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# Glial cell line-derived neurotrophic factor expression in the brain of adult zebrafish (*Danio rerio*)

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**Summary.** In mammals, glial cell line-derived neurotrophic factor (GDNF) is a growth factor of many neuronal populations in the central, peripheral and autonomous nervous system. GDNF may also function as a morphogen during kidney development and may regulate spermatogonial differentiation. GDNF has been characterised in zebrafish embryos and was demonstrated experimentally to be critical for the development of the enteric nervous system. However, in adult zebrafish, no data exist regarding GDNF expression and localisation in the brain and in different organs. Thus, the aim of the present study was to investigate the expression of GDNF in the brain of adult zebrafish (*Danio rerio*).

Transcripts of GDNF mRNA were observed in brain extracts by a standard RT-PCR. The presence of the protein in the brain homogenates was confirmed by SDS-PAGE electrophoresis and Western blotting analysis. Immunohistochemistry and in situ hybridization experiments showed that GDNF protein and mRNA were localised in various nuclei of the telencephalon, diencephalon, mesencephalon, cerebellum and medulla oblongata of the zebrafish brain. In conclusion, this study showed that the expression of GDNF was not restricted to developmental periods but it seems that this factor might be involved in adult zebrafish brain physiology, as observed in mammals.

**Key words:** Teleost fish, GDNF, Neurotrophic factors

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## Introduction

Glial derived neurotrophic factor (GDNF) is a growth factor of many neuronal population in the central, peripheral and autonomous nervous system. Outside the nervous system, GDNF functions as a morphogen in kidney development and regulates spermatogonial differentiation. GDNF acts, together with the related growth factors neurturin, artemin and persephin, through RET receptor tyrosine kinase. The ligand-binding specificity is determined by a glycosyl phosphatidylinositol (GPI)-linked ligand-binding subunit known as GDNF family receptor α (GFRα). GFRα1, GFRα2, GFRα3 and GFRα4 receptors bind to GDNF, neurturin, artemin and persephin respectively. However a crosstalk between GDNF and GFRα2 is reported. GDNF can also signal RET independently through GFRα1, and the NCAM-GFRa1 complex (for a review see Sariola and Saarma, 2003).

GDNF has been characterised in zebrafish embryos. It shows structural motifs similar to those functionally identified in mammals. Transcripts of GDNF mRNA are expressed in the ventral half of somites, in the intermediate mesoderm where the pronephric duct condenses, in the ventral trunk mesoderm and endoderm (Shepherd et al., 2001). Moreover, GDNF was demonstrated experimentally by Shepherd et al. (2004) to be critical for the development of the enteric nervous system. In adult zebrafish there are no data regarding GDNF expression and localization in the different organs, except for the description of GDNF immunoreactivity in endocrine cells of gut epithelium (Lucini et al., 2005).

The adult central nervous system of fish shows marked regenerative capabilities, generating and further

developing new neurons (for a review see Zupanc and Clint, 2003). On the other hand, previously, GDNF was seen to play in mammals a neuroprotective role in the adult nervous system following brain injury (Wang et al., 2002; Tolbert and Clark, 2003, Zhao et al., 2004). Thus, the aim of this study was to investigate the presence and distribution of GDNF in the brain of adult zebrafish (*Danio rerio*), in order to contribute to future studies devoted to the involvement of GDNF in the brain regeneration of this widely used model species.

## Material and methods

### *Animals*

Six month-/one year-old zebrafish were obtained from a local supplier. The experimental protocols were conducted within the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by institutional committes of the University of Naples and Padua. All efforts were made to minimize the number of animals used and their suffering. Zebrafish were deeply anaesthetized by 0.1% ethyl 3-aminobenzoate, methanesulfonate (Sigma Chemicals Co, St Louis, MO, USA).

### *RT-PCR, product cloning and sequencing*

The brain was dissected from twenty adult zebrafish and quickly immersed in RNA-later® buffer (Ambion, Heidelberg, Germany). Samples were processed immediately as described below or stored at -20°C in the same buffer. Total RNA was extracted from 100 mg of tissues using Trizol® reagent (Gibco-BRL, Gaithersburg, MD, USA). To assess the integrity and the amount of the RNA extracted, denaturating agarose gel electrophoresis and spectrophotometric $A_{260/280}$ readings were performed. Two micrograms of Total RNA were reverse-transcribed (RT) into cDNA, using a mixture of random hexamers, with the Superscript RT II kit (Invitrogen) after treatment with DNAse I (Invitrogen) to remove contaminating genomic DNA. The obtained cDNAs were used as a template for PCR expression analysis. Different PCR reactions were conducted using specific primers that amplify a 334 base pair fragment of the GDNF gene (forward 5' CCT GAA ACC TCA AAC CCA AA 3'; reverse 5' TCC TGT AAC CCA AGT CCA GG 3'), designed by means of Primer Express software (Applied Biosystem) on the AF329853 sequence (Shepherd et al., 2001). A ß-actin fragment of 406 bp (forward primer: 5' - CTG GGA TGA CAT GGA GAA GA – 3'; reverse primer: 5' – CTT GAT GTC ACG CAC GAT TT – 3') was amplified to exclude a genomic contamination and to validate the purity of cDNAs (Kreuzer et al., 1999). The PCR product was cloned into the pCR-II® plasmid vector using a TOPO-TA cloning kit (Invitrogen Life Technologies) and sequenced by the CRIBI (http://bmr.cribi.unipd.it/) sequencing service to confirm the identity of the GDNF fragment. The nucleotide sequence of our GDNF fragment was used to perform BLASTN and the BLASTP analysis (http://www.ncbi.nlm.nih.gov/blast) on the complete, non-redundant GenBank database sequences, using default settings, in order to confirm its identity.

### *Western blotting*

For Western blotting analysis, the brain from twenty adult zebrafish of both sexes were homogenized using an Ultraturrax L-407 at 4°C with 5 ml/1.5 g tissue of buffer containing 10 mM PBS, pH 7.2, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 0.01% phenylmethylsulfonyl fluoride (PMSF), 3% aprotinin, 10 µg/ml leupeptin and 1 mM $Na_3VO_4$. Homogenates were centrifuged at 25,000 g for 20 min at 4°C. Supernatants were divided into small aliquots, and stored at -80°C until use. The amount of total proteins in each sample was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Samples containing equal amounts of protein were boiled for 5 min in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% ß-mercaptoethanol) and run on 18% SDS/polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose using a Mini trans-blot apparatus (Bio-Rad Laboratories) CA, USA) according to manufacturer's instructions. Membranes were blocked for 1 h at RT with blocking buffer containing 5% milk in Tris/NaCl/phosphate buffer (Pi) and washed with Tris/NaCl/Pi/Tween (150 mM NaCl, 20 mM Tris HCl, pH 7.4, 0.1% Tween 20). The blots were incubated overnight with rabbit polyclonal antibodies (GDNF1 and GDNF2 described above) diluted 1:100 in Tris/NaCl/Pi, 3% milk. After incubation, the membranes were washed three times with Tris/NaCl/Pi/Tween and incubated for 1 h at RT with horseradish peroxidase conjugated anti-rabbit IgG (Sigma Chemical Co) diluted 1:3000 in Tris/NaCl/Pi, 3% milk. The proteins were visualized by enhanced chemiluminescence (Amersham Corp., Little Chalfont, UK).

### *Immunohistochemistry*

Six adult zebrafish of both sexes were fixed by immersion in Bouin's fluid for 24-48 h at room temperature (RT), dehydrated in an ethanol series and embedded in paraffin wax. Sagittal and horizontal 5-7 µm thick sections were cut from four animals fixed in toto. Transverse 5-7 µm thick sections were obtained from two zebrafish, cut in half at the level of the posterior edge of the operculum before the fixation.

Immunohistochemical staining was performed using the peroxidase-antiperoxidase (PAP) method (Sternberger, 1986). After dewaxing in xylene, sections were rinsed in distilled water and subjected to microwave oven treatment to unmask the antigens (0.01 M sodium citrate buffer, pH 6.0, for 10 min at 750 W)

(Reynolds et al., 1994). Then, sections were rinsed in distilled water and treated with 3% $H_2O_2$ (20 min) to block endogenous peroxidase activity. After rinsing in phosphate saline buffer (PBS), pH 7.4, containing 0.2% Triton X-100 and 0.1% bovine serum albumin (BSA), background blocking was achieved by incubating the sections with 1:5 normal rabbit serum (S-1000; Vector, Burlingame, CA, USA) for 30 min at RT. Then, the sections were incubated in a humid chamber for 24 h at 4°C with the following primary antibodies: a) rabbit polyclonal antibody (GDNF1) against an epitope mapping near the C-terminus of human GDNF (D-20, sc-328; Santa Cruz Biotechnology, Santa Cruz, CA, USA; the synthetic peptide used to raise the antibody is NLVSDKVGQACCRPIAFDDD); b) rabbit polyclonal antibody (GDNF2) against C-terminus of human GDNF (ab27570; Abcam plc, Cambridge, UK; the synthetic peptide used to raise the antibody is DDLSF LDDNLVYHILRKHSA). After incubation, the sections were washed in PBS, incubated with antiserum raised in goat against rabbit IgG (GAR, 1:50; Z0421, Dako, Santa Barbara, CA, USA) for 30 min at RT, then washed in PBS and incubated with rabbit PAP complex (1:100; Z0113, Dako) for 30 min at RT. The sections were rinsed again, and the immunoreactive sites were visualized using a fresh solution of 10 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co) in 15 ml of a 0.5 M Tris buffer, pH 7.6, containing 1.5 ml of 0.03% $H_2O_2$ . Sections were lightly counterstained with Mayer's haematoxylin in order to ascertain structural details.

### *Controls*

The specificity of the immunoreactivity was tested by successively substituting either the primary, secondary antisera, or the PAP complex with PBS or normal serum, in repeated trials.

Adsorption controls were performed by using GDNF1 and GDNF2 antibodies pre-adsorbed with an excessive amount of its homologous (25µg/ml) and heterologous (50 µg/ml) antigens. The control peptides were GDNF (sc-328 P), persephin (sc-8686 P), neurturin (sc-6362 P) and artemin (sc-9330 P) (Santa Cruz Biotechnology).

For dot blotting analysis, strips of nitrocellulose were cut, and 2 ml drops of control peptides at varying concentrations (50 µg, 25 µg, 12.5 µg, 6.25 µg, 3.125 µg) were spotted on the strips and allowed to dry. The strips were fixed with Bouin's fluid for 1 h. The procedure includes the following steps: 1) 25 min washing in PBS and 1% Triton X (PBS-T); 2) blocking with normal goat serum (1/5) for 1 h at RT ; 3) 5 min PBS-T washing; 4) incubation with GDNF1 and GDNF2 antibody overnight at 4°C; 5) 30 min washing in PBS-T; 6) 30 min incubation with GAR (1/100); 7) 30 min PBS-T washing; 8) 30 min incubation with PAP (1/200); 9) 30 min PBS-T washing; and 10) 10-45 min incubation with DAB.

### *Synthesis of RNA probes and in situ hybridization*

A 700 bp cDNA of zebrafish GDNF, cloned into pBluescript KS-(Clontech, Mountain View, CA, USA), was kindly provided by Prof. Shepherd and it was used as a template to generate sense and anti-sense RNA probes. The plasmid was linearized with Not1 using T7 and T3 RNA polymerases (as suggested by Prof. Shepherd, personal communication). The reagents were added at RT in the following order: 10ml sterile distilled water, 4ml 5x transcription buffer (Promega, Madison, WI, USA), 2 ml 0.1 M 1,4-Dithiothreitol (DTT; Promega), 2 ml digoxigenin (DIG) nucleotide mix pH 8.0 (Roche, Nutley, NJ, USA), 1 ml linearized GDNF plasmid (1 mg/ml), 0.5 ml ribonuclease inhibitor (100 U/ml) (Roche), 1 ml T3 or T7 RNA polymerase (10 U/ml, Promega). After incubation at 37°C for 2 h, 2 ml of ribonuclease-free DNase I (Roche) were added, and incubation was continued for an additional 15 min. Precipitation of the synthesized RNA was achieved by adding 100 ml TE (10 mM Tris-HCl, 1 mM EDTA), 10 ml of 4 M lithium chloride and 300 ml ethanol, and storing the tube at -20°C for 30 min. The RNA was then centrifuged at 13,000 rpm for 10 min, the pellet was washed with 70% ethanol and air-dried. The RNA was reconstituted in 50 ml TE, and 5 ml were run on a 1% agarose/TBE gel to assess its quality and concentration. The RNA probe was diluted to 0.1 mg/ml and stored at -80 ºC.

Frozen sections of four adult zebrafish were processed for in situ hybridization experiments as described by Radaelli et al. (2005). Briefly, riboprobes (500 ng/ml final concentration) were resuspended in the following hybridization buffer: 50% (deionized) formamide, 1 x SSC (Saline-Sodium Citrate buffer), 10% dextran sulfate, 1x Denhardt solution, 0.67 M NaCl, 0.1 mg/ml yeast tRNA, and 0.1 mg/ml herring sperm DNA. Approximately 50 ml of hybridization buffer containing the probe were used per slide, overlaid with a coverslip. Hybridization was performed overnight at 48°C in a humidified incubator. Coverslips were removed by rinsing in 6x SSC, followed by two high-stringency steps at 50°C for 20 min in 0.5x SSC and 20% formamide and two rinses in 2x SSC at RT. Unhybridized probe was digested with 2 mg/ml RNase A in 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, at 37°C for 30 min, followed by five washes in 2x SSC at RT and another high-stringency wash for 10 min. The sections were rinsed twice with 2x SSC and maleic buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5), and blocked twice for 15 min and once for 60 min with 5% inactivated BSA in maleic buffer at RT. Methods for the detection of hybridized probes were adapted from the manufacture's protocols using the anti-DIG antibody conjugated with fluorescein (FITC) (Roche).

### *Histological photographies*

Immunocytochemical stainings were photographed

using a Leica microscope DM RA2 (Leica Camera AG, Solms, Germany) attached to a Leica DC 300 F camera and stored in Leica IM1000 archive. In situ hybridization stainings were photographed using a Leica TCS-SP2 confocal laser scanning microscope (CLSM). Photomicrograph processing and lettering was carried out with the CorelDRAW software (version 10; Corel Corporation, Ottawa, Canada). Color balance, contrast, and brightness of the images were adjusted to a variable extent and most of the color images were converted to gray-scale.

## Results

### *RT-PCR and Western blotting analysis*

Transcripts of GDNF mRNA were observed in the brain extracts of adult specimens of Danio rerio (Fig. 1A), by means of a standard RT-PCR (band of 334 bp). The band consisting of 334 nucleotides belongs to a conserved portion of the molecule (Shepherd et al., 2001). The nucleotidic identity of our GDNF fragment (100% identity to the GDNF zebrafish sequence AF329853) was assessed performing the BLASTN and the BLASTP analysis on the complete, non-redundant GenBank database sequences. A ß-actin fragment of 406 bp was also amplified on the same brain samples as a control (Fig. 1B).

Adult zebrafish brain homogenates subjected to SDS-PAGE electrophoresis and Western blotting analysis showed the presence in the brain of one protein with a molecular weight corresponding to 27 kDa (Fig. 1C).

### *Immunohistochemistry*

Immunoreactivity (IR) to GDNF was observed in all regions of the brain of all studied animals. No different localizations were obtained by using antibody GDNF1 and GDNF2.

Controls obtained by substituting the primary antibodies with PBS, normal serum or antibody adsorbed by their antigens showed the lack of reaction. On the contrary, substitution of primary antibodies with antibodies adsorbed by correlated antigens did not modify the reaction. Dot blot analysis demonstrated the lack of any cross-reaction of the employed antibodies.

The description of the brain is based on the topographical atlas of Wullimann et al. (1996). The nomenclature of the optic tectum layers is according to Vanegas et al. (1974).

The distribution of positive nuclei are summarized in Fig. 2A-I. In the nuclei, positive neurons showed varying staining intensities. Fibres positive to GDNF antiserum were seen in numerous regions of the brain.

Telencephalon

The telencephalon includes rostral paired olfactory bulbs and adjacent dorsal and ventral areas. The olfactory bulbs, and especially fibres of their glomerular layers were strongly immunoreactive to GDNF antiserum. Fibres and small neurons with scarce cytoplasm in the lateral and medial zone of dorsal telencephalic area were also immunoreactive to GDNF antiserum (Fig. 2A,B, 3A).

Diencephalon

The diencephalon includes the area preoptica, the epithalamus, the dorsal thalamus, the ventral thalamus,

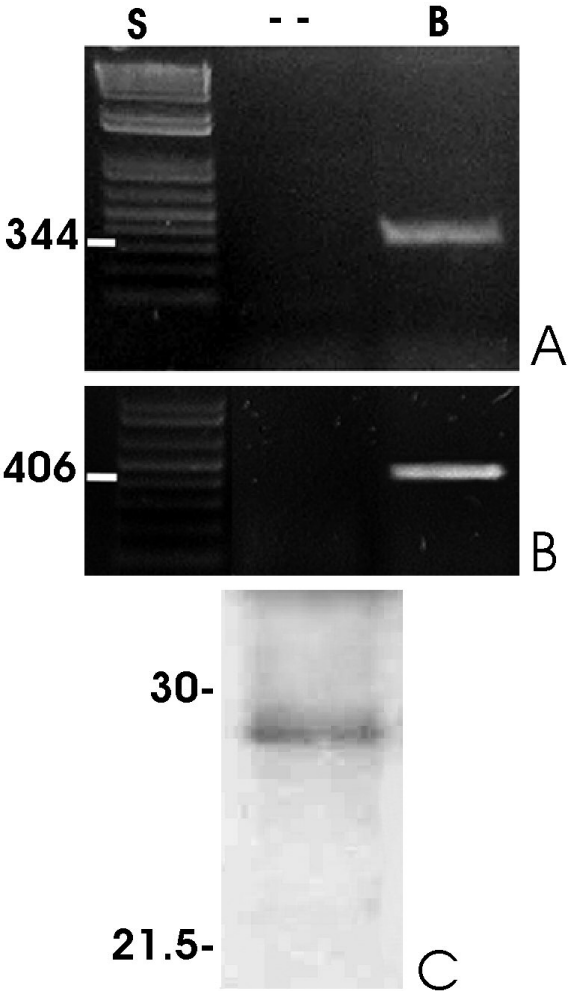


**Fig. 1.** RT-PCR and Western blotting profiles. **A.** GDNF mRNA expression in adult zebrafish brain. (s) molecular size standard (1Kb Plus DNA Ladder, Invitrogen), (--) negative control. Specific primers amplified a band of 334 base in the lane of brain extracts. **B.** a ß-actin 406 base pars fragment was amplified as a control to exclude a genomic contamination and to validate the purity of cDNAs. **C.** electrophoretic profile of homogenates from adult zebrafish brain. Molecular marker sizes are indicated on the left. A band corresponds to a 27 kDa protein. Similar results were obtained in at least four experiments of identical design.

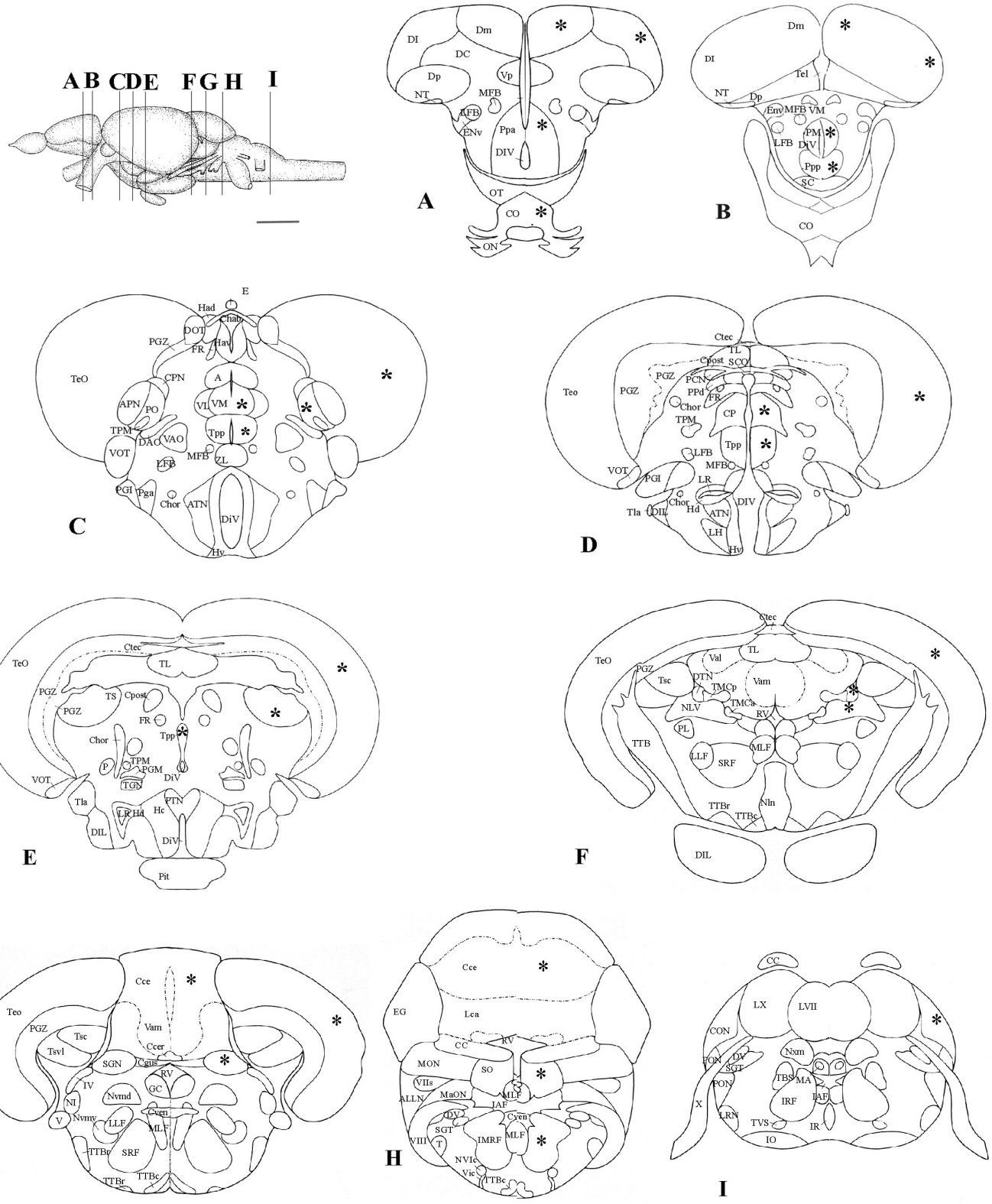


**Fig. 2.** Schematic drawings of transverse sections of adult zebrafish brain. The complete drawings show, on the left side, the cytoarchitecture of the zebrafish brain and, on the right, the distribution of GDNF positive neurons (asterisks). For abbreviations, see Table 1. Scale bar: 200 μm. Modified by Wullimann et al., (1996).

the posterior tuberculum, the hypothalamus (further divided into dorsal, ventral and caudal zones), the synencephalon and the pretectum. In the area preoptica, GDNF neurons were seen in the parvocellular and magnocellular preoptic nucleus (Fig. 2A-B, 3B-C). They often showed a single process. In the dorsal thalamus,

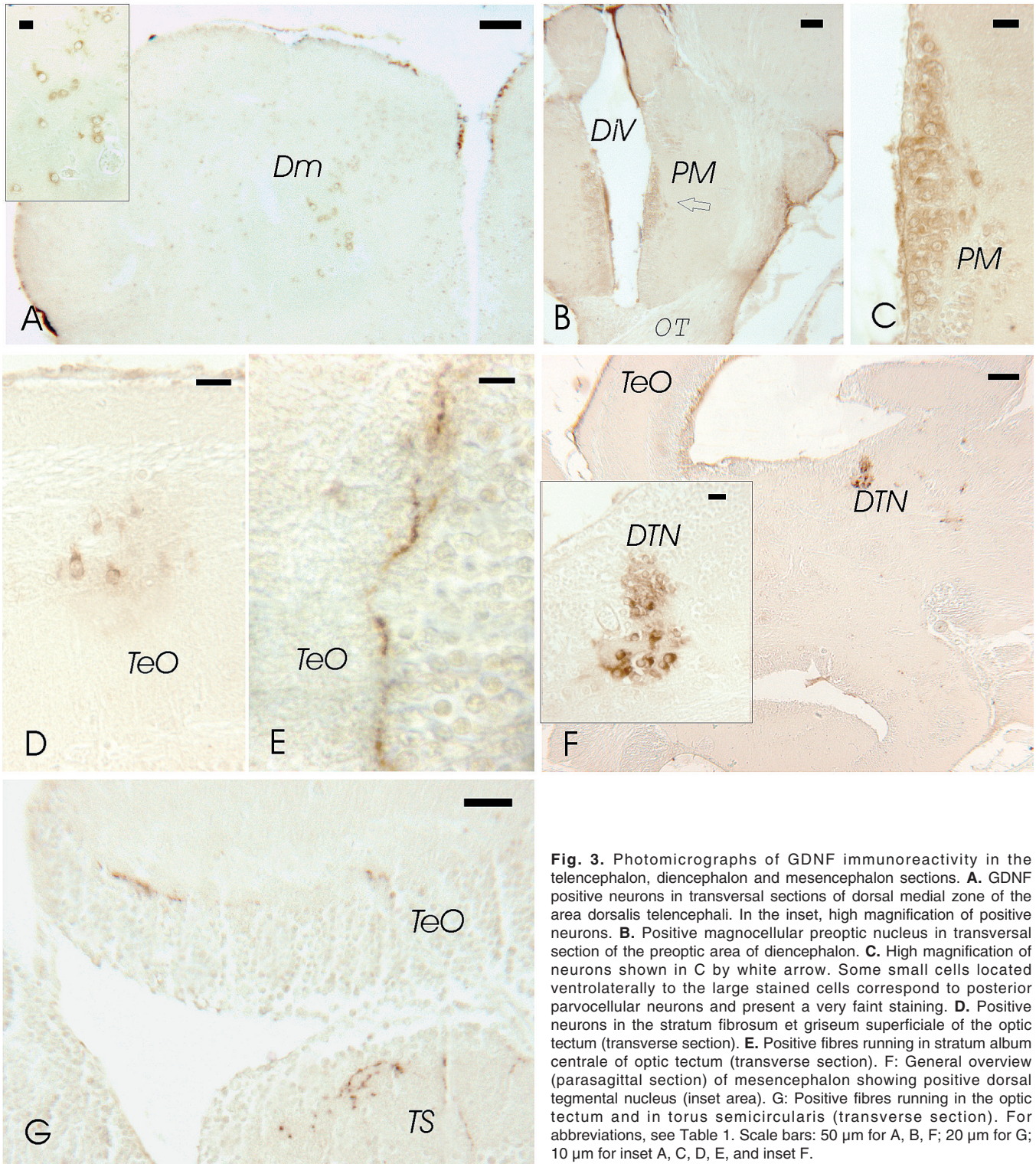


**Fig. 3.** Photomicrographs of GDNF immunoreactivity in the telencephalon, diencephalon and mesencephalon sections. **A.** GDNF positive neurons in transversal sections of dorsal medial zone of the area dorsalis telencephali. In the inset, high magnification of positive neurons. **B.** Positive magnocellular preoptic nucleus in transversal section of the preoptic area of diencephalon. **C.** High magnification of neurons shown in C by white arrow. Some small cells located ventrolaterally to the large stained cells correspond to posterior parvocellular neurons and present a very faint staining. **D.** Positive neurons in the stratum fibrosum et griseum superficiale of the optic tectum (transverse section). **E.** Positive fibres running in stratum album centrale of optic tectum (transverse section). F: General overview (parasagittal section) of mesencephalon showing positive dorsal tegmental nucleus (inset area). G: Positive fibres running in the optic tectum and in torus semicircularis (transverse section). For abbreviations, see Table 1. Scale bars: 50 µm for A, B, F; 20 µm for G; 10 µm for inset A, C, D, E, and inset F.

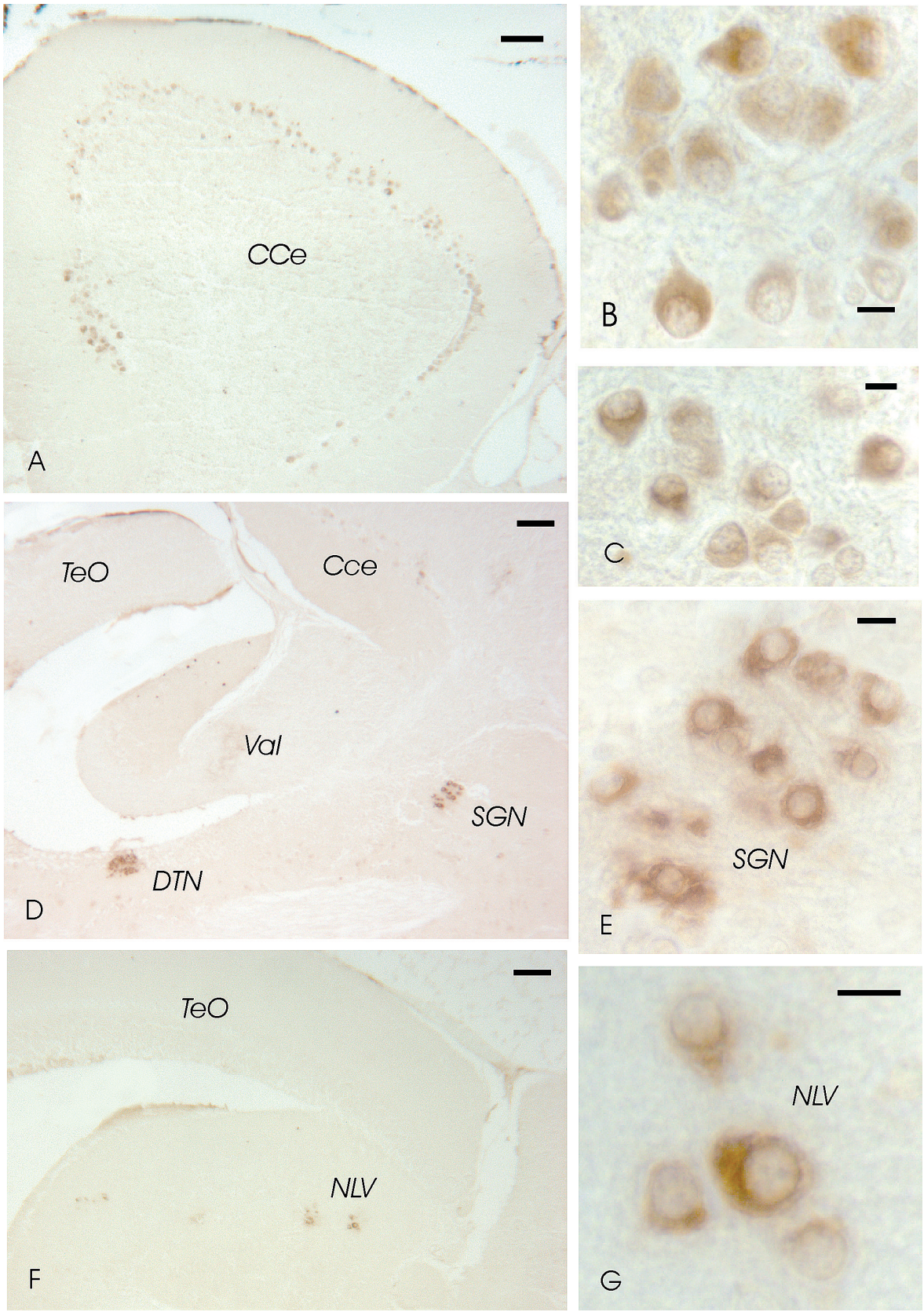


**Fig. 4.** Photomicrographs of GDNF immunoreactivity in mesencephalon and rhombencephalon sections. **A.** Corpus cerebelli showing positive Purkinje cells. **B, C.** Purkinje cells at higher magnification. **D.** Overview of the mesencephalon and the rombencephalon showing the secondary gustatory nucleus. **E.** Secondary gustatory nucleus shown in D at higher magnification. **F.** Section of valvula cerebelli and tectum opticum showing nucleus lateralis valvulae. **G.** Nucleus lateralis valvulae shown in F at higher magnification. For abbreviations, see Table 1. Scale bars: 50 µm for A, D and F; 5 µm for B, C, E, G.

small GDNF positive neurons were seen in the central posterior nucleus (Fig. 2D). In the ventral thalamus, positive neurons were seen in the ventromedial thalamic nucleus (Fig. 2C). Most neurons exhibited one process toward the diencephalic ventricle. In the posterior tuberculum, positive neurons were observed in the periventricular nucleus (Fig. 2C-E). GDNF immunoreactive fibres were scattered throughout all the hypothalamus and in the pretectum, and some intensely stained neurons were seen in the posterior pretectal nucleus (Fig. 2C).

Mesencephalon

The mesencephalon dorsoventrally includes the optic tectum, the torus semicircularis and the tegmentum. Some GDNF positive neurons were seen in the stratum fibrosum et griseum superficiale of the optic tectum (Fig. 2C-G, 3D). The somata of these cells showed their long axis perpendicularly oriented to tectum strata. The IR was preferentially localized in the supranuclear region. Some positive fibres were observed in the stratum album centrale of the optic tectum (Fig. 2C-G, 3E, 3G). GDNF positive neurons were seen in the dorsal tegmental nucleus (Fig. 2F, 3F, 4D). Positive fibres were additionally observed in the torus semicircularis (Fig. 2E, 3G).

Rhombencephalon

In fish, the rhombencephalon includes the cerebellum and the medulla oblongata. In the cerebellum, mostly Purkinje cells showed strong GDNF IR (Fig. 2H, 4A-C). In the medulla oblongata, GDNF

**Table 1.** List of abbreviations.

A: antherior thalamic nucleus
ALLN: anterior lateral line nerve
APN: accessory pretectal nucleus (of Wulliman and Meyer 90)
ATN: anterior tuberal nucleus
CC: cerebellar crest
CCe: cerebellar corpus
Ccer: cerebellar commissure
Cgus: commissure of the secondary gustatory nuclei
Chab: habenular commissure
Chor: horizontal commissure
CO: optic chiasm
CON: caudal octavolateralis nucleus
CP: central posterior thalamic nucleus
CPN: central pretectal nucleus
Cpost: posterior commissure
Ctec: tectal commissure= intertectal commissure
Cven: ventral rhombencephalic commissure
DAO: dorsal accessory optic nucleus
Dc: central zone of dorsal telencephalic area
Dl: lateral zone of dorsal telencephalic area
DIL: diffuse nucleus of the inferior lobe
DiV: diencephalic ventricle
Dm: medial zone of dorsal telencephalic area
DON: descending octaval nucleus
DOT: dorsomedial optic tract
Dp: posterior zone of dorsal telencephalic area
DTN: dorsal tegmental nucleus
DV: descending trigeminal root
E: epiphysis=pineal organ
EG: granular eminence
ENv: entopeduncular nucleus, ventral part
FR: habenulo-interpeduncular tract
GC: central gray
Had: dorsal habenular nucleus
Hav: ventral habenular nucleus
Hc: caudal zone of periventricular hypothalamus
Hd: dorsal zone of periventricular hypothalamus
Hv: ventral zone of periventricular hypothalamus
IAF: inner arcuate fibres
IMRF: intermediate reticular formation
IO: inferior olive
IR: inferior raphe
IRF: inferior reticular formation
IV: trochlear nerve
LCa: caudal lobe of cerebellum
LFB: lateral forebrain bundle
LH: lateral hypothalamic nucleus
LLF: lateral longitudinal fascicle= lateral lemniscus
LR: lateral recess of diencephalic ventricle
LRN: lateral reticular nucleus
LVII: facial lobe
LX: vagal lobe
MA: Mauthner axon
MaON: magnocellular octaval nucleus
MFB: medial forebrain bundle
MLF: medial longitudinal fascicle
MON: medial octavolateralis nucleus
NI: isthmic nucleus
Nln: interpeduncular nucleus
NLV: nucleus lateralis valvulae
NT: nucleus taeniae
NVlc: abducens nucleus, caudal part
NVmd: trigeminal motor nucleus, dorsal part
NVmv: trigeminal motor nucleus, ventral part
NXm: vagal motor nucleus
ON: optic nerve
OT: optic tract
P: posterior thalamic nucleus
PCN: paracommissural nucleus
PGa: anterior preglomerular nucleus
PGl: lateral preglomerular nucleus
PGm: medial preglomerular nucleus
PGZ: periventricular gray zone of optic tectum
Pit: hypophysis= pituitary
PL: perilemniscular nucleus
PM: magnocellular preoptic nucleus
PO: posterior pretectal nucleus
PON: posterior octaval nucleus
PPa: parvocellular preoptic nucleus, anterior part
PPd: periventricular pretectal nucleus, dorsal part
PPp: parvocellular preoptic nucleus, posterior part
PTN: posterior tuberal nucleus
RV: rhombencephalic fourth ventricle
SC: suprachiasmatic nucleus
SCO: subcommissural organ
SGN: secondary gustatory nucleus
SGT: secondary gustatory tract
SO: secondary octaval population
SRF: superior reticular formation
T: tangential nucleus
TBS: bulbo-spinal tract
Tel V:telencephalic ventricles
TeO: optic tectum
TGN: tertiary gustatory nucleus
TL: longitudinal torus
TLa: lateral torus
TMCa: anterior mesencephalo- cerebellar tract
TMCp: posterior mesencephalo- cerebellar tract
TPM: pretecto-mammillary tract
TPp: periventricular nucleus of posterior tuberculum
TS: torus semicircular
TSc: central nucleus of semicircular torus
TSvl: ventrolateral nucleus of semicircular torus
TTB: tecto-bulbar-tract
TTBc: crossed tecto-bulbar tract
TTBr: direct tecto-bulbar tract
TVS: vestibulo-spinal tract
V: trigeminal nerve
Val: lateral division of valvula cerebelli
Vam: medial division of valvula cerebelli
VAO: ventral accessory optic nucleus
VIc: caudal root of the abducens nerve
VIIs: sensory root of the facial nerve
VIII: octaval nerve
VL: ventrolateral thalamic nucleus
VM: ventromedial thalamic nucleus
VOT: ventrolateral optic tract
Vp: postcommissural nucleus of ventral telencephalic area
X: vagal nerve
ZL:zona-limitans

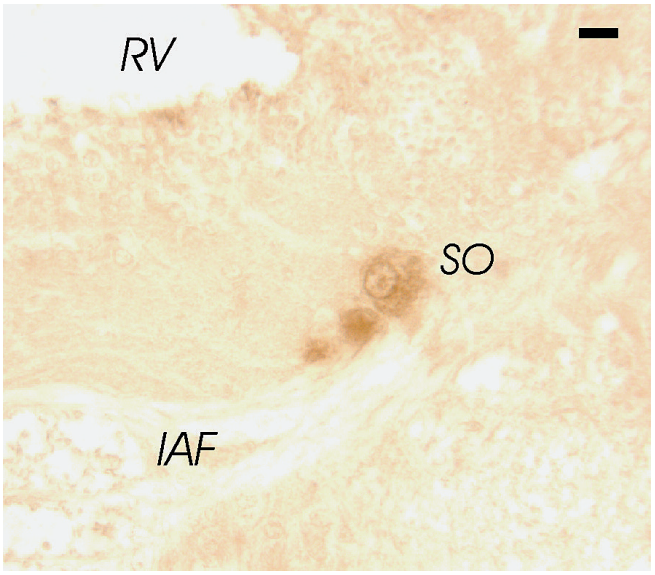


**Fig. 5.** GDNF positive large neurons in the secondary octaval population (transverse section). For abbreviations, see Table 1. Scale bar: 10 µm.

positive neurons were seen in the secondary gustatory nucleus (Fig. 2G, 4D-E). Large neurons immunoreactive to GDNF were observed in caudal octavolateralis nucleus (Fig. 2I). GDNF positive neurons belonging to the intermediate reticular formation (Fig. 2H) showed a single process. Furthermore, intensely stained neurons were seen in the nucleus lateralis valvulae (Fig. 2F, 4F-G). Some large neurons belonging to the secondary octaval population displayed IR to GDNF (Fig. 2H, 5).

### *In situ hybridization*

In order to confirm the presence of GDNF protein in neurons of the zebrafish brain, in situ hybridization analysis was performed. In the lateral and central zone of the dorsal telencephalic area, mRNA transcripts for GDNF were found in small neurons (Fig. 6A). In the area preoptica, GDNF expressing neurons were seen in the magnocellular preoptic nucleus (Fig. 6B). In the ventral thalamus, GDNF was expressed in neurons of the ventromedial thalamic nucleus (Fig. 6C). In the tegmentum, GDNF expression was seen in neurons of the dorsal tegmental nucleus (Fig. 6D). In the medulla oblongata, GDNF expressing neurons were seen in the secondary gustatory nucleus (Fig. 6E).

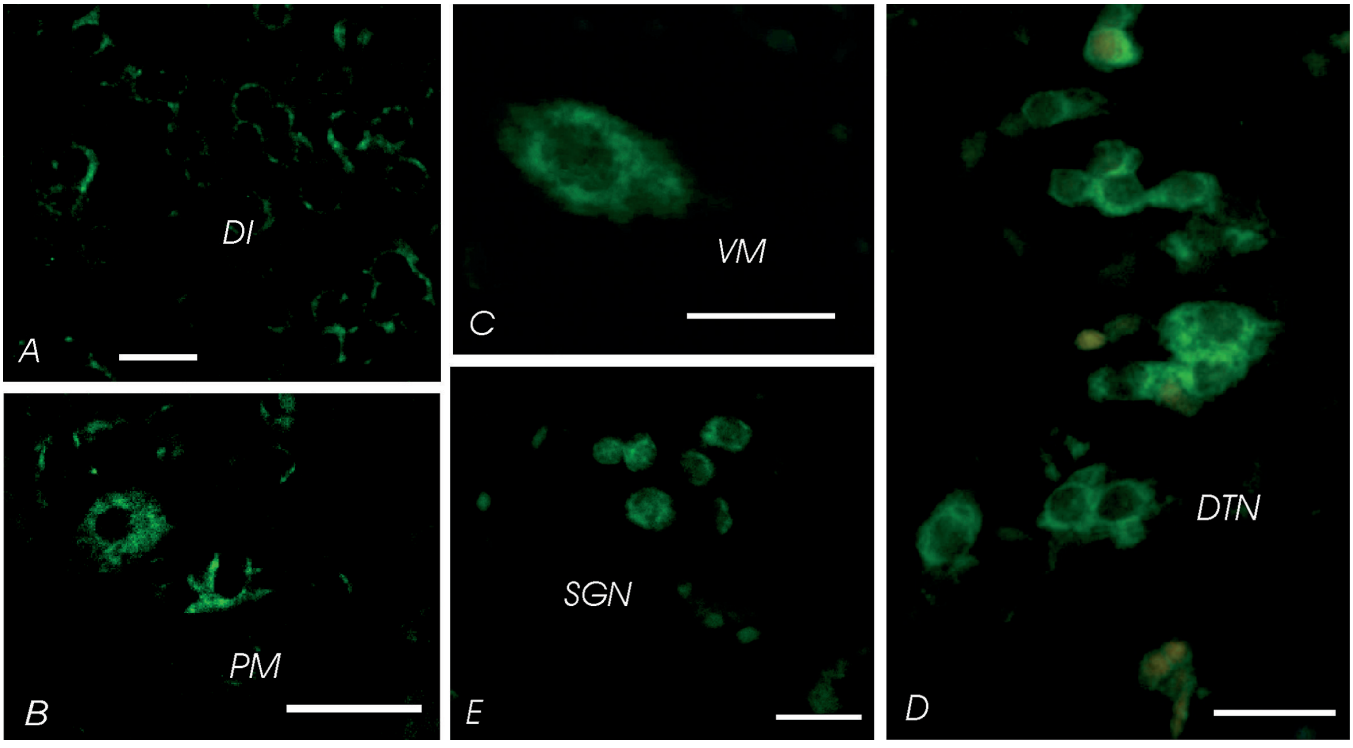


**Fig. 6.** Photomicrographs of GDNF in situ hybridization. **A.** GDNF expression in lateral zone of the dorsal telencephalic area. **B.** GDNF expression in the magnocellular preoptic nucleus of the preoptic area of diencephalon. **C.** GDNF expression in the ventromedial thalamic nucleus of the ventral thalamus. **D.** GDNF expression in the dorsal tegmental nucleus. **E.** GDNF expression in the secondary gustatory nucleus of the rhombencephalon. For abbreviations, see Table 1. Scale bars: 30 µm for C, 50 µm for E, 40 µm for A and 25 µm for B and D.

## Discussion

In this study the presence and the distribution of GDNF in the brain of adult zebrafish were demonstrated by exploiting different experimental approaches, including RT-PCR, Western blotting, immunohistochemistry and in situ hybridization. These findings are, to our knowledge, the first description of extensive GDNF localization in the brain of an adult teleostean fish. Previously, GDNF mRNA and protein distribution has been extensively described only in adult and developing central nervous system of man, rat and mouse (Choi-Lundberg and Bohn, 1995; Tomac et al., 1995; Nosrat et al., 1996; Golden et al., 1998; Kawamoto et al., 2000a,b; Del Fiacco et al., 2002; Serra et al., 2002).

The results obtained by RT-PCR and Western blotting analysis suggested the expression of both GDNF mRNA and the correspondent protein in the brain of adult zebrafish. The PCR product was cloned and sequenced in order to confirm its identity as a GDNF molecule. The nucleotidic sequence of our fragment appeared to be identical (100% of identity) to the GDNF sequence submitted in GenBank by Sheperd et al. (2001). The electrophoretic profile of brain extracts showed a band of 27 kDa, a molecular weight in accordance with that predicted by the study of Shepherd et al. (2001). Regarding the distribution of GDNF, it occurs in various regions of the adult zebrafish brain involved in sensory neurotransmission and integrative roles. Regarding the sensory systems, the presence of GDNF IR in the olfactory bulbs and in the lateral and medial zones of the dorsal telencephalic area suggests that it might functionally contribute to the processing of olfactory stimuli. The staining was also seen to occur in the caudal octavolateral nucleus, which receives primary projections of lateral line mechanoreceptors (signals from hair cells in neuromasts of the lateral line system). Furthermore, GDNF IR was observed in neurons belonging to the central posterior thalamic nucleus and in the secondary octaval population of neurons which projects to the auditory torus semicircularis. These findings lead to the proposal that GDNF is involved in mechanoreceptive and auditory reception. GDNF also seems to be involved in the optic/somatosensorial pathway because of its presence in the ventromedial thalamic nucleus and in the gustatory centers of the secondary gustatory nucleus.

The occurrence of GDNF protein in integrative fish brain regions, such as the dorsal telencephalic area, cerebellar Purkinje cells and mesencephalic optic tectum, suggests an involvement of this neurotrophic factor in the regulation of complex neuronal interactions. In particular, Purkinje cells of rat and human cerebellum were shown to contain GDNF mRNA (Golden et al., 1998) and protein (Kawamoto et al., 2000a). Furthermore, in experimental studies GDNF was seen to promote the survival and morphological differentiation of Purkinje cells (Mount et al., 1995; Tolbert et al., 2001). Thus, it may be presumed that GDNF is produced and localized in the Purkinje cells and, according to Kawamoto et al., 2000a, also acts on the Purkinje cells themselves. In zebrafish, Purkinje cells were strongly immunopositive to GDNF. However, the self-regulation of Purkinje cells by GDNF needs further investigations, including the analysis of GDNF receptor distribution in the cerebellum. Moreover, the mesencephalic optic tectum showed few GDNF positive neurons in the superficial grey layer, with GDNF positive fibres running periventricularly to the cortex as well as among cortical layers. These fibres could originate from neurons in the optic tectum, although they could also represent branches of GDNF positive neurons belonging to the ventromedial thalamic nucleus, which is known to project to the optic tectum (Meek, 1990). In this respect, it is worth noting that the mesencephalic dorsal tegmental nucleus and nucleus lateralis valvulae, which in this study showed some GDNF positive neurons, in the goldfish resulted projecting to the cerebellum (Wullimann and Northcutt, 1989).

Numerous nuclei involved in sensitive and integrative functions of the zebrafish brain contained, besides GDNF protein, GDNF mRNA transcripts. Thus, these findings further confirm immunohystochemical data and suggest that numerous neuronal population of zebrafish brain synthesise GDNF. It is tempting to argue that in the central nervous system of zebrafish GDNF interaction could be transynaptic, allowing the coordination of different region responses.

In regions of the zebrafish brain involved in effector function, GDNF IR was not detected. However, GDNF IR was observed in neurons of the reticular formation, whose nuclei are involved in pre-motor function.

In conclusion, this study demonstrates that the presence of GDNF is not limited to developmental periods and it seems that this factor might be involved in the adult zebrafish brain, as previously suggested in mammals. Similarly to roles reported in adult mammalian brain, GDNF in zebrafish could play a neuronal maintenance and neurotransmiter regulation role as suggested by the presence of GDNF receptor RET in numerous neuronal populations of adult zebrafish brain (our unpublished data). Furthermore, the central nervous system of teleost fish was seen to have particular regenerative properties (Zupanc and Clint, 2003). However, the presence and distribution of GDNF in zebrafish brain and spinal cord did not appear particularly more abundant and widespread than in mammals. On the other hand, the fundamental role of GDNF in neuronal repairs is well known. Thus, GDNF could be involved in regenerative processes only successively tissue damages, although further studies are needed on this subject and the present findings might represent a basic starter point.

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## References

Choi-Lundberg D.L. and Bohn M.C. (1995). Ontogeny and distribution of glial cell line-derived neurotrophic factor (GDNF) mRNA in rat. Brain Res. Dev. Brain Res. 85, 80-88.

Del Fiacco M., Quartu M., Serra M.P., Follesa P., Lai M.L. and Bachis A. (2002). Topographical localization of glial cell line-derived neurotrophic factor in the human brain stem: an immunohistochemical study of prenatal, neonatal and adult brains. J. Chem. Neuroanat. 23,29-48.

Golden J.P., Baloh R.H., Kotzbauer P.T., Lampe P.A., Osborne P.A., Milbrandt J. and Johnson E.M. Jr (1998). Expression of neurturin, GDNF, and their receptors in the adult mouse CNS. J. Comp. Neurol. 398, 139-150.

Kawamoto Y., Nakamura S., Matsuo A. and Akiguchi I. (2000a). Glial cell line-derived neurotrophic factor-like immunoreactivity in the cerebella of normal subjects and patients with multiple system atrophy. Acta Neuropathol. 100, 31-137.

Kawamoto Y., Nakamura S., Matsuo A., Akiguchi I. and Shibasaki H. (2000b). Immunohistochemical localization of glial cell line-derived neurotrophic factor in the human central nervous system. Neuroscience 100, 701-712 .

Kreuzer K.A., Lass U., Landt O., Nitsche A., Laser J., Ellerbrok H., Pauli G., Huhn D. and Schmidt C.A. (1999). Highly sensitive and specific fluorescence reverse transcription-PCR assay for the pseudogene-free detection of beta-actin transcripts as quantitative reference. Clin. Chem. 45, 297-300.

Lucini C., Maruccio L., Tafuri S., Bevaqua M., Staiano N. and Castaldo L. (2005). GDNF family ligand immunoreactivity in the gut of teleostean fish. Anat. Embryol. 210, 265-274.

Meek J. (1990). Tectal morphology: connections, neurons and synapses. In: The visual system of fish. Douglas R.H. and Djamgoz M.B.A (eds). Chapman and Hall. London. pp 239-277.

Mount H.T., Dean D.O., Alberch J., Dreyfus C.F. and Black I.B. (1995). Glial cell line-derived neurotrophic factor promotes the survival and morphologic differentiation of Purkinje cells. Proc. Natl. Acad. Sci. USA 92, 9092-9096. Erratum in: Proc. Natl. Acad. Sci. USA 92, 1145.

Nosrat C.A., Tomac A., Lindqvist E., Lindskog S. and Stromberg I. (1996). Cellular expression of GDNF mRNA suggests multiple functions inside and outside the nervous system. Cell Tissue Res. 286, 191-207.

Radaelli G., Patruno M., Rowlerson A., Maccatrozzo L. and Funkenstein B. (2005). Cellular localisation of insulin-like growth factor binding protein-2 (IGFBP-2) during development of the marine fish, Sparus aurata. Cell Tissue Res. 319, 121-131.

Reynolds G.M., Young F.I., Williams A. and Rowlands D.C. (1994). Microwave-oven antigen retrieval applied to the immunostaining of cytopathology specimens. Cytopathology 5, 345-358.

Sariola H. and Saarma M. (2003). Novel functions and signalling pathways for GDNF. J. Cell Sci. 116, 3855-3862.

Serra M.P., Quartu M., Lai M.L., Follesa P. and Del Fiacco M. (2002). Expression of glial cell line-derived neurotrophic factor mRNA in the human newborn and adult hippocampal formation. Brain Res. 928, 160-164.

Shepherd I.T., Beattie C.E. and Raible D.W. (2001). Functional analysis of zebrafish GDNF. Dev. Biol. 231, 420-435.

Shepherd I.T., Pietsch J., Elworthy S., Kelsh R.N. and Raible D.W. (2004). Roles for GFRalpha1 receptors in zebrafish enteric nervous system development. Development 131, 241-249.

Sternberger L.A. (1986). Immunocytochemistry. 3rd. John Wiley and Sons. New York.

Tolbert D.L., Bradley M.W., Tolod E.G., Torres-Aleman I. and Clark B.R. (2001). Chronic intraventricular infusion of glial cell line-derived neurotrophic factor (GDNF) rescues some cerebellar Purkinje cells from heredodegeneration. Exp. Neurol. 170, 375-379.

Tolbert D.L. and Clark B.R. (2003). GDNF and IGF-I trophic factors delay hereditary Purkinje cell degeneration and the progression of gait ataxia. Exp. Neurol. 183, 205-219.

Tomac A., Widenfalk J., Lin L.F., Kolmo T., Ebendal T., Hoffer B.J. and Olson L. (1995). Retrogade axonal transport of glial cell line-derived neurotrophic factor in the adult nigrostriatal system suggests a trophic role in the adult. Proc. Natl. Acad. Sci. USA 92, 8274-8278.

Wang Y., Chang C.F., Morales M., Chiang Y.H. and Hoffer J. (2002). Protective effects of glial cell line-derived neurotrophic factor in ischemic brain injury. Ann. NY Acad. Sci. 962, 423-437.

Wullimann M.F. and Northcutt R.G. (1989). Afferent connections of the valvula cerebelli in two teleosts, the common goldfish and the green sunfish. J. Comp. Neurol. 289, 554-567.

Wullimann M.F., Rupp B. and Reichert H. (1996). Neuroanatomy of the zebrafish brain. A topological atlas. Birkhäuser Verlag. Berlin.

Vanegas H., Laufer M. and Amat J. (1974). The optic tectum of a perciform teleost. I General configuration and cytoarchitecture. J. Comp. Neurol. 154, 43-60.

Zhao Z., Alam S., Oppenheim R.W., Prevette D.M., Evenson A. and Parsadanian A. (2004). Overexpression of glial cell line-derived neurotrophic factor in the CNS rescues motoneurons from programmed cell death and promotes their long-term survival following axotomy. Exp. Neurol. 192, 356-372.

Zupanc G.K. and Clint S.C. (2003). Potential role of radial glia in adult neurogenesis of teleost fish. Glia 43, 77-86.

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