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Cytoarchitectonic and neurochemical differentiation of the visual system in ethanol-induced cyclopic zebrafish larvae

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ABSTRACT

Embryonic exposure to ethanol leads to malformations such as cyclopia. Cyclopic embryos present fused eyes and lack of the ventral specification of the brain, with physiological and morphological defects in the visual system, which provides a useful model for teratology and neurotoxicity assessments.

We analysed the differentiation of the visual areas in the ethanol-induced cyclopic animals. For this purpose we exposed zebrafish embryos to 1.5% ethanol from 4 hours post-fertilisation (hpf) to 24 hpf in order to get cyclopic embryos. We monitored cytoarchitecture and quantified both the proliferation rate and cell differentiation from 2 days post-fertilisation (dpf) onwards, focusing on the main components of the visual system (retina, optic nerve and optic tectum) of normal and cyclopic zebrafish embryos.

The visual system of the zebrafish embryos is affected by exposure to ethanol; two optic nerves that fuse before leaving the eyes are present in cyclopic specimens but an optic chiasm is not evident. Cell differentiation is severely delayed throughout the visual system at 2 dpf. At 5 dpf, lamination in the cyclopic retina and optic tectum is completed, but they are filled with pyknotic nuclei demonstrating cell death. At this stage the proliferation rate and expression patterns are unaltered and glial and neuronal neurochemical differentiations are similar to untreated animals. We found that the alterations produced by exposure to ethanol are not only cell-selective, but also tissue-selective.

Cyclopia is the most severe phenotype induced by ethanol, although cell differentiation and proliferation can reach normal patterns after a certain period of time, which points to a neural plasticity process. Zebrafish embryos may possess a compensation mechanism against the ethanol effect, which would account for their use for pharmacogenetic and chemical screenings in the analysis of new molecules that could improve visual problems.

1. Introduction

The zebrafish provides a good model for studying the development of the visual system of vertebrates. At 12 hours post-fertilisation (hpf) the eyes are flattened evaginations of the prospective diencephalon. Around 36 hpf retinogenesis starts and at 5 days post-fertilisation (dpf) the differentiation of the retinal layers and the establishment of a topographical map of retinotectal projections is easily observable (Bilotta et al., 2002; Karlstrom et al., 1996). Several studies have shown that zebrafish constitute an effective genetic (Dasmahapatra et al., 2001; Loucks and Carvan, 2004), anatomical (Arenzana et al., 2006b; Blader and Strähle, 1998) and physiological (Bilotta et al., 2002; Bilotta et al., 2004; Matsui et al., 2006) *in vivo* model system for the analysis of the teratogenic effect of ethanol on visual system development. Research focussed on the early exposure of zebrafish embryos to ethanol has demonstrated the impairment of the ventralisation of the Central Nervous System (CNS) (Blader and Strähle, 1998) and the fusion of the optic vesicles leading to cyclopia (Arenzana et al., 2006a). Nonetheless, the specific effect of the early exposure to ethanol on signalling pathways involved in the differentiation of the visual areas is still unknown. Moreover, ethanol not only induces alterations in zebrafish but also in mouse (Hirabayashi et al., 2004), rat (Crews et al., 2006) and human (Ribeiro et al., 2007) development.

Ethanol-treated larvae show alterations in the processing of visual information (Bilotta et al., 2004), although they are stimulated by light at later stages. Moreover, ethanol reduces the cell proliferation rate in the CNS (Guizzetti et al., 1997) and increases apoptosis (Carvan et al., 2004). The effect of ethanol on the zebrafish visual system depends on several factors: strain, sex, dose and time of administration (Arenzana et al., 2006a; Blader and Strähle, 1998; Dlugos et al., 2010; Gerlai et al., 2008; Kashyap et al., 2007), producing a wide range of effects such as cyclopia. Previous work by our group demonstrated that ethanol produces a delay in development: the lamination of the

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retina and the optic tectum (OT) is not completed in cyclopic larvae at 3 dpf (Arenzana et al., 2006a).

In this work, we studied the teratogenic effects of ethanol exposure (1.5%) on cytoarchitectonic, cellular and neurochemical differentiation of the main components of the zebrafish visual pathway (retina, optic nerves, OT). For this purpose we analysed semi-thin sections, incorporation of BrdU and immunodetection of different molecular markers involved in the early morphogenesis (specification of pigmented epithelium and neural retina, spatial axes of OT) and late morphogenesis (tissular and cellular differentiation of neural retina, retinotopic map of OT). We have used this approach in the cyclopic zebrafish visual system to provide valuable neuroanatomical data regarding the nature of teratogenic and neurotoxic effects of ethanol.

Morphology and gene expression patterns of cyclopic zebrafish larvae at late development stages have not been studied and might be useful to further the knowledge of the effects of ethanol in visual system development.

2. Methods

2.1. Animals

AB strain zebrafish embryos were generated by natural pair-wise mating. They were staged and reared according to standard procedures (Westerfield, 1995). Specimens were anaesthetised with ethyl 3-aminobenzoate methanosulphonate salt (Sigma, St Louis, MO, U.S.A.) before they were fixed.

All of the following procedures were carried out in untreated control embryos and embryos presenting cyclopia induced by ethanol, treated embryos which did not display cyclopia were not used. The stages used were 2, 3 and 5 dpf.

All procedures and experimental protocols were in accordance with the guidelines of the European Communities Directive (86/609/EEC and 2003/65/EC) and the current Spanish legislation for the use and care of animals in research (RD 1201/2005, BOE 252/34367-91, 2005) and conformed to NIH guidelines.

2.2. Ethanol treatment and identification of cyclopic embryos

Embryos were treated following the protocol previously described (Arenzana et al., 2006a; Loucks et al., 2007), where a dose-dependent response of ethanol was assessed. They were exposed to 1.5% ethanol during the period from dome/30% epiboly to 24 hpf. Groups of fifty embryos were exposed in a Petri dish covered with parafilm to minimise evaporation. The ethanol solution was made using 100% ethanol diluted with E3 medium. During the second day of development embryos were assessed for cyclopia, as indicated by fused-eye vesicles. Cyclopic embryos were isolated, counted and grown to 5 dpf in E3 medium. Dead embryos were removed every day. Results provided by embryos from different Petri dishes were always consistent.

The period of exposure was chosen in order to affect all the early eye development in the zebrafish (Schmitt and Dowling, 1994). The concentration of ethanol (1.5%) was chosen because previous studies show a high survival rate and provide enough amounts of cyclopic embryos for the experiments (Arenzana et al., 2006a; Loucks et al., 2007).

Untreated embryos were used as control specimens.

2.3. Semi-thin sections

Ten 5 dpf untreated and 10 cyclopic specimens were anaesthetised and fixed by immersion with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 (PB) for 24 h at 4 °C, and postfixed in osmium tetroxide containing 1% potassium ferricyanide for 1 h. Specimens were dehydrated using a graded series of cold ethanol and embedded with EMbed-812 (#14120 Electron Microscopy Science, Fort Washington, PA, U.S.A.). Parasagittal serial sections of 1 μm-thickness were cut on an ultramicrotome Reichert-Jung Ultracut E (Nussloch, Germany). Sections were stained with 1% toluidine blue solution.

2.4. Immunohistochemistry

For immunodetecting proteins and BrdU, embryos of each stage were anaesthetised and fixed by immersion with 4% paraformaldehyde for 24 h at 4 °C. Embryos were maintained in methanol at −20 °C until their use.

2.4.1. Cell differentiation

The acetylated tubulin wholemount immunohistochemical detection was performed in 10 control and 10 cyclopic embryos at 3 dpf. After rehydration, embryos were permeabilised with a solution of 0.02 μg/μl of protein kinase (Sigma) for 30 min. Standard fluorescence immunohistochemistry was carried out using an antibody against anti-acetylated tubulin made in mouse and diluted 1/100 (Sigma) for one night at 4 °C and anti-mouse IgG conjugated with cy3 (Jackson). Nuclei were labelled with DAPI (Sigma). The immunospecifity of the monoclonal anti-acetylated tubulin used in this report has already been proved (Kim et al., 2008).

Other markers were analysed in cryostat sections which were obtained as follows: fixed embryos were washed in 0.1 M phosphate buffer pH 7.4 (PB) and were embedded in 1.5% agar and 10% sucrose. Blocks were cryoprotected in 30% sucrose for 48 h at 4 °C. Sections (10 μm) were obtained on a cryostat and stored at −20 °C until their final use. The immunohistochemistry in sections was performed as previously described (Arenzana et al., 2005, 2006b; Clemente et al., 2004). The following antibodies were used: rabbit anti-Calretinin (CR, 1:10000, Swant, Bellinzona, Switzerland), mouse anti-TH (1:1000, Chemicon, Billerica, MA, USA), rabbit anti-Pax-6 (1:500, Covance), rabbit anti-Pax2 (1:500, Abcam), rabbit anti-Prox1 (1:1000, Covance, Princeton, NJ, USA), mouse anti-glutamine synthetase (GS, 1:1000, Chemicon), mouse anti-Zpr1 (1:500, ZIRC, University of Oregon, USA) and mouse anti-Zpr3 (1:500, ZIRC). All the antibodies have been previously used in zebrafish and other teleosts (CR: Castro et al., 2006; TH: Arenzana et al., 2006a; Pax6: Sánchez-Simon et al., 2010; Pax2: Sánchez-Simon et al., 2010; Prox1: Cid et al., 2010; GS: Cid et al., 2010; Zpr1 and Zpr3 are specific of zebrafish). As secondary antibody either anti-mouse or antirabbit IgG conjugated with cy2 was used. Nuclei were labelled with DAPI (Sigma).

2.4.2. BrdU incorporation

BrdU incorporation was performed as previously described (Laguerre et al., 2005). Briefly, at 2, 3 and 5 dpf, 25 untreated and 25 cyclopic larvae were mechanically dechorionated and incubated in a 10 mM solution of BrdU (Sigma) with 12% dimethyl sulphoxide/E3 medium at 28.5 °C for 2 h. Control and cyclopic animals at the same stage were exposed in the same Petri dish. After two washes in E3 medium, the specimens were fixed and maintained as described above.

For detecting BrdU incorporation, cryostat sections were obtained as described above. Sections were washed in HCl 2 N at 37 °C for 30 min and then in borate buffer pH = 8. Standard fluorescence immunohistochemistry was performed after maintaining the sections in rat anti-BrdU IgG diluted 1/5000 (Roche, Barcelona, Spain) overnight at room temperature. Anti-rat IgG conjugated with cy2 (Jackson, Suffolk, UK) was used for labelling. Nuclei were labelled with DAPI (Sigma).