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Development of the cerebellum in turbot (*Psetta maxima*): Analysis of cell proliferation and distribution of calcium binding proteins

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ABSTRACT

The morphogenesis, cell proliferation and neuronal differentiation of the turbot (*Psetta maxima*) cerebellum has been studied using conventional histological techniques and immunohistochemical methods for proliferating cell nuclear antigen and calcium binding proteins. As in other vertebrates, the cerebellar anlage emerges as proliferative plates of neural tissue during the embryonic period. The anlage of the cerebellum persists without morphological changes until the end of the larval life when the mantle zone is differentiated. The major ontogenetic changesthat drive the formation of the cerebellar subdivisions begin in late premetamorphic larvae when cerebellar plates growth and merge medially. This transformation is accomplished by the reorganization of proliferative zones as well as by the onset of cell differentiation. The cerebellum becomes fully differentiated during metamorphosis when parvalbumin and calretinin were detected in Purkinje and eurydendroid cells. Sustained proliferation is maintained in all subdivisions of the cerebellum and this support the robust growth of this part of the brain that takes place during the metamorphic and juvenile periods.The location and histological organization of the proliferative activity in the turbot mature cerebellum are described and their functional significance was analyzed in light of the information available for other teleosts.

1. Introduction

The cerebellum of vertebrates represents a brain center associated with movement control. In teleosts, the cerebellum includes three subdivisions: the corpus cerebelli, the valvula cerebelli and the vestibulolateral lobe, all of which display a typical gross morphology (Meek and Nieuwenhuys, 1998). The corpus cerebelli constitutes a prominent protrusion behind the optic tectum and this extends in a ribbon structure, the valvula cerebelli, into the mesencephalic ventricle. The vestibulolateral lobe includes the eminentia granularis and the caudal lobe, which flank the corpus laterally and caudally, respectively. The major types of neurons in the cerebellum of teleosts are distributed in three layers according to a pattern common to most vertebrates (Butler and Hodos, 2005; Meek and Nieuwenhuys, 1998). Stellate cells lie in the most external, molecular layer of the cerebellum, while the inner, granular layer contains granule and Golgi cells. Purkinje cells soma are arranged in the ganglionic layer, between the molecular and granular layers. Basket cells have not been described in the cerebellum of teleosts, which contain a specific neuronal type, the eurydendroid cells, whose somata are intermingled with those of the Purkinje cells. Eurydendroid cells are the main output of the cerebellum, in a way similar to the deep cerebellar nuclei in mammals (Heap et al., 2013; Ikenaga et al., 2006; Meek and Nieuwenhuys, 1998). A useful approach to identify some cell types in the cerebellum of teleosts involves the use of calcium binding proteins (CaBPs) as neuronal markers, since several reports have revealed a specific neurochemical profile by which parvalbumin (PV) identifies Purkinje cells and calretinin (CR) labels a subpopulation of eurydendroid cells (Bae et al., 2009; Castro et al., 2006; Díaz-Regueira and Anadón, 2000; Kaslin et al., 2009; Meek et al., 2008).

During ontogenesis, the cerebellum arises from the upper rhombencephalic alar plate. A detailed analysis of the morphogenesis of the cerebellum in the developing trout was provided by Pouwels (1978a,b), who described the early pattern of cell proliferation, the migrating cells paths associated with the neuronal differentiation as well as the establishment of secondary proliferative zones. Different aspects of this pioneering work were subsequently confirmed in other teleosts (Candal et al., 2005;

Abbreviations: Cc, canalis cerebelli; CL, caudal lobe; GE, granular eminences; M, mesencephalon; OT, optic tectum; VC, valvula cerebelli.

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Haugedé-Carré et al., 1977, 1978; Ishikawa et al., 2010; Kage et al., 2004; Lannoo et al., 1990, 1991) and more recent genetic studies have identified various genes that underlie cerebellar morphogenesis in both zebrafish and medaka (Ishikawa et al., 2008; Kani et al., 2010; Kaslin et al., 2013). As in other parts of the brain, continuous growth of the cerebellum occurs in adult teleosts and this involves continued neurogenesis (Candal et al., 2005; Delgado and Schmachtenberg, 2011; Ekström et al., 2001; Kuroyanagi et al., 2010; Margotta et al., 2004; Olivera-Pasilio et al., 2014; Zikopoulos et al., 2000; Zupanc and Horschke, 1995; Zupanc et al., 1996).

Turbots (Order Pleuronectiformes) are teleosts that have an indirect development (Flegler-Balon, 1989). Their life cycle includes a dramatic metamorphic process in which animals lose their bilateral symmetry to transform into asymmetric benthic flatfish juveniles (Al-Maghazachi and Gibson, 1984). Overall, the timing of developmental events in the brain and sensory organs are slower in premetamorphic turbots (Cid et al., 2013; De Miguel Villegas et al., 1997; Doldán et al., 2011, 2000, 1999; Prego et al., 2002). The aim of the present paper was to exploit the protracted development in *P. maxima* in order to study the morphogenesis of the cerebellum by analyzing the progression of its citoarchitecture and proliferative activity at consecutive developmental stages. Additionally, the labeling patterns of PV and CR in the different subdivisions of the cerebellum were evaluated in order to understand how neuronal differentiation progresses during ontogeny.

2. Material and methods

Turbots used in this study (28 embryos, 32 prolarvae, 60 larvae and 12 juvenile specimens) were obtained from the High Technology Center (Bergen, Norway) and Piscicola del Morrazo (Nerga, Spain). Developing fishes were grouped into four major stages: embryonic, prolarval (a yolk-feeding period equivalent to the free embryo stage defined by Balon (1985)), larval and juvenile (see Table 1). In the culture conditions (water temperature around 13 °C), most embryos hatched 140 h after fertilization, while the prolarval and larval periods lasted around 4 and 50 days, respectively. During the protracted larval period the animals lose their bilateral symmetry and undergo a drastic metamorphosis in which the right eye migrates to the left side of the body. Therefore, we established the following four subdivisions of the larval stage: premetamorphic larvae (with bilateral symmetry and staged by days post-hatching -dph-) and early, middle and late metamorphic larvae characterized by the progression of the eye migration, as defined by Bejarano-Escobar et al. (2010) and Cid et al. (2013). All experiments were conducted according to the guidelines on animal care and experimentation of the University of Vigo and Spanish laws.

Animals were anesthetized with 0.1% tricaine methanesulfonate (MS-222, Sigma) and then fixed by immersion in Bouin's fluid for 24 h. The specimens were then dehydrated in a graded series of alcohols and embedded in paraffin. Serial transverse, horizontal and sagittal sections ranging from 7 to 12 μm were obtained with a rotary microtome (Leica RM2145) and mounted on gelatin-coated slides. Then, sections were dewaxed and stained with hematoxylin-eosin or processed for immunohistochemistry.

The initial step for all immunohistochemistry procedures consisted of the treatment of sections with 3% hydrogen peroxide in phosphate-buffered saline (PBS), 0.1 M, pH 7.3, followed by blocking with 1% bovine serum albumin (BSA) in PBS with 0.25% Triton X-100 for 1 h to localize CaBPs or with 10% normal goat serum (NGS) in PBS with 0.5% Triton X-100 (PBS-T), pH 7.2, to localize PCNA. The sections were then incubated overnight at room temperature with primary antisera (rabbit anti-CR, Swant, code 7699/4; rabbit anti-PV, Swant, code PV 27; mouse anti-PCNA, clon PC10, Dako, code M0879) diluted either 1:1000 in PBS-TBSA (CaBPs antisera) or 1:500 in PBS-T with 3% NGS (PCNA antiserum). After rinsing in PBS, the samples were incubated for 1 h with the secondary antibody (goat anti-rabbit IgG or goat anti-mouse IgG, Dako) diluted 1:100 in PBS containing 0.25% Triton X-100 (PBS-T). Rabbit or mouse peroxidase-antiperoxidase complex (Dako) diluted 1:100 or 1:50 in PBS-T was used for CaBPs or PCNA detection, respectively. The immunoreaction was visualized by using 0.05% diaminobenzidine (DAB, Sigma) in 0.05 M Tris–HCl buffer (pH 7.6) containing 0.01% hydrogen peroxide. Intensification of the DAB immunoreaction was achieved by adding 1% nickel ammonium sulfate and 1% cobalt chloride to the reaction medium. Slides were counterstained with hematoxylin for 1 min. The sections were then dehydrated, cleared in xylene, and coverslipped with DPX (VWR Chemicals). Immunostaining was not observed when primary antisera were omitted from the protocol.

Images were captured with a Nikon Eclipse NiE microscope equipped with a Nikon DS-Ri2 camera and NIS-element software. Contrast and brightness were adjusted using Corel DRAW Software. The final figures were composed and labeled with CorelDRAW Software.

The nomenclature used for the developing cerebellum follows that proposed by Pouwels (1978a) and Wullimann and Knipp (2000).

3. Results

In the following description, the development of the turbot cerebellum has been divided into two main stages. The initial stage includes changes in the cerebellar primordium in the embryonic, prolarval and most of the larval period; the second stage comprises the morphogenetic transformations which occur from the late larvae onwards, leading to the formation of the differentiated cerebellum.

3.1. Stage I: early cerebellar development

During turbot embryogenesis, a small constriction in the outer surface of the neural tube defined the isthmic region at the midbrain-hindbrain boundary. Shortly after the formation of the isthmus, the IVth ventricle widened to give rise to a profound, horizontally oriented recess in the rostral aspect of the dorsal

Table 1
Periods of turbot development and main traits for staging. The approximate duration of some stages/substages is shown in brackets.

Stage	Substage	Symmetry	Description
Embryonic (140 h)		X	Onset of differentiation of primary organ systems
Prolarval (4 days)		X	Yolk sac (endogenous feed)
Larval (50 days)	Premetamorphosis (11 days)	X	Mouth opening (exogenous feed)
	Early Metamorphosis (15 days)		Onset of the right eye migration
	Middle Metamorphosis (12 days)		Migrated right eye at the top of the head
	Late Metamorphosis (12 days)		Migrated right eye twisted in the left side of the body
Juvenile			Eye migration completed