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Brain-derived neurotrophic factor (BDNF)-like immunoreactivity localization in the retina and brain of *Cichlasoma dimerus* (Teleostei, Perciformes)

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Abstract

Brain-derived neurotrophic factor (BDNF) is a neurotrophin involved in the development and maintenance of vertebrate nervous systems. Although there were several studies in classical animal models, scarce information for fish was available. The main purpose of this study was to analyze the distribution of BDNF in the brain and retina of the cichlid fish *Cichlasoma dimerus*. By immunohistochemistry we detected BDNF-like immunoreactive cells in the cytoplasm and the nuclei of the ganglion cell layer and the inner nuclear layer of the retina. In the optic tectum, BDNF-like immunoreactivity was detected in the nucleus of neurons of the stratum periventriculare and the stratum marginale and in neurons of the intermediate layers. In the hypothalamus we found BDNF-like immunoreactivity mainly in the cytoplasm of the nucleus lateralis tuberis and the nucleus of the lateral recess. To confirm the nuclear and cytoplasm localization of BDNF we performed subcellular fractionation, followed by Western blot, detecting a 39 kDa immunoreactive-band corresponding to a possible precursor form of BDNF in both fractions. BDNF-like immunoreactivity was distributed in areas related with photoreception (retina), the integration center of retinal projections (optic tectum) and the control center of background and stress adaptation (hypothalamus). These results provide baseline anatomical information for future research about the role of neurotrophins in the adult fish central nervous system.

Keywords: Neurotrophins; Teleost; Retina; Optic tectum; Hypothalamus

1. Introduction

Neurotrophins are important regulators of development and maintenance of vertebrate nervous systems (Fariñas and Reichardt, 1996). They also play critical roles in synaptic activity and plasticity in many groups of mature neurons (Lu, 2003). Neurotrophins comprise a family of growth factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, NT-6 and NT-7 (Heinrich and Lum, 2000). These last two neurotrophins are typical fish neurotrophins (Götz and Schartl, 1994; Lai et al., 1998; Nilsson et al., 1998). The primary amino acid sequence of BDNF is highly conserved among all species. Gotz et al. (1992) demonstrated in the teleost fish *Xiphophorus maculatum* that the DNA-deduced amino acid sequence of the processed mature fish BDNF showed a 90% identity with the mouse sequence. Also, the primary amino acid sequences of zebrafish (*Danio rerio*) and human BDNF are 91% identical (Hashimoto and Heinrich, 1997).

Using a mammalian BDNF antiserum, Caminos et al. (1999) found BDNF immunoreactivity (-ir) in neuronal cell bodies and Müller cell processes in the retina of the Tench *Tinca tinca*. On the other hand, in embryo and larvae of zebrafish, Lum et al. (2001) and Hashimoto and Heinrich (1997) observed BDNF-ir in the retina, otic vesicle, olfactory organ, muscle layer of the pectoral fin and the hair cells of the neuromasts among other structures, that were consistent

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with results obtained using *in situ* hybridization. Although BDNF-ir was seen in the nerve cells of mouse intestine, in very few endocrine cells of mouse and duck intestine (Lucini et al., 2002), it was not observed in the gut of several teleost species in spite of the presence of both NGF and NT-3 like immunoreactivity. However, BDNF-ir was observed in the brain of goldfish and trout (Lucini et al., 2003).

Many types of brain injuries examined to date are able to induce modifications in neurotrophin mRNA expression in rats (Lindvall et al., 1994). In rats, stress applications modify BDNF mRNA expression in the hippocampus, hypothalamus and pituitary gland (Barbany and Persson, 1992; Smith et al., 1995; Givalois et al., 2001, 2004). Also, proneurotrophin was involved in Alzheimer and Parkinson disease (Fahnestock et al., 2001, 2002). Moreover, in human brains, studies have shown a decrease in the precursor of BDNF (proBDNF) protein in Alzheimer's disease (Michalski and Fahnestock, 2003).

Cichlasoma dimerus is a South American cichlid fish and represents an interesting experimental model due to its high survival and reproductive rates under laboratory conditions. *C. dimerus* is a Perciform fish within the Superorder Acanthopterygii, where two of the best known fish models, *Oryzias latipes* and *Tetraodon nigroviridis*, are located. *C. dimerus* shows a marked color display during stress, mating and environmental changes. In our laboratory, several studies were performed reporting the localization of the prolactin, somatolactin and growth hormone-ir expressing cells in the adult pituitary, as well as their ontogeny (Pandolfi et al., 2001a,b), the expression of gonadotropin-releasing hormone (GnRH), melanin concentrating hormone, (MCH) and melanocite stimulating hormone (αMSH) cells along different developmental stages and in adults (Pandolfi et al., 2002, 2003, 2005), and the involvement of the pituitary hormone somatolactin in background color adaptation (Cánepa et al., 2006).

The main purpose of this study was to analyze the distribution of BDNF in the brain and retina of the cichlid fish *C. dimerus*. This result will provide baseline information for future investigations about the role of BDNF in the adult fish nervous system.

2. Materials and methods

2.1. Fish and tissue samples

Adult *C. dimerus* (20) used for this study (10–11 cm) were laboratory-reared descendants of specimens captured in Esteros del Riachuelo, Corrientes, Argentina (27°25′S 58°15′W). They were maintained in glass tanks, well aerated and with external filtration at 27 ± 1 °C and with a 12:12 h photoperiod and fed with commercial pellet. Fish were anesthetized with 0.1% benzocaine, killed by decapitation and processed for immunohistochemistry (IHC) or Western blot techniques. Principles of laboratory animal care (NIH Guide for the Care and Use of Laboratory Animals 1996 (7th ed.) Washington, DC: National Academy Press, aka National Research Council Guide) were followed.

2.2. Immunohistochemistry

Five brains were fixed for 18 h in Bouin's solution, embedded in paraplast, coronally sectioned at 7 μm intervals and mounted on gelatin-coated slides.

Sections were then deparaffinized in xylene, rehydrated through graded ethanol up to phosphate buffered saline (PBS, pH 7.4) and incubated for 30 min in PBS containing 5% non-fat dry milk at room temperature (RT). Next, they were incubated overnight (ON) at 4 °C with a 1:1000 dilution of primary rabbit anti-BDNF against a peptide mapping at the amino terminus of the mature form of BDNF of human origin (sc-546, Santa Cruz Biotechnology, Inc., USA), or with the primary antiserum preadsorbed with the antigen (see Section 2.5). In order to confirm the immunostaining, a different antiserum against recombinant BDNF was used (1:1000 dilution of sheep anti-BDNF polyclonal antibody AB1513P, Chemicon International, Inc.). After that, sections were washed in PBS and incubated at RT for 40 min in a horse biotynilated anti-rabbit IgG diluted 1:600 or horse biotynilated anti-sheep IgG diluted 1:500. Amplification of the signal was achieved by using the tyramide based catalyzed signal amplification (CSA) System (Dako, Carpinteria, CA) at RT and following the datasheet instructions. The staining was developed with 0.1% 3,3′-diaminobenzidine in Tris buffer (pH 7.6) and 0.03% H_2O_2. Sections were slightly counterstained with hematoxylin, mounted, examined with a Nikon Microphot FX microscope and digitally photographed (Coolpix 5400, Nikon). Some sections were stained by Nissl technique in order to describe the morphology of the ir-cells.

2.3. Western blot analysis

Ten Fish were anesthetized with 0.1% benzocaine and killed by decapitation. Each brain was homogenized in 500 μl of 50 mM Tris–HCl buffer (pH 7.4) with 10 μl of Protease inhibitor cocktail (Sigma, Saint Louis, MO) and centrifuged at 10,000 rpm for 20 min at 4 °C. Homogenate (15 μl) was diluted in 5 μl sample buffer (120 mM Tris–HCl, pH 6.8, 3% dodecylsulfate, 10% glycerol, 1% glycerol and 1% β-mercapto-ethanol) and heated at 95 °C for 5 min before being loaded in the 15% SDS-PAGE. After electrophoresis, which was performed on 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot, proteins and molecular markers (SeeBlue Plus2 Pre-Stained Standard, Invitrogen Corp.) were transferred to a nitrocellulose membrane (Amersham Biosciences, UK) for 75 min at 100 V. Then, the membranes were washed in TBST pH 7.5 (100 mM Tris–HCl, 0.9% NaCl, 0.1% Tween-20) and blocked with TBST containing 3% non-fat dry milk and 3% BSA at 4 °C ON. After that, they were