Immunoreactivity to glial cell line-derived neurotrophic factor and its receptors in the trout pancreas: a further endocrine-exocrine relationship?

C. Lucini, L. Maruccio, R. Antonucci, L. Castaldo
Dip Strutture, Funzioni e Tecnologie Biologiche, Università di Napoli Federico II, Napoli, Italy

Glial cell line-derived neurotrophic factor (GDNF) is a growth factor promoting the survival of several neuronal populations 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 exerts its roles by binding to glial cell line-derived neurotrophic factor receptor (GFR) α1, which forms a heterotetramic complex with rearranged during transfection (RET) proto-oncogene product, a tyrosine kinase receptor.
In this study we report the presence of GDNF-, RET- and GFRα1-like immunoreactivity in the pancreas of juvenile trout. GDNF immunoreactivity was observed in the islet cells, while GFRα1- and RET- immunoreactivity was observed in the exocrine portion. These findings suggest a paracrine role of GDNF towards exocrine cells showing GDNF receptors GFRα1 and RET. The relationship could reflect physiological interactions, as previously indicated in mammalian pancreas, and/or a trophic role by endocrine cells on exocrine parenchyma, which shows a conspicuous increase during animal growth.

Key words: Teleost fish, growth factors, digestive apparatus.

Correspondence: Carla Lucini,
Dip Strutture, Funzioni e Tecnologie Biologiche,
Università di Napoli Federico II, Napoli, Italy
E-mail: carla.lucini@unina.it

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In mammals, glial cell line-derived neurotrophic factor (GDNF) was initially characterized as a growth factor promoting the survival of dopaminergic neurons in the midbrain and successively was seen to support several neuronal populations 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 exerts its roles by binding to glial cell line-derived neurotrophic factor receptor (GFR) α1, which forms a heterotetramic complex with rearranged during transfection (RET) proto-oncogene product, a tyrosine kinase receptor. RET receptor also mediates, by means of other three GFRs α2-4 receptors, the signals of the other ligands related to GDNF: neurturin (GFRα2), artemin (GFRα3) and persephin (GFRα4). However, GDNF can also signal RET independently through GFRα1 (for a review see Sariola and Saarma 2003).

The zebrafish genome encodes two GDNF, two GFRapha1 and one RET (Shepherd *et al.,* 2001, 2004; Bisgrove *et al.,* 1997; Marcos-Gutierrez et al 1997; Hätinen *et al.,* 2007). In zebrafish embryos, GDNF expression was detected in the ventral somitic muscles, in the intermediate mesoderm where the pronephric duct differentiates, in the ventral trunk mesoderm and endoderm and then in developing gut tube (Shepherd *et al.,* 2001). RET expression was seen in the brain, spinal cord, cranial ganglia, pharyngeal arches, in the pronephric duct and in cells throughout the length of the intestine (Bisgrove *et al.,* 1997; Marcos-Gutierrez *et al.,* 1997). GFRα1 expression was reported in the brain, peripheral ganglia and enteric nervous system precursors (Shepherd *et al.,* 2004). Depletion of GDNF in zebrafish embryos by means of morpholino antisense oligos demonstrated that GDNF is critical for the development of the zebrafish enteric nervous system,

similarly to results obtained in mammals (Shepherd *et al.,* 2001, 2004).

However, no findings exist regarding the presence of GDNF and their receptors in the pancreas of fish embryos. In adult mammals GDNF, together with other neurotrophic factors, was reported to be localized in islets and, after induction of pancreatitis, also in acinal and ductal cells (Toma *et al.,* 2002). Moreover, GDNF was seen to promote tumour cell proliferation and invasion in pancreatic cancer cell lines (Ito *et al.,* 2005).

In adult teleost fish species, artemin was detected in adult zebrafish pancreas (Lucini *et al.,* 2004), suggesting that GFLs could be involved in the biology of fish pancreas. Thus, the aim of the present study was to investigate the presence and localization of GDNF, and its receptors in the pancreas of the trout.

Materials and Methods

Six young trouts (*Salmo gairdneri;* 30 day olds; length 1-1,5 cm), from "Centro ittiogenico" of Campania Region (Guardia dei Lombardi, AV) were obtained. The experimental protocols were conducted within the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by institutional committees of the University of Naples. Every effort was made to minimize the number of animals used and their suffering. The trouts were deeply anaesthetized by 0.1% ethyl 3-aminobenzoate, methanesulfonate (Sigma Chemicals Co, St Louis, MO, USA) before fixation by immersion in Bouin's fluid for 24-48 h at room temperature (RT). They were then 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 trouts, cut in half at the level of the posterior edge of the operculum before 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 (005-000-121, Jackson ImmunoResearch, Baltimore Pike, PA, 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) GDNF rabbit polyclonal antibody against an epitope mapping near the C-terminus of human GDNF (D-20, sc-328; Santa Cruz Biotechnology, Santa Cruz, CA, USA; b) RET rabbit polyclonal antibody against a peptide mapping at the C-terminus of RET isoform C of human origin. It recognizes both isoform A and C; c) GFRα1 rabbit polyclonal antibody against epitope corresponding to amino acids 368-437 mapping near the C-terminus of GFRα-1 of human origin (H-70, sc-10716; Santa Cruz Biotechnology, CA, USA). After incubation, the sections were washed in PBS, incubated with antiserum raised in goat against rabbit IgG (GAR, 1:50; 111-035-003 Jackson ImmunoResearch, Baltimore Pike, PA, USA) for 30 min at RT, then washed in PBS, and incubated with rabbit PAP complex (1:100; 323-005-025 Jackson Immuno Research, Baltimore Pike, PA, USA) for 30 min at RT. The sections were rinsed again, and the immunoreactive sites were visualized using a fresh solution of 10 μg 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. To estimate the relative frequency of cells imunoreactive to RET and GFRα1 antisera, the mean number of cells/0.08 mm^2 was calculated in 5 areas for each animal.

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 primary antibodies pre-adsorbed with an excessive amount of its homologous (25 μg/mL) and heterologous (50 μg/mL) antigens.

Results

The pancreas of 30 day-old trouts is a solid mass of islet tissue surrounded by exocrine tissue.

All three employed antisera against GDNF, RET and GFRα-1 intensely stained numerous cells in the trout pancreas. The GDNF antiserum showed cords of immunoreactive cells in the islet, intermingled with the other endocrine cells. The immunoreactivity was diffused in the whole cytoplasm (Figure 1 A, B). The RET antiserum strongly stained 95% of the exocrine cells. The immunoreactivity was preferentially localized in the basal pole of the cells. (Figure 1 C, D). The GFRα-1 antiserum stained 72% of the exocrine cells. The immunoreactivity was localized at the apical pole of the cells (Figure 1 E, F, G).

The replacement of all three primary antibodies with PBS, normal rabbit serum or primary antibody pre-adsorbed with homologous antigen led to a negative reaction, while the replacement of the primary antibodies with pre-adsorbed primary antibodies with heterologous antigens did not affect the reaction.

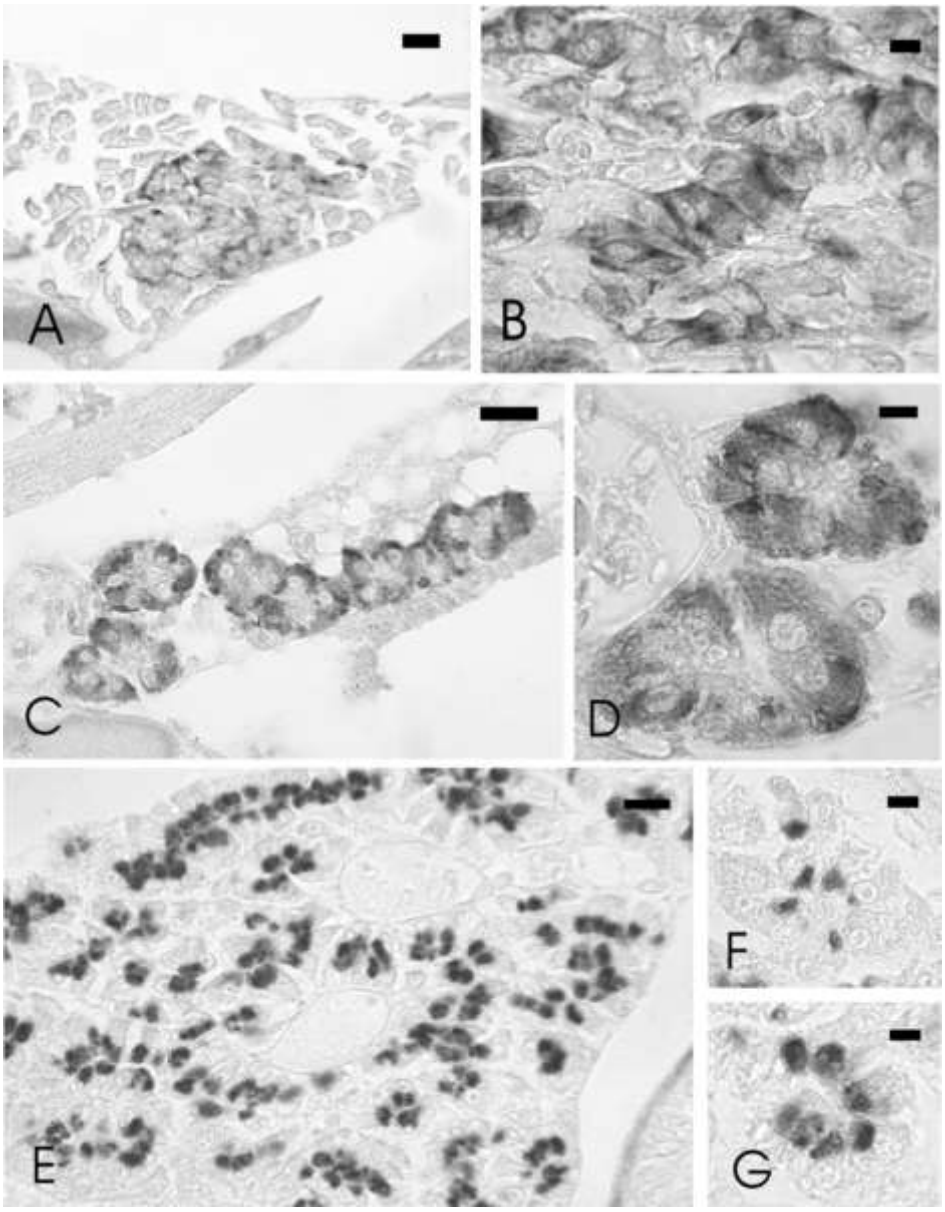

Figure 1. GDNF-, RET- and GFRα1- immunoreactivity in the trout pancreas. A, B: GDNF immunoreactivity localized in endocrine cells. The positive cells formed cordons intermingled with other negative endocrine cells. C, D: RET immunoreactivity localized at the basal pole of exocrine cells; E, F, G: GFRα1 in exocrine cells showing immunoreactivity at apical pole of the cells. Scale bars: 50 mμ (A); 20 mμ (C, E); 5 mμ (B, D, F, G).

Discussion

In this study GDNF-, RET- and GFRα-1-like immunoreactivity was observed in exocrine and endocrine pancreas of the trout. The antisera employed are against human antigens, however the accurate control experiments suggest that the proteins identified in this study are really GDNF, RET and GFRα-1. Moreover, the detection of GDNF in the endocrine pancreas and the detection of RET and GFRα-1 in the exocrine pancreas, differently localized at cytoplasmatic level, indicates the lack of cross-reactivity among antisera employed. Furthermore, in previous studies Western blotting analysis on gut tissue extracts indicated that the GDNF antiserum recognized a protein of 27 kDa (Lucini *et al.,* 2005), consistently with molecular weight predicted by sequencing analysis of *gdnf* gene (Shepherd *et al.,* 2001). At the amino acid level zebrafish GDNF has 49%, GFRα1a has 78.5% , GFRα1b has 78% of identity with human GDNF, GFRα1a and GFRα1b, respectively (Shepherd *et al.,* 2001). Zebrafish RET corresponds to the ret51 isoform (also named isoform A) present in mammals and its intracellular part has 88% of identity with human RET (Bisgrove *et al.,* 1997; Marcos-Gutierrez *et al.,* 1997). Presumably, the antiserum against GFRα1 cannot discriminate between the two zebrafish isoforms, whose sequences are very similar. Moreover GFRα1a and GFRα1b were seen to be equivalent in zebrafish embryos, as suggested by the phenotypes rescue by the introduction of mRNA for *gfrα1a* and *gfrα1b* in embryos treated by injection of antisense morpholinos against both *gfrα1* (Shepherd *et al.,* 2004).

This study is the first report, to our knowledge, regarding the presence of GDNF in fish pancreas. Previously, GDNF was seen in islets of Langerhans of the rat and its upregulation during experimentally induced pancreatitis led to hypothesize that GDNF is a critical component of the response of the pancreas to injury (Toma *et al.,* 2002). Moreover, GDNF, detected in intrapancreatic nerves and islet cells, promotes pancreatic cancer cell proliferation and intrapancreatic neural invasion. Thus, RET in cancer cells may be a useful clinical marker for pancreatic cancer and a potential target for anti-invasion therapy (Ito *et al.,* 2005).

The presence of GDNF in the trout pancreatic islets further extends the list of insular substances. In addition to the four classic islet hormones, islets of Langerhans secrete other non classic hormones: amylin, adrenomedullin, calcitonin gene-related peptide, C- peptide, pancreastatin, secretoneurin, ghrelin, cortistatin, orexin A, peptide YY, resistin, urocortin III and the corticotrophin-releasing factor (CRF) (for a review see Bertelli and Bendayan 2005). Also some neurotrophic factors have been described in endocrine pancreas: NGF (Vidaltamayo *et al.,* 2003), BDNF (Lucini *et al.,* 2003a), neurotrophin-3 (Lucini *et al.,* 2003b), and artemin (Lucini *et al.,* 2004).

In the trout pancreas GDNF immunoreactivity was observed in the islet cells, while GFRα1 and RET were observed in the exocrine portion. These findings suggest a paracrine role of GDNF towards exocrine cells showing GDNF receptors GFRα1 and RET. This hypothesis is supported by previous studies reporting that major islet hormones regulate acinar secretions via a continuous insulo-acinar venous portal system connecting the islets of Langerhans to the surrounding acinar tissue (for a review see Williams and Goldfin, 1993). Moreover, during growth, the exocrine portion of the pancreas strongly increased in mass, invading the islet tissue to such an extent that the latter is subdivided into small groups of islets (Wagner and McKeon, 1981). Thus, because in the present study we have investigated young trouts of 30 days, GDNF could exert a trophic role on exocrine cells which showed GDNF receptors. Thus, in conclusion, these findings may suggest a close relationship between endocrine/exocrine cells in the pancreas of juvenile trout.

References

Bertelli E, Bendayan M. Association between endocrine pancreas and ductal system. More than an epiphenomenon of endocrine differentiation and development. J Histochem Cytochem 2005; 53:1071-86.

Bisgrove BW, Raible DW, Walter V, Eisen JS, Grunwad DJ. Expression of c-ret in the zebrafish embryo: potential roles in motoneuronal development. J Neurobiol 1997;33:749-68.

Håtinen T, Holm L. Airaksinen MS. Loss of neurturin in frog-Comparative genomics study of GDNF family ligand-receptor pairs. Mol Cell Neurosci 2007;34:155-67.

Ito Y, Okada Y, Sato M et al. Expression of glial cell line-derived neurotrophic factor family members and their receptors in pancreatic cancers. Surgery 2005;138:788-94.

Lucini C, Maruccio L, Girolamo P, Castaldo L. Brain-derived neurotrophic factor in higher vertebrate pancreas: immunolocalization in glucagon cells. Anat Embryol 2003a;206:311-8.

Lucini C, Costagliola C, Borzacchiello G, Castaldo L. Neurotrophin 3

and its receptor TrkC immunoreactivity in glucagon cells of buffalo pancreas. Anat Histol Embryol 2003b;32: 253-6.

Lucini C, Maruccio L, Tafuri S, Bevaqua M, Staiano N, Castaldo L. GDNF family ligand immunoreactivity in the gut of teleostean fish. Anat Embryol 2005;210:265-74.

Lucini C, Maruccio L, Tafuri S, Staiano N, Castaldo L. Artemin-like immunoreactivity in the zebrafish, Danio rerio. Anat Embryol 2004; 208:403-10.

Marcos-Gutierrez CV, Wilson SW, Holder N, Pachnis V. The zebrafish homologue of the ret receptor and its pattern of expression during embryogenesis. Oncogene 1997; 14:879-89.

Reynolds GM, Young FI, Williams A, Rowlands DC. Microwave-oven antigen retrieval applied to the immunostaining of cytopathology specimens. Cytopathology 1994; 5: 345-58.

Sariola H, Saarma M. Novel functions and signalling pathways for GDNF J Cell Sciences 2003;116:3855-62.

Shepherd IT, Beattie CE, Raible DW. Functional analysis of zebrafish GDNF Dev Biol 2001;231:420-35.

Shepherd IT, Pietsch J, Elworthy S, Kelsh RN, Raible DW. Roles for GFRα1 receptors in zebrafish enteric nervous system development. Development 2004;131:241-9.

Sternberger LA. Immunocytochemistry. John Wiley & Sons 3rd ed. New York, 1986.

Toma H, Winston JH, Micci M-A, Li H, Hellmich HL, Pasricha PJ. Characterization of the neurotrophic response to acute pancreatitis. Pancreas 2002;25:31-8.

Vidaltamayo R, Mery CM, Angeles-Angeles A, Robles-Diaz G, Hiriart M. Expression of nerve growth factor in human pancreatic beta cells. Growth Factors 2003;21:103-7.

Wagner GF, McKeon BA. Immunocytochemical localization of hormone –producing cells within the pancreatic islets of the rainbow trout (Salmo gairdneri). Cell Tissue Res 1981; 221:181-92.

Williams JA, Goldfine ID.The insulin-acinar relationship. In Go VLW, Lebenthal E, DiMagno EP, Reber HA, Gardner JD, Scheele GA, eds. The Pancreas Biology, Pathobiology, and Disease. 2nd ed. New York, Raven Press 1993;301-50.