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NERVE GROWTH FACTOR IS EXPRESSED AND STORED IN CENTRAL NEURONS OF ADULT ZEBRAFISH

Pietro Cacialli¹, ⁴, Claudia Gatta¹, Livia D’Angelo¹, ², Adele Leggieri¹, Antonio Palladino³, Paolo de Girolamo¹, Elisabeth Pellegrini⁴, Carla Lucini¹

¹ Dept. of Veterinary Medicine and Animal Productions, University of Naples Federico II, Naples, Italy; ² Stazione Zoologica Anton Dohrn, Napoli, Italy; ³ Centro Ricerche Interdipartimentali sui Biomateriali, University of Naples Federico II, Naples, Italy; ⁴ Univ Rennes, Inserm, EHESP, Irset (Institut de recherche en santé, environnement et travail) - UMR_S 1085, F-35000 Rennes, France

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ABSTRACT

Nerve Growth Factor (NGF), a member of the neurotrophin family, was initially described as neuronal survival and growth factor, but successively has emerged as an active mediator in many essential functions in the central nervous system of mammals. NGF is synthesized as a precursor pro-NGF and is cleaved intracellularly into mature NGF. However, recent evidence demonstrates proNGF is not a simple inactive precursor, but is also secreted outside the cells and can exert multiple roles. Despite the vast literature present in mammals, studies devoted to NGF in the brain of other vertebrate models are scarce. Zebrafish is a teleost fish widely known for developmental genetic studies and is well established as model for translational neuroscience research. Genomic organization of zebrafish and mouse NGF are highly similar, and zebrafish NGF protein has been reported in a mature and two precursors forms. To add further knowledge on neurotrophic factors in vertebrate brain models, we decided to determine the NGF mRNA and protein distribution in the adult zebrafish brain and to characterize the phenotype of NGF positive cells.

NGF mRNA was visualized by in situ hybridization on whole mount brains. NGF protein distribution was assessed on microtomic sections by using an antiserum against NGF, able to recognize proNGF in adult zebrafish brain as demonstrated also in previous studies. To characterize NGF positive cells, anti-NGF was employed on microtomic slides of aromatase B transgenic zebrafish (where radial glial cells appeared fluorescent) and by means of double immunolabelling against NGF/PCNA (proliferation marker) and NGF/MAP2 (neuronal marker).

NGF mRNA and protein were widely distributed in the brain of adult zebrafish and their pattern of distribution of positive perikaryal was overlapping, both in males and females, with few slight differences. Specifically, the immunoreactivity to the protein was observed in fibers over the entire encephalon. MAP2 immunoreactivity was present in the majority of NGF positive cells, throughout the zebrafish brain. PCNA and aromatase B cells were not positive to NGF, but they were closely
intermingled with NGF cells. In conclusion, our study demonstrated that mature neurons in the
zebrafish brain express NGF mRNA and store proNGF.

Key words: fish; neurotrophins; brain; encephalon; zebrafish neurons;
INTRODUCTION

Nerve Growth Factor (NGF) is the first identified factor belonging, together with Brain Derived Neurotrophic Factor (BDNF), Neurotrophin (NT) 3 and NT 4/5, the neurotrophin family. As all members of the family, NGF is synthesized as a precursor form (pro-NGF) and either secreted outside the cells, as pro- and mature NGF, or cleaved intracellularly to mature NGF. Active forms of pro- and mature NGF are homodimers. To date, three types of NGF receptors are known: TrkA, p75 and sortilin. TrkA, the high affinity receptor, is a member of receptor tyrosine kinases family which includes receptor TrkB (for BDNF and NT 4/5) and TrkC (for NT 3). p75NTR is a member of the tumor necrosis factor receptor (TNFR) superfamily and can transduce signals of all neurotrophins. Sortilin is a member of the family of Vps10p-domain transmembrane receptors, and was earlier characterized as a receptor for neurotensin. While TrkA mediates trophic signalling of the mature form of NGF, p75NTR bifunctionally mediates: (a) a signal to neuronal survival, specially when binding to mature NGF and acting together with TrkA; or (b) the induction of neuronal death when forming a receptor complex with sortilin and mediating proNGF signal (for a review see Niewiadomska et al., 2011).

NGF, initially described as neuronal survival and growth factor, encompasses roles regarding the density of innervation, synthesis of neurotransmitters and neuropeptides and cell body size, axonal sprouting and dendritic arborization (for a review see Minnone et al 2017).

The brain of fish possesses, beyond structural organization similar to all vertebrates, extremely high adult neurogenesis and astonishing regenerative properties. These peculiarities make the fish brain suitable for discovering regenerative mechanisms probably suppressed in mammals during evolutive adaptation (Panula et al 2010; Cacialli et al 2018b). Among teleost fish, zebrafish (Danio rerio) is widely used as model species for developmental genetic studies and functional mechanisms of numerous genes responsible of human diseases (D’Angelo et al., 2016). In addition, zebrafish brain has been largely utilized in numerous studies devoted to adult neurogenesis (Pellegrini et al 2007;
Diotel et al 2013; Coumailleau et al 2015; Than-Trong and Bally-Cuif 2015; Anand et al 2017) and regenerative ability after injury (Cosacak et al 2015; Alunni and Bally-Cuif 2016; Cacialli et al 2018 a,b).

In fish species, homologs of mammalian neurotrophins have been identified, and an additional member, named NT6/7, probably originated by a duplication of the ray-finned fish NGF (Dethleffsen et al 2003). NT6/7 has been identified also in zebrafish (Nilsson et al., 1998). Genomic organization of zebrafish and mouse NGF are highly similar, and zebrafish NGF protein has been reported in a mature form of 194 aminoacid. (Dethleffsen et al 2003). In addition, two proNGF isoforms have been described in zebrafish: isoform 1 (NP_001338647.1) and isoform 2 (NP_954680.2), respectively of 224 and 261 a.a.. (Dethleffsen et al 2003).


In the present study, we evaluated NGF mRNA and protein distribution in the brain of zebrafish and identified neurons as the NGF source cells. Thanks to the conserved adult neurogenesis in fish brain and the potential involvement of neurotrophin in the regenerative ability, the identification of NGF neurons in the adult brain of zebrafish could represent a useful tool to evaluate its involvement in the regenerative process after injury or chemical/genetic induced degeneration.
MATERIALS AND METHODS

Animals and brain dissection

Animals used in this study were housed in zebrafish facilities (INRA LPGP, BIOSIT, Rennes, France, agreement number: B 35-238-6) under standard conditions of photoperiod (14/10) and temperature (28°C). This project was approved by the local animal care and Westerfield ethics committee (Comité Rennais d'Ethique en matière d'Expérimentation Animale, Rennes, France), under the number EEA B-35-040. Zebrafish did not receive medical treatment prior or during the experience. No deaths occurred in the facilities before the euthanasia of animals used for in situ hybridization and immunohistochemistry experiments. Fish were suppressed with an overdose of tricaine methanesulfonate (MS-222).

In situ hybridization (ISH)

Oligonucleotide primers used to amplify and clone cDNA for the production of NGF ISH probes are:

forward 5'-ACATGTACCATGAGGAGCAC-3'; reverse 5'-GTCGCTGGTGTGTGGAAAAT-3' (708 bp; NM_001351718.1). For preparation of NGF digoxigenin (DIG) labeled antisense riboprobe, the vector pCRII-TOPO containing NGF was linearized by BamHI digestion and digoxigenin-labeled riboprobe was prepared using in vitro transcription with T7 RNA polymerase. For sense riboprobe the vector containing NGF was linearized by NotI and digoxigenin-labeled riboprobe was prepared using in vitro transcription with SP6 RNA polymerase.

ISH was performed on whole mount brains as previously described by Adolf et al. (2006); Diotel et al. (2015). Briefly, six zebrafish brains (males and females) were excised and fixed in paraformaldehyde (PFA 4%) dissolved in phosphate-buffered saline (PBS), for 24 hours (h) at 4°C. Then, the brains were dehydrated in a methanol/PBS concentration (25%; 50%; 75%; 100%) and stored at -20°C. After rehydrating through methanol/PBS gradient series and washed in PBS, the brains were incubated for 40 minutes (min) in PBS containing proteinase K (10 µg/ml) at room temperature (RT). After post-fixation in 4% PFA for 20 min and washes in PBS, brains were then
prehybridized for 1 h and incubate overnight at 65°C in the hybridization buffer (pH 6) containing the DIG-labeled probe. Then, brains were washed in SCC 2x/formamide 50% and SSC 0.2x, and pre-incubated with blocking buffer for 3 h and then overnight with antidigoxigenin-AP, Fab fragments (1:5,000; Roche, NJ; Cat# 11093274910, RRID: AB_514497) at 4°C. The next day the brain sections were washed with PBS before staining with NBT/BCIP buffer (pH 9.5).

Whole mount stained brains were embedded in agar 2% and photographed with a Digital camera equipped on Zeiss Stemi. Then, the embedded whole mount stained brains were transversally sectioned with a razor blade or vibratome and the sections were mounted on the slide.

The specificity of the ISH labelling was demonstrated by using sense riboprobe, that showed absence of any staining.

Immunohistochemistry (IHC)

Imunohistochemical procedures were performed following detailed suggestions reported by de Girolamo and Lucini (2011). Six adult zebrafish brains (male and female) were fixed in Bouin’s solution for 24 h and processed for paraffin embedding. Transverse microtome sections were mounted on poly-lysine slides. Sections were deparaffinised in xylene, rehydrated through graded ethanol, treated with 3% H₂O₂ for 30 min and rinsed in PBS (pH 7.4) followed by antigen retrieval in sodium citrate buffer (pH 6; 80 °C) for 30 min.

Single immunohistochemistry

After rinsing 2 times in 0.2% Triton PBS (PBT), non-specific binding was blocked by treating sections with 1/5 normal goat serum (Vector, Burlingame, CA, USA, cod S-1000-20) for 30 min at RT. Then, sections were incubated over night at RT in a humidified chamber with rabbit antibody against NGF (Santa Cruz Biotechnology, CA, USA, cod sc-549. It recognizes N-terminus of the mature chain of NGF of human origin) diluted 1/100 or 1/300, respectively depending on the secondary antibody specified at following point a) or b). The next day, the sections were washed several times in PBT and alternatively incubated with a) Alexa Fluor® goat anti-rabbit 488 (1:200;
Invitrogen Molecular probes, Eugene, OR, REF: A-11037; RRID: AB_10561549) for 2 h at RT in a
dark and humidified chamber or b) EnVision-horseradish anti-peroxidase (HRP)-system (Dako,
Santa Barbara, CA, cod. K4002). This system is based on a HRP labeled polymer conjugated with
goat anti-rabbit IgG.

Sections treated with Alexa Fluor® goat anti-rabbit 488, after three washes in PBT, were mounted
with the medium Vectashield (Vector) containing 4,6-diamino-2-phenylindole (DAPI), to visualize
cell nuclei. Sections treated with EnVision-HRP-system were immersed in a fresh solution of 10 μg
of 3,3′-diaminobenzidine tetrahydrochloride (Sigma–Aldrich Corporation, St. Louis, MO, USA, cod.
D5905) in 15 ml of a 0.5 mol, Tris buffer, pH 7.6, containing 1.5 ml of 0.03% H₂O₂. Then, sections
were dehydrated and mounted.

For immunohistochemistry on cyp19a1bGFP transgenic zebrafish (glial cell marker, Pellegrini et al.,
2007; Tong et al., 2008), NGF antibody was detected with Alexa Fluor® goat anti-rabbit 594 (1:200;
Invitrogen Molecular probes, Eugene, OR, USA, REF: A-11037; RRID: AB_10561549).

The specificity of IHC, assessed by substitution of NGF antiserum, secondary antibody fluorescent
dye -conjugated or the EnVision with PBS or normal serum, achieved no specific immunostaining.

Moreover, the incubation of NGF antiserum preincubated with its homologous antigen showed no
immunoreactivity, and NGF antiserum preincubated with its heterologous antigens did not modify
the normal pattern of immunostaining.

Double immunolabelling

Double immunocytochemical staining NGF/PCNA and NGF/MAP2 was performed as follows:
dewaxed and rehydrated consecutive sections were rinsed in PBS, and incubated for 48 h at RT.

PCNA (proliferative cell nuclear antigen), antibody at diluted 1:100, was used to detect proliferative
cells (Clone PC10; Dako, Glostrup, Denmark; REF: M0879; RRID: AB_2160651). This antibody is
a marker of proliferating cells in vertebrate species, including zebrafish (Pellegrini et al., 2007; Marz
et al., 2011; Cacialli et al., 2016; 2018). MAP2 (Microtube-Associated Protein2) antibody, diluted
1:100, was used to detect neurons, (sc-74422 MAP-2 (A-8) Santa Cruz Biotechnology, Santa Cruz, CA, USA). After rinsing in PBS, the sections were incubated for 2 h at RT with mixture of the secondary antibodies directed against rabbit and mouse IgG. a) Alexa fluor® goat anti-mouse 594 (1:200; Invitrogen Molecular probes, Eugene, OR, REF: A-11005; RRID: AB_10561507; b) Alexa Fluor® goat anti-rabbit 594 (1:200; Invitrogen Molecular probes, Eugene, OR, USA, REF: A-11037; RRID: AB_10561549). Tissue sections were washed in PBS-Triton 0,2%, and slides were mounted with the Vectashield medium containing DAPI for nuclei counterstaining (Vector Laboratories, Burlingame, CA). Controls for double immunolabelling were performed by incubating the sections with one of the two primary antisera and with the mismatched secondary antibodies.

Microscopy

The stained sections were photographed using a Nikon Eclipse 90i microscope, an epifluorescence microscope Olympus equipped with a DP71 digital camera and Leica DM6B, SN: 449492. The digital raw images were optimized for image resolution, contrast, evenness of illumination, and background by using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA, USA).
RESULTS

NGF mRNA (Fig 1A) and protein were widely distributed in the brain of adult zebrafish (Tab. 1). The pattern of distribution of positive perikarya was overlapping, both in males and females, with few slight differences (Table 1). Specifically, the immunoreactivity to the protein was observed in fibers over the entire encephalon. Thus, regions characterized only by presence of fibers, such as the glomerular layer of olfactory bulbs and deep and white zone of the optic tect, showed positivity to the protein. Based on these general considerations, for sake of the simplicity, the term NGF in place of NGF mRNA and NGF protein was used in the following description of results. The anatomical terminology follows “Neuroanatomy of the zebrafish brain” by Wulliman et al. (1996).

Telencephalon

The olfactory bulbs showed moderate quantity of NGF (Fig 1C, D). The cells of both external and internal cellular layer and fibers of the glomerular layer resulted positive.

In the whole telencephalon, more intense NGF positivity was seen in the ventral telencephalon (Fig 1C, D) and in the posterior zone dorsal telencephalic area (Fig 1B, C). Positive cells were distributed in the medial (Fig 3B, B¹), dorsal (Fig 8B, D), lateral (Fig 2A¹, 3C, C¹, 10B, D), central (Fig 11B, D) and posterior part of dorsal part of telencephalic area. In the ventral telencephalic area, small round cells were seen in the dorsal and ventral part.

Diencephalon

Numerous intensely stained cells were seen in the anterior parvocellular preoptic nucleus (Fig 1C, 2B¹), and in the posterior parvocellular preoptic nucleus (Fig 9 B, D). Weak signal was detected in few cells of magnocellular preoptic nucleus. In dorsal and ventral habenular nucleus few positive cells were detected. High density of NGF positive cells were observed in the ventro-medial and ventro-lateral thalamic nuclei (Fig 9B, D). Few and weak positive cells were seen in the central posterior thalamic nucleus. Numerous positive cells were detected in the posterior tuberal nucleus. In the lateral and medial preglomerular nuclei, positive cells and some fibers were detected.
In the hypothalamus, the ventral zone of periventricular hypothalamus showed intense positivity in the whole mount brain (Fig 1E) and numerous NGF positive cells were seen in histological sections (Fig 2C, 5B-C). In the dorsal (Fig 1E) and caudal zone of periventricular hypothalamus moderate positivity was seen. Large NGF positive cells belonging to the diffuse nucleus of the inferior lobe were seen (Fig 6 A–B). Few NGF positive cells were also present in the mammillary body and in the nucleus of the medial longitudinal fascicle.

Mesencephalon

NGF positive fibers were present in the longitudinal tori and the optic tect, particularly in the deep white zone and superficial white zone. Numerous small NGF positive cells appeared scattered in the periventricular grey zone (Fig 4, B, B2).

In the tegmentum, NGF was observed in cells of central nucleus of semi-circular torus and superior reticular formation.

Rhombencephalon

The cerebellar body was intensely reactive (Fig 1F). NGF positivity was observed in numerous large cells of the Purkinje layer and in few cells of the molecular layer of valvula and body (Fig. 7A –C). Few weak stained cells were observed in the granular eminence.

In the medulla oblongata, few large cells containing NGF and belonging to the inferior reticular formation were seen.

Characterization of NGF containing cells

In order to identify the nature of NGF positive-cells in the brain of adult zebrafish, we carried out immunohistochemical staining against NGF on slides of aromatase B transgenic fish (cyp19a1b GFP), where radial glial cells appeared green fluorescent. Also, we performed double labelling using antibodies against NGF/PCNA (proliferation marker) and NGF/MAP2 (neuronal marker). Aromatase positive cells, distributed along ventricles, were closely intermingled with NGF positive cells along telencephalic (Fig 8 B -D) and diencephalic (Fig 9B–D) ventricles. PCNA-positive cells were
positioned along the ventricular lining of the brain, and NGF was detected in the cytoplasm of cells very close to PCNA labelled cells (Fig 10B-D). MAP2 immunoreactivity was present in the majority of NGF positive cells, throughout the zebrafish brain (Fig 11B-D).
DISCUSSION

This study documents the neuroanatomical distribution of NGF mRNA and protein in zebrafish. The serum raised against NGF was previously characterized (Gatta et al 2016), where brain homogenates of adult zebrafish showed only a band of 25 kDa, corresponding to the molecular weight of proNGF isoform 1 (NP_001338647.1) (Gatta et al 2016). Accordingly, the presence of proNGF was also reported in the brain of the teleost *N. furzeri*, by employing the same antiserum (D’Angelo et al 2014). On the other hand, this antiserum detected the mature form of NGF in different organs of fish, such as gut (Lucini et al 2003) and kidney (Arcamone et al., 2005). Taking into consideration that the employed NGF antiserum is able to detect both pro- and mature NGF in fish tissues, our results suggest that only the proNGF form is present in the brain of zebrafish. Remarkably, in mammalian brain, precursor and intermediate forms of NGF are expressed (Fahnestock et al 2001, 2004), and has been demonstrated to be actually the predominant form of NGF in central nervous system (CNS) (Fahnestock et al., 2001), whereas mature NGF appears to be lacking.

The co-presence of pro-NGF and the neuronal marker MAP2 immunoreactivity throughout the brain of adult zebrafish demonstrates that pro-NGF containing cells are neurons. This result was further confirmed by the absence of the glial marker aromatase B and proliferative marker PCNA. Consistently with our observations, in the teleost fish *N. furzeri*, NGF was morphologically detected in neurons widespread throughout all brain regions (D’Angelo et al., 2014). Only some glial cells lining the mesencephalic and rhomboencephalic ventricles of *N. furzeri* seemed to express NGF. At opposite, in goldfish NGF was almost totally localized in radial glia cells lining the ventricles (Benowitz and Shashoua, 1979). Species specific characteristics could explain the different results achieved in zebrafish, *N. furzeri* and goldfish. In agreement with our results, in the rat brain, NGF production was reported in neurons, predominantly localized in GABAergic neurons of the cortex, hippocampus, striatum and basal forebrain (Lauterborn et al 1993, 1995; Pascual et al 1998; Bizon et al 1999; Sofroniew et al 2001; Biane et al 2014). NGF was also reported in neuronal populations of adult monkey brain (Hayashi et al 1993; Zhang et al 2007). However, oligodendroglial progenitors
derived from human embryonic stem cells are a source of NGF (Zhang et al 2006; Althaus et al 2000).

Remarkably in adult zebrafish, another member of neurotrophin family, BDNF, was also expressed in neuronal populations of the whole brain (Cacialli et al 2016) and of telencephalon after injury (Cacialli et al 2018).

NGF expression was comparable between zebrafish and *N. furzeri* (D’Angelo et al 2014), despite some slight differences in the neuroanatomy, whereas substantial differences in NGF cell localization and distribution were seen between goldfish and zebrafish as previously described. In rat brain, NGF levels resulted consistent in all regions with the highest presence in cortical areas (Hoener et al., 1996; Sakamoto et al., 1998). Specifically, the pattern of distribution of both NGF mRNA (Shelton and Reichardt, 1986) and protein (Nishio et al., 1992) was described throughout the rat brain, with the highest intensity in the neocortex, the hippocampal pyramidal layer and striatum (Gall and Isackson, 1989, Rylett and Williams, 1994). Notably, observations in adult zebrafish brain do not support the fact that the forebrain is the NGF prevalent containing region.

In our study, both NGF mRNA and protein were detected in the perikaryon, and only proNGF protein was distributed along neuronal prolongations. Although the cellular co-presence of NGF mRNA and protein was not investigated, the overlapping pattern of distribution of NGF mRNA and protein throughout the zebrafish brain suggests that NGF expression and translation take place in mature neurons. The presence of NGF protein along neuronal prolongments could be retrogradely transported, according to the classical view on neurotrophins considered as target-derived retrogradely transported substances, but also anterogradely transported, accordingly to a vast literature. The idea that NGF may be produced locally was suggested since two decades ago (Lauterborn et al 1991, Conner and Varon 1992) and activity-dependent release of NGF and its effect on synaptic plasticity was postulated (Blöchl and Thoenen, 1995, 1996; Wu et al., 2004). Finally, Guo and collaborators (2012) demonstrated, by immunohistochemical, ELISA and electrophysiological analyses, anterograde delivery of NGF in hippocamposeptal system of mice.
ProNGF in the CNS is released in the extracellular space (Bruno and Cuello 2006) and induces activation of the apoptotic machinery with subsequent death of different neuronal populations, mostly after injury and neurodegenerative disorders (for a review see Costa et al 2018).

The present results demonstrate that also in zebrafish brain NGF is synthesized in perykaria, however future studies are necessary to test whether proNGF is anterogradely transported and released in the extracellular space, at terminal ending of NGF positive fibers.

In conclusion, our study demonstrated that mature neurons of the zebrafish brain express NGF mRNA and store proNGF. Experimental studies reported proNGF as inhibition factor of the proliferation of neural stem cells isolated from postnatal mouse hippocampus, causing cell cycle arrest in the G0/G1 phase (Guo et al 2013). Thus, it is tempting to speculate that proNGF in the zebrafish brain, where cell proliferation is considerably high and persist along the entire lifespan, could represent a key negative regulator factor of this process.

Author’s contribution

PC conceived and planned the experimentation, acquired and analyzed data; CG, AL, AP acquired and analyzed data; LDA, PdG, EP critically revised the manuscript; CL analyzed data and wrote the paper.


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Fig. 1 NGF mRNA in whole mount-brain of adult zebrafish.

NGF mRNA distribution in whole mount brain (A); telencephalon (B); preoptic area and olfactory bulbs (C, D); hypothalamus (E), cerebellum (F). CCe: cerebellum; DIL: diffuse nucleus of the inferior lobe; Hv: ventral zone of periventricular hypothalamus; Hc: caudal zone of periventricular hypothalamus; Hd: dorsal zone of periventricular hypothalamus; Mo: medulla oblongata; OB: olfactory bulbs; PPa: preoptic area; Tel: telencephalon; TeO: optic tect; Scale bar: (A) 500 μm; (B, C, E, F) 250 μm; D: 150 μm.

Fig. 2 NGF mRNA in cross-section of adult zebrafish brain.

(A – C) Representative section taken from the zebrafish atlas (Wullimann et al., 1996). NGF positive cells are represented by blue dots. (A\textsuperscript{1} – C\textsuperscript{1}) NGF positive cells in the dorsal part of telencephalon (A\textsuperscript{1}), in the anterior parvocellular preoptic nucleus (B\textsuperscript{1}) and along the ventral zone of periventricular hypothalamus (C\textsuperscript{1}). Hv: ventral zone of periventricular hypothalamus; PPa: anterior parvocellular preoptic nucleus; Tel: telencephalon. Scale bar: 50 μm.

Fig. 3 NGF protein in the telencephalon.

(A) Representative section taken from the zebrafish atlas (Wullimann et al., 1996). NGF positive cells are represented by green dots. (B - C) Cells positive to NGF in the medial (B) and lateral (C) part of dorsal telencephalic area. (B\textsuperscript{1}, C\textsuperscript{1}) NGF positive cells co-marked with DAPI. Dl: lateral zone of dorsal telencephalic area; Dm: medial zone of dorsal telencephalic area. Scale bar: (C, C\textsuperscript{1}) 100 μm; (B, B\textsuperscript{'}) 50 μm.

Fig. 4 NGF protein in the forebrain and midbrain.
NGF positive cells are represented by green dots. Cells positive to NGF in the posterior zone of dorsal telencephalic area (A1) and in periventricular gray zone of optic tect (B1). (A2, B2) NGF positive cells co-marked with DAPI. TelV: telencephalic ventricle; Scale bar: 50 μm.

Fig. 5 NGF protein in the hypothalamus.

(A) Representative section taken from the zebrafish atlas (Wullimann et al., 1996). NGF positive cells are represented by green dots. (B – C) NGF protein (green) along the ventral hypothalamus at low (B) and high (C) magnification. (C1) NGF positive cells co-marked with DAPI. Hv: ventral zone of periventricular hypothalamus. Scale bar: (B) 100 μm; (C, C1) 50 μm.

Fig. 6 NGF protein in the hypothalamus.

NGF positive cells in the diffuse nucleus of inferior lobe (DIL) at low (A) and high (A1, B) magnification. In B1 NGF positive cells co-marked with DAPI. Scale bar: (A) 120 μm; (A1) 30 μm; (B, B1) 20 μm.

Fig. 7 NGF protein in the cerebellum.

(A) Representative section taken from the zebrafish atlas (Wullimann et al., 1996). NGF positive cells are represented by green dots. (B, C) NGF positive cells in the Purkinje layer of cerebellum. In C’ NGF positive cells co-marked with DAPI. CCe: cerebellar body; Scale bar: 50 μm.

Fig. 8 NGF positive cells are close to aromatase B cells along telencephalic ventricle.

(A) Representative section of dorsal and ventral telencephalon taken from the zebrafish atlas (Wullimann et al., 1996). NGF positive cells are represented by red dots and Aromatase B is represented by black dots with thin lines indicating radial glia cytoplasmic processes. (B – F) Cross sections of dorsal telencephalic area. Double staining for NGF (red) (B), Aromatase-B (green) (C)
and merge with DAPI (D). High magnification of NGF and aromatase B cells, closely intermingled (E, F). Scale bar: (B – D) 40 μm; (E) 20 μm; (F) 10 μm.

Fig. 9 NGF positive cells are close to aromatase B cells along diencephalic ventricle.

(A) Representative section of diencephalon taken from the zebrafish atlas (Wullimann et al., 1996). NGF positive cells are represented by red dots and Aromatase B is represented by black dots with thin lines indicating radial glia cytoplasmic processes. (B – D) Double staining for NGF (red) (B), Aromatase-B (green) (C) and merge with DAPI (D) on cross-section of area surrounding diencephalic ventricle. (E -F) high magnifications of B and C images. Scale bar: (B – D) 50 μm; (E _F) 20 μm.

Fig. 10 NGF positive cells are intermingled with PCNA positive cells.

(A) Representative section of dorsal and ventral telencephalon taken from the zebrafish atlas (Wullimann et al., 1996) NGF positive cells are represented by green dots and PCNA positive cells by red dots. (B – D) Double staining for NGF (green) (B), PCNA(red) (C), merge with DAPI and high-magnification of a zoom area (D) on cross-sections through the telencephalon. Scale bar: 50 μm, and particular of the region in (D) 30 μm.

Fig. 11 NGF immunoreactivity is colocalized with MAP2 in cells of central zone of dorsal telencephalic area.

Representative section dorsal and ventral telencephalon taken from the zebrafish atlas (Wullimann et al., 1996). NGF positive cells are represented by red dots and MAP2 positive cells by green dots. (B – D) Double staining for NGF (red) (B), MAP2 (green) (C) and merge (D) on cross-sections through the telencephalon. Scale bar: 20 μm

Supplementary material
Negative controls performed by NGF antiserum preincubated with its homologous antigen did not show any reactivity. Scale bar: (A) 100 μm; (B) 50 μm
Table 1. Distribution of NGF mRNA and protein in the brain of adult zebrafish. The scheme was done following qualitative and not quantitative criteria.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>NGF mRNA</th>
<th>NGF protein</th>
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<tbody>
<tr>
<td><strong>Olfactory bulbs</strong></td>
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<tr>
<td>Glomerular layer</td>
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<td>External cellular layer</td>
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<td>Internal cellular layer</td>
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<td><strong>Dorsal telencephalic area</strong></td>
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<tr>
<td>Medial zone of dorsal telencephalic area</td>
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<tr>
<td>Dorsal zone of dorsal telencephalic area</td>
<td>+</td>
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</tr>
<tr>
<td>Lateral zone of dorsal telencephalic area</td>
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</tr>
<tr>
<td>Central zone of dorsal telencephalic area</td>
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</tr>
<tr>
<td>Posterior zone of dorsal telencephalic area</td>
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<tr>
<td><strong>Ventral telencephalic area</strong></td>
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</tr>
<tr>
<td>Ventral-dorsal part</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Ventral-central part</td>
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<td>++</td>
</tr>
<tr>
<td><strong>Preoptic area</strong></td>
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</tr>
<tr>
<td>Magnocellular preoptic nucleus</td>
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</tr>
<tr>
<td>Parvocellular preoptic nucleus, anterior part</td>
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</tr>
<tr>
<td>Parvocellular preoptic nucleus, posterior part</td>
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<tr>
<td><strong>Epithalamus</strong></td>
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</tr>
<tr>
<td>Dorsal habenular nucleus</td>
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</tr>
<tr>
<td>Ventral habenular nucleus</td>
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<tr>
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<tr>
<td>Central posterior thalamic nucleus</td>
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<tr>
<td><strong>Ventral thalamus</strong></td>
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<td></td>
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<tr>
<td>Ventromedial thalamic nucleus</td>
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<tr>
<td>Ventrolateral thalamic nucleus</td>
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<tr>
<td><strong>Posterior Tuberculum</strong></td>
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</tr>
<tr>
<td>Posterior tuberal nucleus</td>
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<tr>
<td>Lateral preglomerular nucleus</td>
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<tr>
<td>Medial preglomerular nucleus</td>
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<tr>
<td><strong>Hypothalamus</strong></td>
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<tr>
<td>Diffuse nucleus of the inferior lobe</td>
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<tr>
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<tr>
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</tr>
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<td>Caudal zone of periventricular hypothalamus</td>
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<td>Mammillary body</td>
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<tr>
<td><strong>Synencephalon</strong></td>
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<tr>
<td>Nucleus of the medial longitudinal fascicle</td>
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<tr>
<td>Deep white zone</td>
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<tr>
<td>Central zone</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Superficial white grey zone</td>
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<tr>
<td>Longitudinal torus</td>
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<tr>
<td><strong>Torus semicircularis</strong></td>
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</tr>
<tr>
<td>Central nucleus of semicircular torus</td>
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<td>+</td>
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<tr>
<td><strong>Tegmentum</strong></td>
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<td></td>
</tr>
<tr>
<td>Superior reticular formation</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><strong>Cerebellum</strong></td>
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<td></td>
</tr>
<tr>
<td>Molecular layer</td>
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<td>+</td>
</tr>
<tr>
<td>Purkinje cell layer</td>
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<tr>
<td><strong>Medulla oblongata</strong></td>
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</tr>
<tr>
<td>Inferior reticular formation</td>
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Fig. 1 Nerve growth factor (NGF) mRNA in whole-mount brain of adult zebrafish. NGF mRNA distribution in (A) whole-mount brain; (B) telencephalon; (C, D) preoptic area and olfactory bulbs; (E) hypothalamus; and (F) cerebellum. CCe, cerebellum; DIL, di nucleus of the inferior lobe; Hv, ventral zone of periventricular hypothalamus; Hc, caudal zone of periventricular hypothalamus; Hs, dorsal zone of periventricular hypothalamus; Mo, medulla oblongata; OB, olfactory bulbs; PPa, preoptic area; Tel, telencephalon; TeO, optic tectum. Scale bars: 500 μm (A); 250 μm (B, C, E, F); 150 μm (D).

Fig. 2 Nerve growth factor (NGF) mRNA in cross-section of adult zebrafish brain. (A–C) Representative section taken from the zebrafish atlas (Wullimann et al., 1999). NGF-positive cells are represented by blue dots. (A1–C1) NGF-positive cells in the dorsal part of the telencephalon (A1), in the anterior panencephalic preoptic nucleus (B1) and along the ventral zone of the periventricular hypothalamus (C1). Hv, ventral zone of periventricular hypothalamus; PPa, anterior panencephalic preoptic nucleus; Tel, telencephalon. Scale bar: 50 μm.
Fig. 3 Nerve growth factor (NGF) protein in the telencephalon. (A) Representative section taken from the zebrafish atlas (Weidman et al., 1996). NGF-positive cells are represented by green dots. (B, C) Cells positive to NGF in the medial (B) and lateral (C) part of the dorsal telencephalic area. (B', C') NGF-positive cells co-marked with 4,6-diamidino-2-phenylindole (DAPI). DL, lateral zone of dorsal telencephalic area; Dm, medial zone of dorsal telencephalic area. Scale bars: 100 μm (C, C'); 50 μm (B, B').
Fig. 4 Nerve growth factor (NGF) protein in the hypothalamus. (A) Representative section taken from the zebrafish atlas (Wullimann et al., 1996). NGF-positive cells are represented by green dots. (B, C) NGF protein (green) along the ventral hypothalamus at low (B) and high (C) magnification. (C') NGF-positive cells co-marked with 4,6-diamino-2-phenylindole (DAPI). Hv, ventral zone of periventricular hypothalamus. Scale bars: 100 μm (B), 50 μm (C, C').

Fig. 5 Nerve growth factor (NGF) protein in the hypothalamus. NGF-positive cells in the diffuse nucleus of interior lobe (DIL) at low (A) and high (A', B) magnification. (B') NGF-positive cells co-marked with 4,6-diamino-2-phenylindole (DAPI). Scale bars: 120 μm (A); 30 μm (A'), 20 μm (B, B').
Fig. 6  Nerve growth factor (NGF) protein in the forebrain and midbrain. (A, B) Representative sections taken from the zebrafish atlas (Willman et al., 1996). NGF-positive cells are represented by green dots. Cells positive to NGF in the posterior zone of the doral telencephalic area (A') and in the periventricular gray zone of optic tect (B'). (A', B') NGF-positive cells co-marked with 4,6-diamino-2-phenylindole (DAPI). TeIV, telencephalic ventricle. Scale bar: 50 μm.

Fig. 7  Nerve growth factor (NGF) protein in the cerebellum. (A) Representative section taken from the zebrafish atlas (Willman et al., 1996). NGF-positive cells are represented by green dots. (B) NGF-positive cells in the Purkinje layer of cerebellum. (C) NGF-positive cells co-marked with 4,6-diamino-2-phenylindole (DAPI). CCo, cerebellar body. Scale bar: 50 μm.
Fig. 8  Nerve growth factor (NGF)-positive cells are close to aromatase B cells along telencephalic ventricle. (A) Representative section of dorsal and ventral telencephalon taken from the zebrafish atlas (Wulliman et al., 1996). NGF-positive cells are represented by red dots, and aromatase B is represented by black dots with thin lines indicating radial glial cytoplasmic processes. (B–F) Cross-sections of the dorsal telencephalic area. Double-staining for (A) NGF (red), (C) aromatase B (green), and merge with (D) 4,6-diamino-2-phenylindole (DAPI). High magnification of NGF and aromatase B cells, closely intermingled (B, F). Scale bars: 40 μm (B–D); 20 μm (E); 10 μm (F).
Fig. 9  Nerve growth factor (NGF)-positive cells are close to aromatase B cells along the diencephalic ventricle. (A) Representative section of the diencephalon taken from the zebrafish atlas (Wulliman et al., 1996). NGF-positive cells are represented by red dots, and aromatase B is represented by black dots with thin lines indicating radial glia cytoplasmic processes. (B–D) Double-staining for (B) NGF (red), (C) aromatase B (green), and merge with (D) 4,6-diamino-2-phenylindole (DAPI) on cross-section of the area surrounding diencephalic ventricle. (E, F) High magnifications of (B) and (C) images. Scale bars: 50 μm (B–D); 20 μm (E, F).
Fig. 10 Nerve growth factor (NGF)-positive cells are intermingled with proliferative cell nuclear antigen (PCNA)-positive cells. (A) Representative section of dorsal and ventral telencephalon taken from the zebrafish atlas (Wulliman et al., 1996). NGF-positive cells are represented by green dots and PCNA-positive cells by red dots. (B-D) Double-staining for (B) NGF (green), (C) PCNA (red), and merge with (D) 4,6-diamino-2-phenylindole (DAPI) and high-magnification of a zoom area on cross-sections through the telencephalon. Scale bar: 50 μm, and particular of the region in (D) 30 μm.

Fig. 11 Nerve growth factor (NGF) immunoreactivity is co-localized with microtubule-associated protein2 (MAP2) in cells of the central zone of the dorsal telencephalic area. Representative section of dorsal and ventral telencephalon taken from the zebrafish atlas (Wulliman et al., 1996). NGF-positive cells are represented by red dots, and MAP2-positive cells by green dots. (B-D) Double-staining for (B) NGF (red), (C) MAP2 (green), and merge (D) on cross-sections through the telencephalon. Scale bar: 20 μm.