, 1998; Pan, Choy, Prober, & Schier, 2012; Morimura, Nozawa, Tanaka, & Ohshima, 2013; Dyer, Linker, Graham, & Knight, 2014; Auer et al., 2015). In particular, groups of *ntrk3a* mRNA-positive cells were found in the telencephalon, pituitary gland, posterior hypothalamus, ventrorostral thalamus, posterior hindbrain, cranial ganglia, otic vesicle and RB neurons (Fig. 7a-c). Contrary to previous evidence, *ntrk3a* was undetectable in the trigeminal ganglia (Pan, Choy, Prober, & Schier, 2012). With some exceptions, *ntrk3a* mRNA pattern is very similar with that of *ntrk2a* (compare Fig. 6b & Fig. 7a), and even more with that of *bdnf* (De Felice et al., 2014). At later developmental stages, *ntrk3a* expression occurred in pectoral motor nerve ganglia (48 hpf) and diffusely in the brain (48 to 96 hpf) (Fig. 7d-f).

3.3.2.5 *ntrk3b*: At 24 hpf, the expression of the *ntrk3b* gene was confined to small groups of neural cell types in the telencephalon, ventral thalamus, ventral tegmentum and anterior margin of the otic vesicle (Fig. 7g-i). This pattern was reminiscent of *ntrk2b* transcript

distribution at same stage (compare Fig. 6i & Fig. 7g), raising the possibility of functional redundancy between *ntrk2b* and *ntrk3b*. At 48 hpf, *ntrk3b* expression was switched on in the olfactory placode, GCL and hindbrain, while it persisted in diencephalon and midbrain (Fig. 7j,k). Finally, *ntrk3b* transcription was detected across the brain at 96 hpf (Fig. 7l). Both *ntrk3a* and *ntrk3b* were expressed in the GCL at 6 dpf (Auer et al., 2015).

3.3.2.6 *bdnf and ntrk2a co-localization*: Based on the expression patterns of zebrafish *ntf* and *ntrk* genes, ligands and receptors could be co-expressed in several subregions of the neural and sensory systems during ìbrain development. In support of this hypothesis, our data indicate that the expression pattern of *ntrk2a* was very similar to that of *bdnf* (compare Fig. 6b and Fig. 2b in De Felice et al., 2014). We thus performed double *in situ* hybridizations of *bdnf* and its specialized *ntrk2a* receptor at 24 hpf. As expected, fluorescent imaging of transcripts showed co-labeling in cranial nerves but not in RB neurons (Fig. 8).

4 DISCUSSION

4.1 *Phylogenetic reconstruction*

Our phylogeny reveals that, differently from NTFs, Ntrk receptors exhibit a complex evolutionary history. Genome duplication events expanded the gene complement in vertebrates, giving rise to three receptor genes (*Ntrk1*, *Ntrk2*, *Ntrk3*) in tetrapods and five in teleosts (*ntrk1*, *ntrk2a*, *ntrk2b*, *ntrk3a*, *ntrk3b*). Independent gene losses have sculpted the modern scenario in which gnathostomes lack a fourth *Ntrk* member and teleosts lost a *ntrk1* duplicate. We have registered other cases of *ntrk* gene loss in holocephalans and early-branching actinopterygians. This phenomenon could be correlated with gene subfunctionalization or, more intriguingly, with evolutionary innovations (Albalat & Cañestro, 2016).

4.2 *Expression patterns*

We used the zebrafish to provide the first comprehensive examination of the expression profiles of neurotrophins and neurotrophin tyrosine kinase receptors in vertebrates (Fig. 9,

Supporting Table 2). Previous findings highlighted the presence of five *ntf* and five *ntrk* receptor genes in the zebrafish genome, but offered incomplete understanding of the transcriptional anatomy during early development. In addition, *ntf4/5* and *ntf6/7* were hitherto not described in terms of expression domains in fish embryo development. Our global survey shows that nearly all patterns of *ntf* and *ntrk* genes exhibit restricted mRNA domains and are expressed in unique combinations, reflecting qualitative differences between tissues and organs. Considering that NTFs have stereotypical cellular functions, their anatomical distribution in zebrafish reveals the physiological and cell biological state in the embryo during development.

4.2.1 *Expression of ntf and ntrk genes in neural and sensory systems of zebrafish embryos*

The results described here hint at *bdnf* as the only neurotrophin gene whose transcription is extensively associated with early neurogenesis in zebrafish. Based on ligand and receptor transcripts co-localization (Supporting Table 2), a potential role may be ascribed to zebrafish Bdnf protein in the development of neurons devoted to a variety of functions and behaviours (this work; Lagler, Bardach, & Miller, 1967; Eaton & Whittemore, 1996; De Felice et al., 2014). As suggested by comparative analysis of expression patterns, Bdnf could exert these various roles by activating its specialized receptor, Ntrk2, but also Ntrk3 (Fig. 9; Supporting Table 2). Similarly, Ngf could bind to both Ntrk1 and Ntrk2a, given its unique expression in optic tectum and trigeminal cell bodies, lending support to a neurotrophic role in the development of neural circuits involved in visually guided behaviour and in sensory coding of environmental stimuli (Robles, Smith, & Baier, 2011; reviewed in Pan, Choy, Prober, & Schier, 2012).

One of the most interesting observations we have made is that, differently from tetrapods, *bdnf* is the only neurotrophin gene expressed in the telencephalon of the zebrafish embryo (Fig. 9, Supporting Table 2). In the classical view of telencephalic evolution, the dorsal pallium of fish has acutely expanded, becoming the isocortex of mammals. This enlargement has paralleled the acquisition of increased complexity in learning and behaviour processes in man (Rowe, Macrini, & Luo, 2011; Mueller, 2012). It is tempting to speculate that expansion of the neurotrophin signaling system, with the activation of *ngf*, *ntf3* and *ntf4/5* expression in the isocortex, was one of the enabling conditions in the

evolution of complex brains (Lauterborne, Isackson, & Gall, 1994; Brunstrom, Gray-Swain, Osborne, & Pearlman, 1997; Jaaro, Beck, Conticello, & Fainzilber, 2001; Jaaro & Fainzilber, 2006). In this view, the abundance of nerve growth factors in integrative centers could have represented the basis for giving rise to higher cognitive functions.

Previous studies indicated that the NTF pathway is highly implicated in the development of neuronal and sensory cell types in the peripheral nervous system. The presence of *ntrk* (*ntrk1*, *ntrk2a*, *ntrk3a*) gene transcripts in RB neurons, in the absence of NTF gene expression (Fig. 9, Supporting Table 2), could reflect a long-range anterograde NTF axonal transport (Martin, Sandell, & Heinrich, 1998; Williams et al., 2000; Knaut et al., 2005; Pan, Choy, Prober, & Schier, 2012; Tanaka et al., 2011; Morimura, Nozawa, Tanaka, & Ohshima, 2013; Palanca et al., 2013; Dyer, Linker, Graham, & Knight, 2014; Auer et al., 2015). We cannot exclude that Ntrk proteins in the spinal cord of the zebrafish embryo are transactivated by alternate ligands without involvement of neurotrophins, *e.g.* via a mechanism based on G-protein-coupled receptor ligands (Daub, Weiss, Wallasch, & Ullrich, 1999; Lee et al., 2001; Rajagopal, Chen, Lee, & Chao, 2004; Kim et al., 2016). Long-distance or alternative signaling mechanisms may also support *ntrk3a* and *ntf3* expression in pineal and pituitary glands, respectively.

The auditory system of vertebrates is composed of the inner ear and the mechanosensory lateral line system (Gasanov, Rafieva, & Korzh, 2015). Intriguingly, transcripts of three neurotrophins (*bdnf*, *ngf*, *ntf6/7*) and two receptors (*ntrk3a*, *ntrk3b*) were detected in the otic vesicle (Fig. 9, Supporting Table 2), an anatomical structure that develops into the auditory (hearing) and vestibular (balance) organ of the fish, equivalent to the inner ear of amniotes. In addition, we found that *ntf3* and *ntf4/5*, like *bdnf* and *ngf* (Germanà et al., 2010; De Felice et al., 2014), are expressed in the lateral line primordium. Collectively, our results highlight the potential role of the NTF pathway in the development of the otic vesicle, and indicate the existence of a complex neurotrophin mechanism involved in migration and maintenance of mechanosensory progenitors (Grave et al., 2013; Gasanov, Rafieva, & Korzh, 2015).

The role of BDNF and NTF3 in retinal neurorestoration, neuroprotection and oxidative stress has been extensively tested over the past two decades. Responsiveness of the ganglion cell layer to neurotrophins coincides with terminal arborisation and patterning of retinal axons (Cohen-Cory & Fraser, 1994; Logan, Ahmed, Baird, Gonzalez, & Berry,

2006; Hackam, 2008; Liu et al., 2009; Santos et al., 2011). Based on results from a previous study, zebrafish Ntf3 controls axon branching in the retinotectal system (Auer et al., 2015). In our work, NTF role in the developing retina of zebrafish appears to be genetically conserved with reference to mammalian retina, with the transcriptional activation of *bdnf*, *ntrk2a*, *ntrk2b* and *ntrk3b* in the ganglion cell layer, and of *ntf3* in the photoreceptor cell layer (this work; Hallböök, Bäckström, Kullander, Ebendal, & Carri, 1996; De Felice et al., 2014; Garcia, Hollborn, & Bringmann, 2017). During retina neurogenesis, the expression of *bdnf* and *ntf3* expands in opposing directions and coincides in the inner nuclear layer (this work; De Felice et al., 2014). Concerted expression of these two zebrafish NTFs may play an important role in the differentiation and maintenance of multiple retinal cell types. In addition, it evokes that Bdnf and Ntf3 anterograde signaling in ganglion cell layer is mediated by Ntrk receptors, and it is temporally regulated (Butowt & von Bartheld, 2005). Also, late activation of *bdnf* and *ntf3* genes in the retina argues against a role in cell proliferation.

4.2.2 *Non-neuronal roles for zebrafish neurotrophins*

In the present study, the spatial expression of *ntf* and *ntrk* genes supports previous findings in zebrafish that imply differential functions in brain development. It also provides additional information on non-neural activities about which little is known (Tessarollo, 1998). NTFs have important functions in the development and physiology of mesoderm and endoderm, with particular combinations of NTFs and Ntrk receptors that these cells express.

BDNF and NTF3 participate in cardiac development regulating embryonic cardiomyocyte proliferation and intramyocardial vessel stabilization in mammals (Donovan, Hahn, Tessarollo, & Hempstead, 1996; Donovan et al., 2000; Tessarollo et al., 1997; Lin et al., 2000; Feng et al., 2015), a function that is mediated by the expression of truncated Ntrk2 receptor (Fulgenzi et al., 2015). We have previously shown that zebrafish *bdnf* is transiently expressed in cardiac cell progenitors (De Felice et al., 2014). Here, *ntf3* mRNA was seen across the whole zebrafish heart field at 24 hpf and 48 hpf. Conversely, no *ntrk* gene expression was switched on in the heart during zebrafish embryogenesis (this work; Hannestad et al., 2000).

The pancreas is a complex organ formed by endocrine islet cells that secrete insulin and glucagon, exocrine cells that secrete digestive enzymes, and a ductal network. In addition, while intrapancreatic blood vessels assure hormone circulation, a pancreas-specific nervous system consisting of ganglia and nerves participate in the regulation of insulin secretion and blood glucose homeostasis (Salvioli et al., 2002; Houtz, Borden, Ceasrine, Minichiello, & Kuruvilla, 2016). In mammals, the classical neurotrophin NGF regulates ß cell survival and function, and there is evidence that altered NTF secretion could contribute to diabetes (Kim et al., 2009). The present study shows that, in zebrafish, the only *ntf* gene to be robustly expressed in pancreatic endocrine cells is *ntf3*, whereas its mammalian ortholog is weakly expressed in the pancreatic islet cells (Ohta et al., 1997; Miralles, Philippe, Czernichow, & Scharfmann, 1998). Besides no evidence of *ntrk* receptor expression in the glandular organ has been collected so far in zebrafish, our findings are consistent with an important role in the development of the pancreas for Ntf3.

In mammalian digestive system, NTF4/5 is thought to be required for proper intestinal sensory innervation (Fox et al., 2001), a role that seems to be conserved in teleosts, as suggested by zebrafish *ntf4/5* expression in the intestinal bulb of the midgut. Noteworthy, *ntrk* transcripts are undetected in gastrointestinal progenitor cells at any developmental stage under scrutiny. The absence of *ntrk* gene expression in non-neural tissues was already reported by Martin and coauthors (1995), who used radioactive riboprobe-based *in situ* hybridization of sections of 4 dpf zebrafish larvae. Evidence that *p75a* and *p75b* ohnologous genes are not transcribed in non-neural tissues of zebrafish embryos (Brösamle & Halpern 2009; Han et al., 2014) extends to mesoderm and endoderm anlagen the view that zebrafish neurotrophins function by activating Ntrk receptors via long-distance signaling or through different local receptors.

4.2.3 *Conclusions*

Within vertebrates, whole-genome duplications led to multiple gene copies of neurotrophins and neurotrophin tyrosine kinase receptors. This duplicative pattern allowed the evolution of novel functions and pleiotropic effects, most of which still unknown, produced by the different associations of NTFs and their ligands in the nervous system. Our work, aimed at defining the precise expression domains of each NTF family member during zebrafish development, is just framed in the perspective of gaining information

about the structure of molecular interactions, thus providing a canvas for targeted functional studies (Fig. 9). This complete developmental profiling indicates differential functions in different regions, offering novel routes for investigations into the biological role of each individual NTF as well as for the study of their combinatorial interaction with neurotrophin receptor tyrosine kinases.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors had full access to all the data in the present study and certify the integrity of the data and the accuracy of data analysis and interpretation. V.N., R.M.S., U.C., Y.D.A. E.D.F., A.P., Q.A.V., P.S. and S.D.A. led the experimental part and acquired the data. P.S. wrote the majority of the article, further completed by all authors; V.N., A.L., P.S., F.R. and S.D.A. led the figure and table preparation. All authors approved the article.

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FIGURE LEGENDS

Figure 1. Three Ntrk receptor classes in vertebrates. Phylogenetic reconstruction using predicted proteins of gnathostome Ntrk receptor family. MrBayes tree shows the presence of three distinct Trk classes in all vertebrates analysed (except cyclostomes): Ntrk1 in red, Ntrk2 in blue, Ntrk3 in purple, and single-copy Ntrk of *Branchiostoma floridae* used as outgroup in yellow. Numbers on nodes represent posterior probabilities for Bayesian Inference. The scale bar represents expected changes per site.

Figure 2. *ngf* mRNA localizes to optic tectum. Whole-mount RNA *in situ* hybridization of *ngf* from 24 to 48 hpf. Embryos are oriented with head toward the left in lateral views (a-c,e), and head toward the left in ventral views (d,f). (a-b) Cells expressing *ngf* in the optic tectum (ot), pharyngeal arches (ph), otic vesicle (ov), lateral line primordium (ll) and posterior somites (ps) at 24 hpf. (c,d) At 36 hpf, *ngf* transcript in pharyngeal arches, trigeminal ganglion (tg) and posterior pharynx (pp). (e,f) Expression at 48 hpf stays in lower jaw structures as paired cell clusters (arrowheads in f) and in the posterior pharynx. Scale bars: 100 μm (a,b-f) and 150 μm (a',a").

Figure 3. Expression of *ntf3* in pituitary gland, retina, heart and pancreas. Whole-mount RNA *in situ* hybridization of *ntf3* from 12 to 72 hpf. Embryos and dissected eyes are oriented with head toward the left in dorsal views (a,c), head toward the left in lateral views (b,d,f,k), head toward the left in ventral views (e), and head toward the top in dorsal views (g,h). Cross (i) and longitudinal (k) sections are shown to identify the *ntf3*-expressing cell layers of the retina. *ntf3* activation in the epidermis surrounding the anterior margin of the neural plate (black arrowheads in a), otic vesicle (ov) and lateral line primordium (12 hpf). (b,c) At 22 hpf, *ntf3* expression is switched on in heart (he), pancreas (pa) and posterior somites (ps). (d,e,g) *ntf3* mRNA in the central retina (cr) and pituitary gland (pg) at 48 hpf. (e') A lateral view of the pancreas at 48 hpf. (f) Small groups of neurons expressing *ntf3* in the brain (white arrowheads in f; eyes have been removed to make brain signals visible). (g,h) Expression in the retina expands from central to peripheral retina (g,h). Whole-mount staining in photoreceptors (pr) out of the plane of focus is indicated by asterisks (g,h). (i,j)

Magnification of a cross section shows expression in the nuclear region (nr) and the outer segment (os) of the photoreceptor cell layer. (k) A second domain of *ntf3* expression spreads to the inner nuclear layer (inl) in the temporal retina (dashed line in h; bracket in k). Abbreviations: gcl, ganglion cell layer; le, lens; di, diencephalon; rh, rhomboencephalon; tm, tegmentum. Scale bars: 100 μm (a-f), 50 μm (g-i) and 25 μm (j).

Figure 4. Expression of *ntf4/5* and *ntf6/7* is highly specific. Whole-mount RNA *in situ* hybridization of *ntf4/5* from 12 to 96 hpf (a-j), and of *ntf6/7* from 16 to 48 hpf (k-o). Embryos are oriented with head toward the left in lateral views (a,d-f,k,m-o), head toward the bottom in dorsal view (b), head toward the left in ventral views (g,h), and in frontal views (c,l). (a-c) *ntf4/5* transcript in the posterior epiblast (ep) at 12 hpf. (d,e) At 24 hpf, clusters of cranial nerves (arrowheads in d), lateral line primordium (ll) and posterior median finfold (mf) express *ntf4/5*. (f,g) Hybridization signal along pharyngeal arches (ph) at 48 hpf (h-j), persisting at its anterior margin (72 hpf). A new signal in the intestinal bulb (ib) at 96 hpf. (k-o) *ntf6/7* expression in the otic vesicle (ov) from 16 to 48 hpf. Abbreviations: hp, hypoblast. Scale bars: 100 μm (a-n) and 50 μm (o).

Figure 5. **Neural and sensory cell types expressing *ntrk1***. Whole-mount RNA in situ hybridization of *ntrk1* from 24 to 72 hpf. Embryos are oriented with head toward the left in lateral views (a,c,f) and head toward the left in dorsal views (b,d,e). (a,b) *ntrk1* mRNA in cranial neurons (arrow), pectoral motor nerve ganglia (arrowhead) and Rohon-Beard (rb) neurons at 24 hpf. (d-f) Expression in trigeminal (white arrowhead), cranial (arrow) and pectoral (black arrowhead) neurons at 48 and 72 hpf. Abbreviations: ov, otic vesicle. Scale bars: 100 μm.

Figure 6. Overlapping and unique expression of *ntrk2a* and *ntrk2b*. Whole-mount RNA *in situ* hybridization of *ntrk2a* (a-g), and *ntrk2b* (h-m) from 12 to 96 hpf. Embryos are oriented with head toward the left in lateral views (a,b,d,e,g-i,k,m) and head toward the left in dorsal views (c,f,j,l). (a) Early *ntrk2a* transcript is localized in the hypoblast along the embryo at 12 hpf. (b-d) At 24 hpf, mRNA labeling in neurons of telencephalon (te), thalamus (th), hypothalamus (hy), tegmentum (tm), hindbrain (hb), cranial nerves (arrowheads in c), Rohon-Beard neurons (rb) and ventral cells in the neural tube that may

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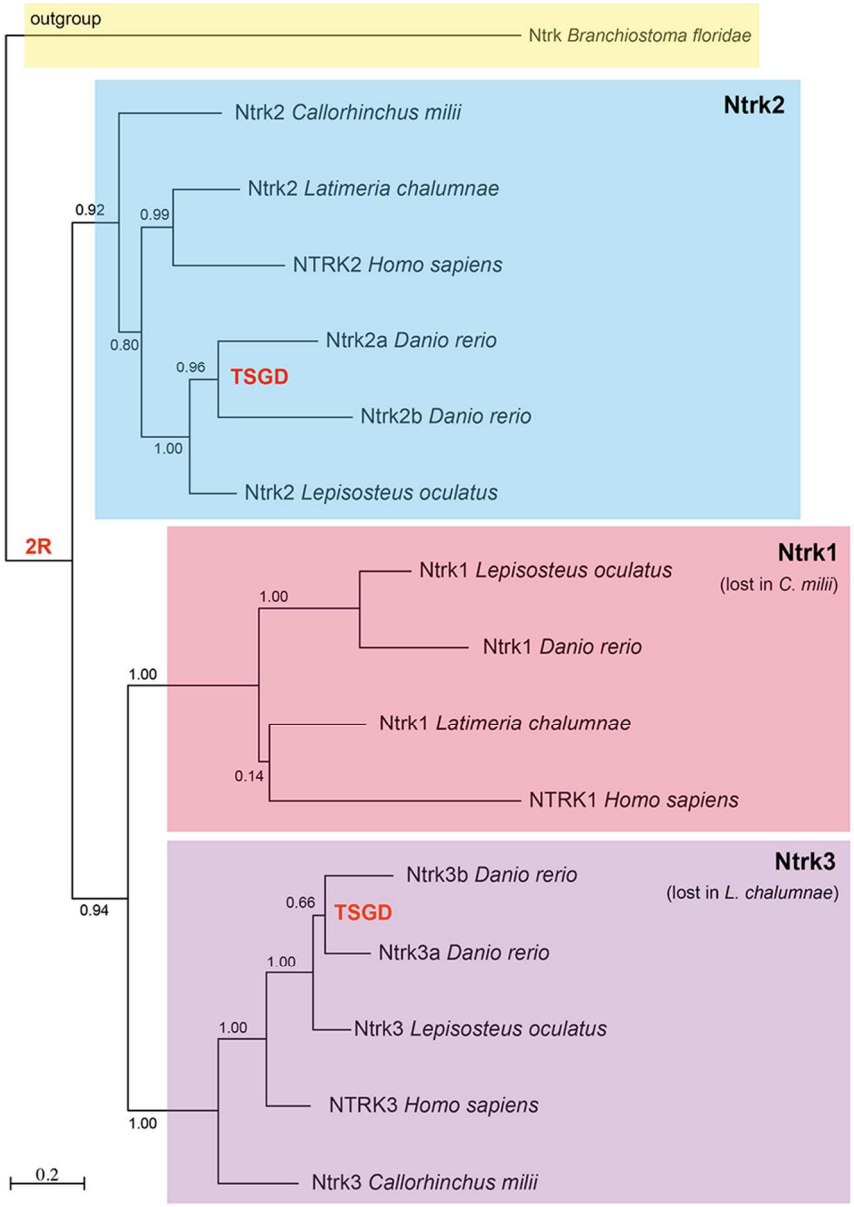
Figure 7. **Overlapping and unique expression of *ntrk3a* and *ntrk3b***. Whole-mount RNA *in situ* hybridization of *ntrk3a* (a-g) from 24 to 72 hpf, and *ntrk3b* (h-m) from 24 to 96 hpf. Embryos are oriented with head toward the left in lateral views (a,b,d,f,g,j,l), head toward the left in dorsal views (c,e,i,k). (a-c) At 24 hpf, *ntrk3a* expression is confined to nervous and sensory regions, including telencephalon (te), pineal gland (pn), posterior hypothalamus (hy) (white arrow in a), ventrorostral thalamus (th), cranial nerve ganglia (white arrowheads in a and c), posterior hindbrain (hb) (black arrow in a and c), otic vesicle (ov) and Rohon-Beard neurons (rb). mRNA across the brain and in pectoral motor nerve ganglia (arrowhead in e) from 48 to 72 hpf. (g-i) Expression of *ntrk3b* in sub-groups of neuronal cell types in telencephalon, ventral thalamus, ventral tegmentum (tm) and otic vesicle (24 hpf). (j-l) Signal along the brain from 48 to 72 hpf, with weak expression in the ganglion cell layer (gcl). Background due to riboprobe trapping in the digestive system (asterisk in l). Scale bars: 100 µm (a-h,j-l) and 50 µm (i).

Figure 8. **Co-localization of *bdnf* and *ntrk2a* mRNAs.** Double whole-mount *in situ* hybridization at 24 hpf. Embryo is oriented with head to the left in lateral view. (a) *bdnf* and *ntrk2a* are co-expressed in cranial nerves (arrows) (a', magnifications) but not in Rohon-Beard neurons (rb). Abbreviations: ov, otic vesicle. Scale bars: 100 µm.

Figure 9. Scheme of *nt* and *ntrk* gene expression patterns in 24 hpf embryos.



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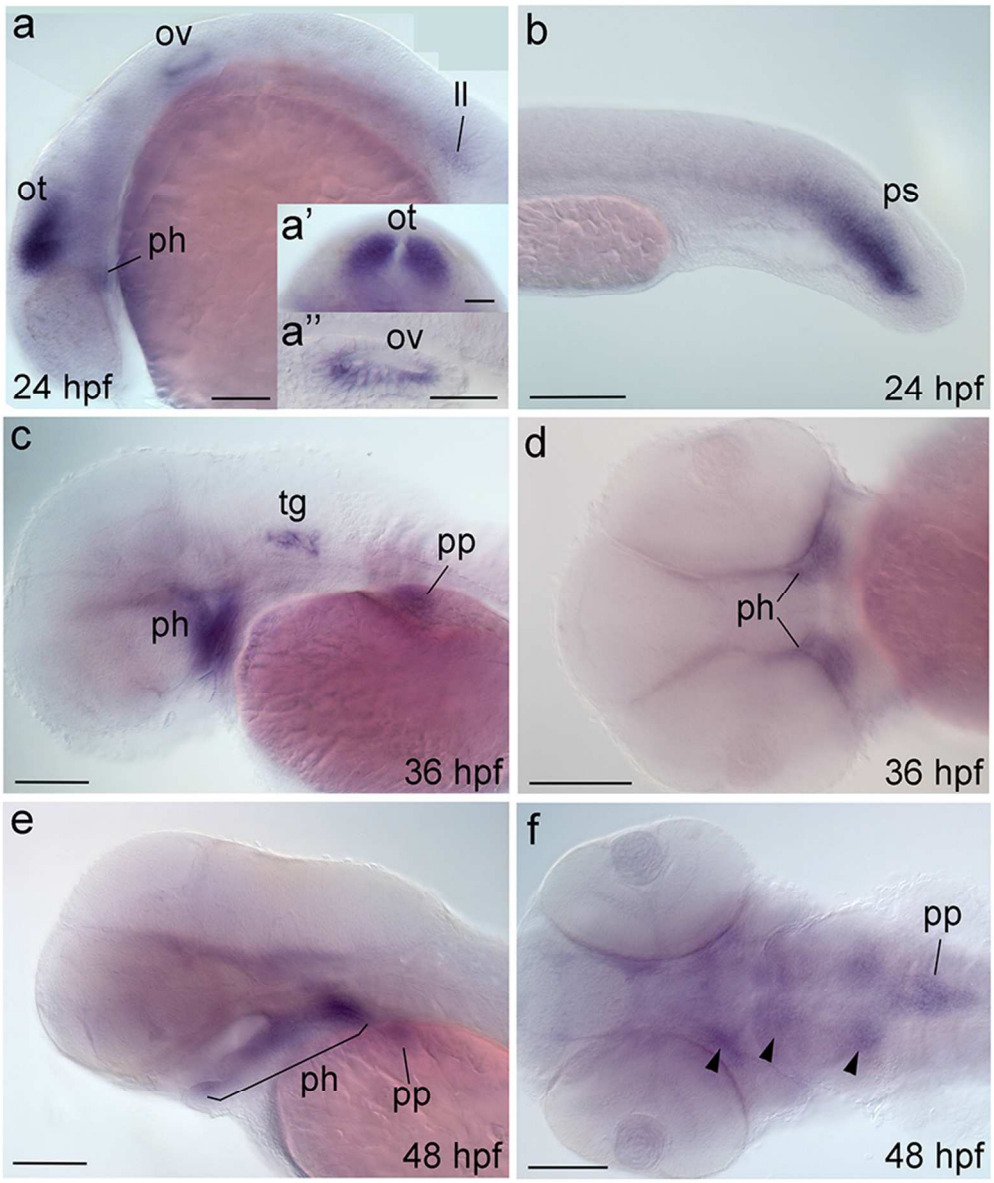

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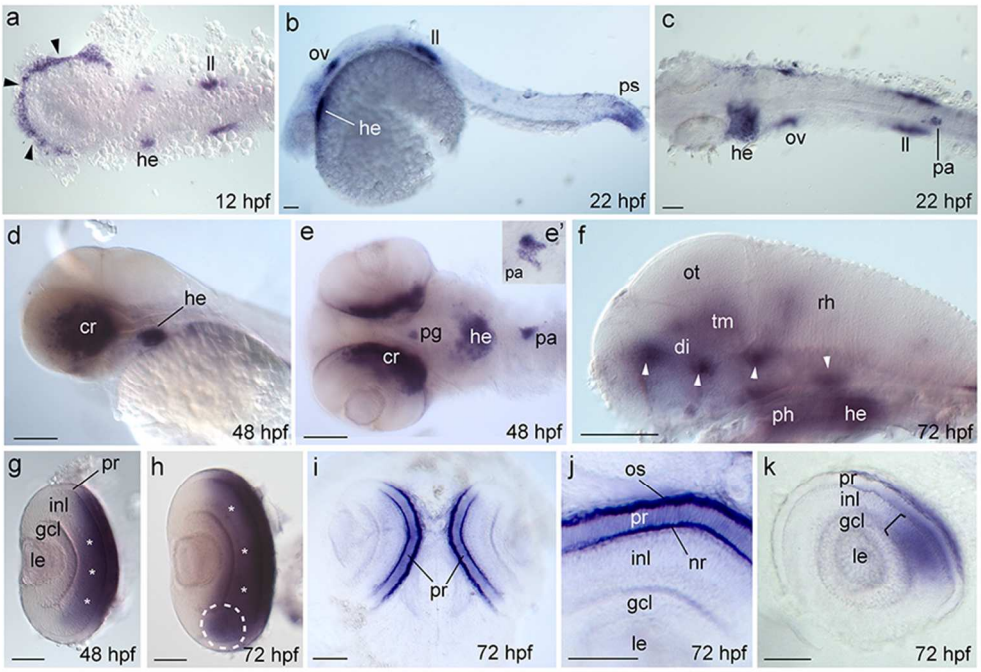

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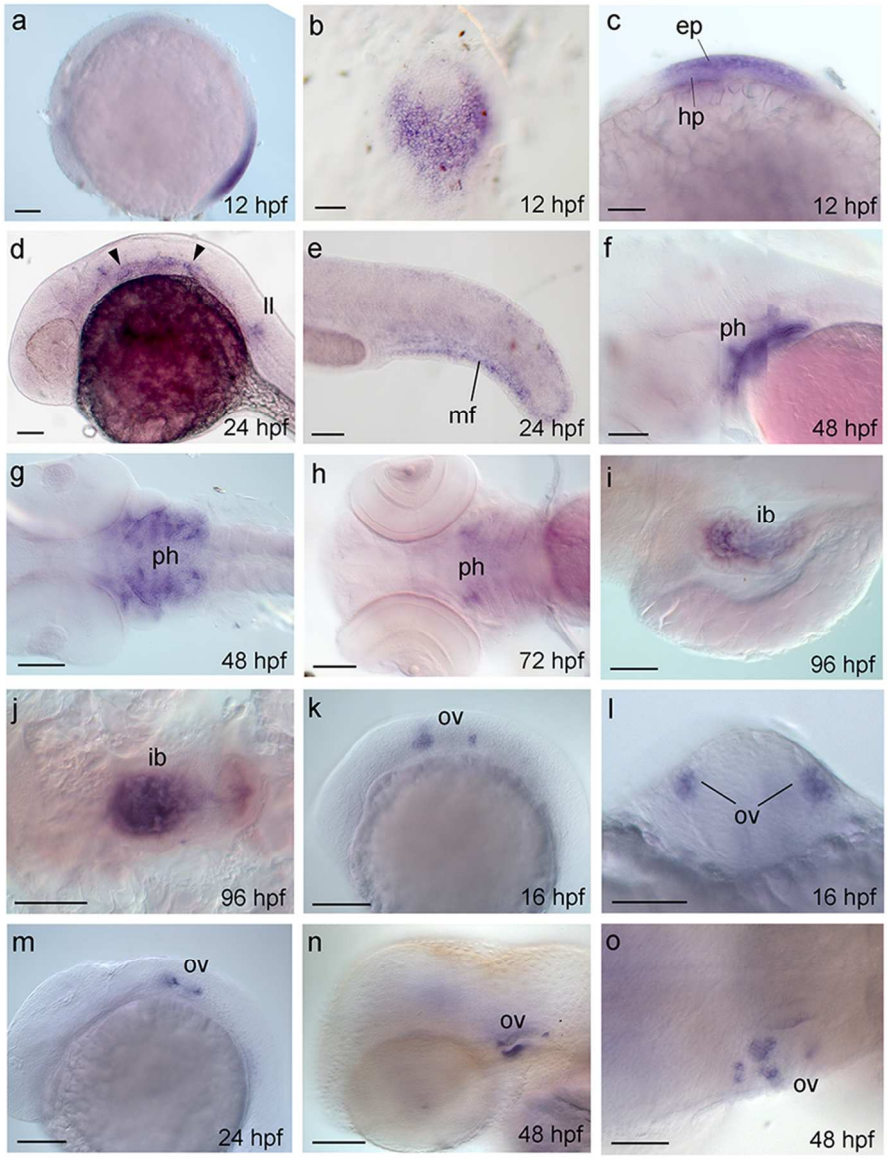

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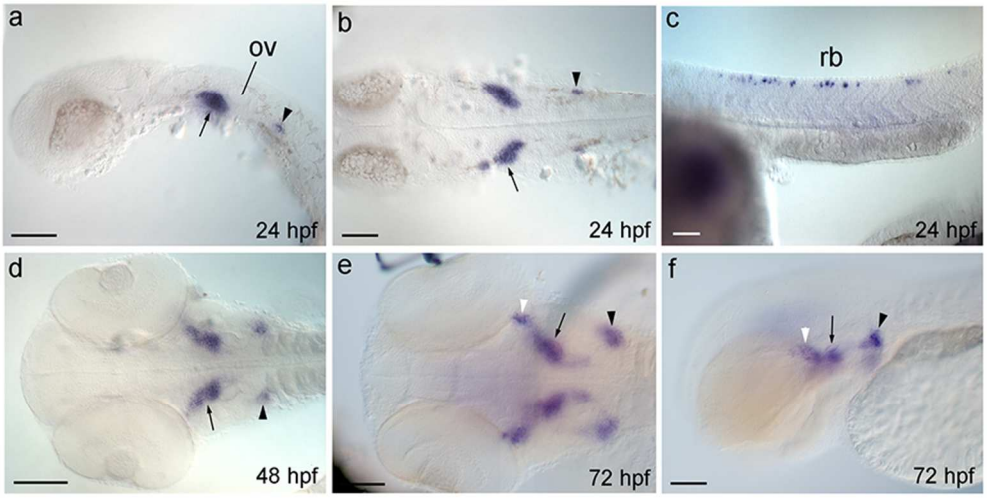

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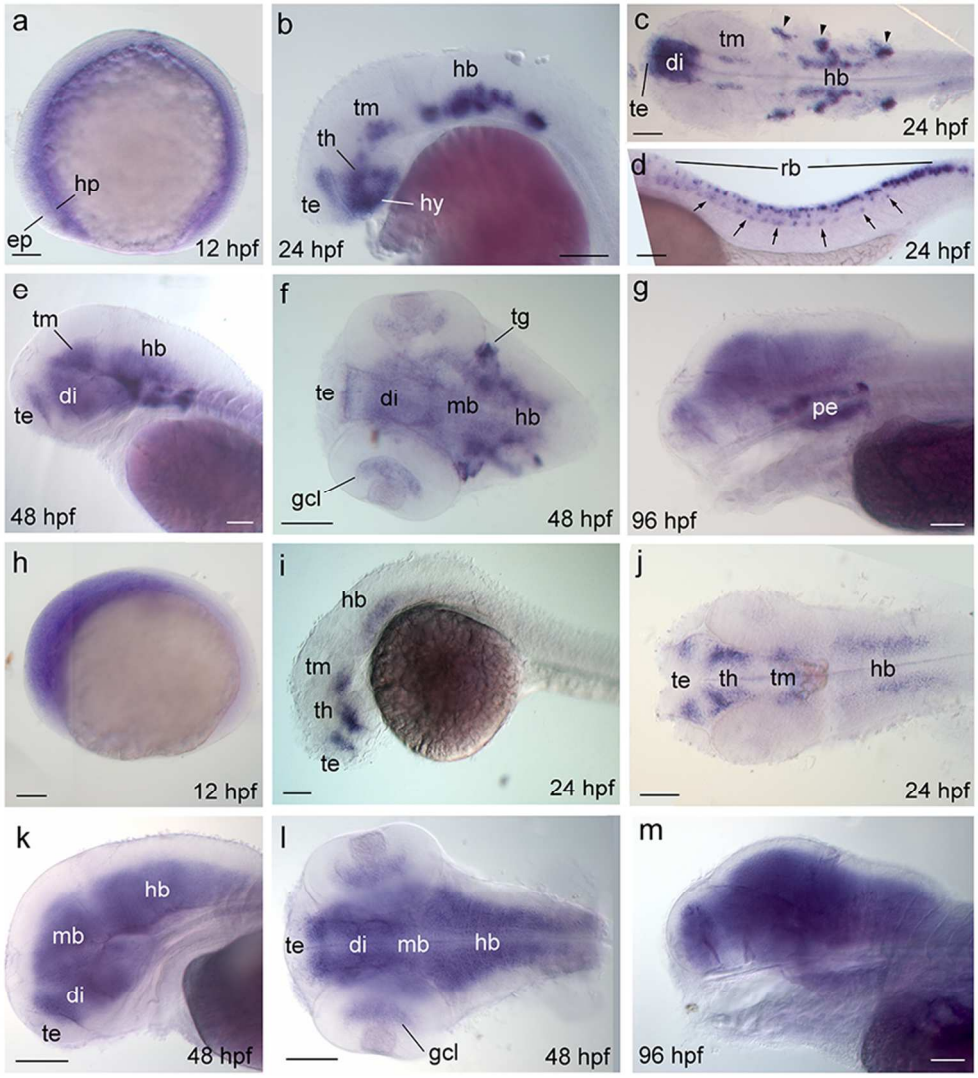

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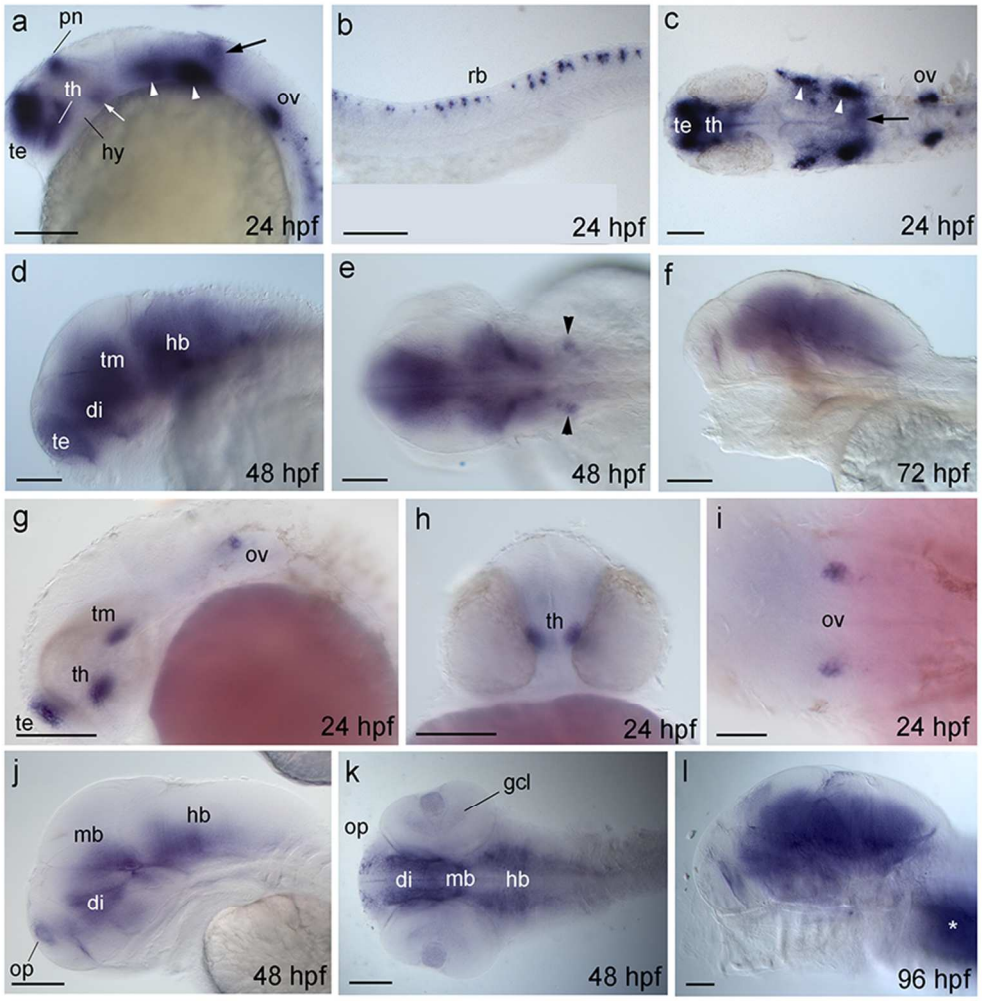

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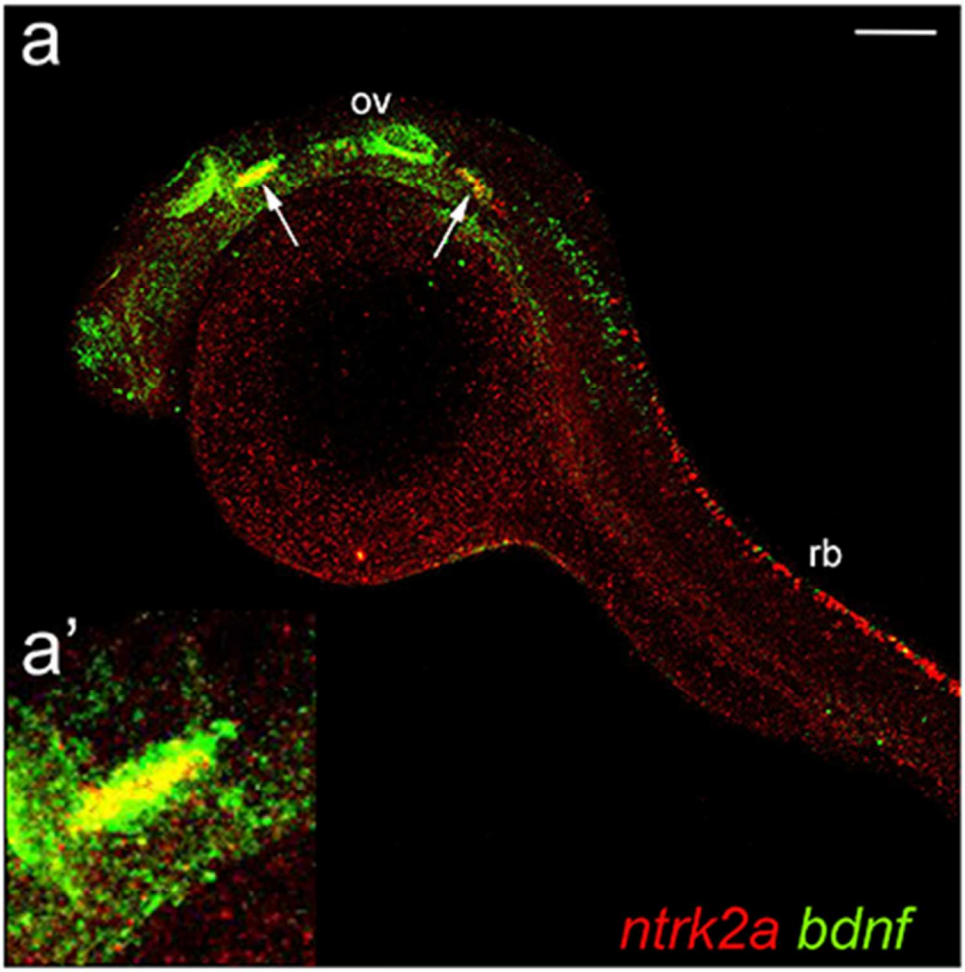

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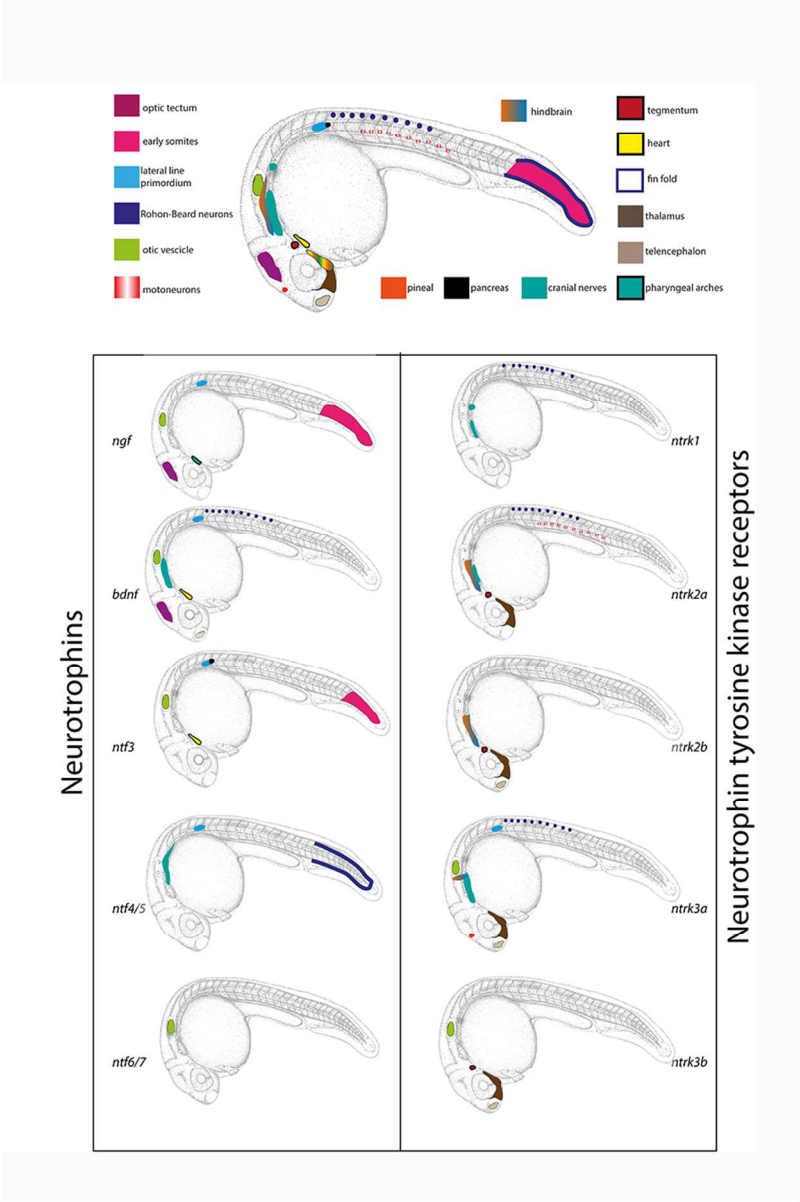

Figure 9. Scheme of nt and ntrk gene expression patterns in 24 hpf embryos.

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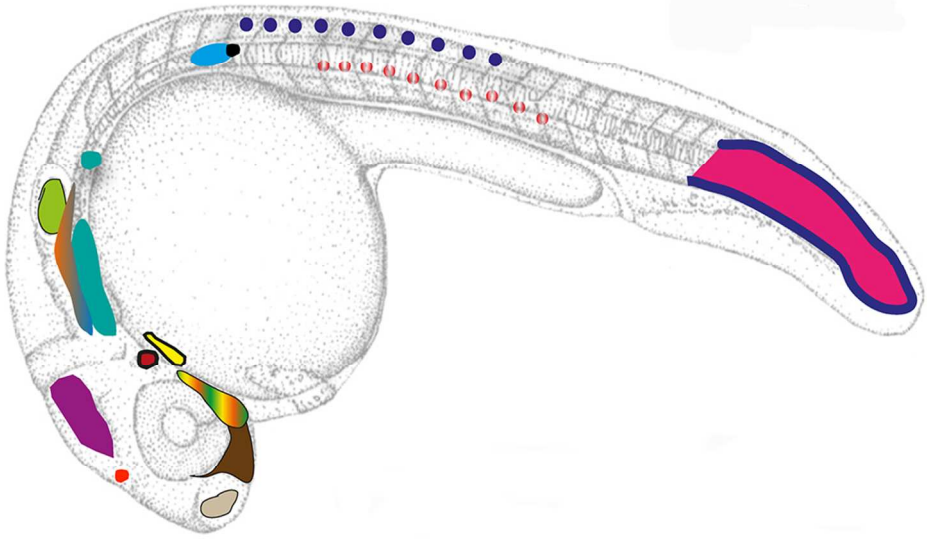

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First examination of the expression profiles of the whole complement of neurotrophin and neurotrophin tyrosine kinase receptor genes in vertebrate embryogenesis. Our study offers novel routes for investigating the biological role of each neurotrophin and their combinatorial interaction with neurotrophin receptor tyrosine kinases.