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ETHANOL ALTERS GENE EXPRESSION AND CELL ORGANIZATION DURING OPTIC VESICLE EVAGINATION

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Abstract—Ethanol has been described as a teratogen in vertebrate development. During early stages of brain formation, ethanol affects the evagination of the optic vesicles, resulting in synophthalmia or cyclopia, phenotypes where the optic vesicles partially or totally fuse. The mechanisms by which ethanol affects the morphogenesis of the optic vesicles are however largely unknown. In this study we make use of *in situ* hybridization, electron microscopy and immunohistochemistry to show that ethanol has profound effects on cell organization and gene expression during the evagination of the optic vesicles. Exposure to ethanol during early eye development alters the expression patterns of some genes known to be important for eye morphogenesis, such as *rx3/1* and *six3a*. Furthermore, exposure to ethanol interferes with the acquisition of neuroepithelial features by the eye field cells, which is clear at ultrastructual level. Indeed, ethanol disrupts the acquisition of fusiform cellular shapes within the eye field. In addition, tight junctions do not form and retinal progenitors do not properly polarize, as suggested by the mis-localization and down-regulation of *zo1*. We also show that the ethanol-induced cyclopic phenotype is significantly different to that observed in cyclopic mutants, suggesting a complex effect of ethanol on a variety of targets. Our results show that ethanol not only disrupts the expression pattern of genes involved in retinal morphogenesis, such as *rx3* and *rx1*, but also disrupts the changes in cell polarity that normally occur during eye field splitting. Thus, ethylic teratology seems to be related not only to modifications in gene expression and cell death but also to alterations in cell morphology.

Key words: eye specification, morphogenesis, cell polarity, cyclopic mutants.

INTRODUCTION

Optic vesicle evagination is the process by which a unique morphogenetic domain, the eye field, gives rise to two symmetric domains. These domains then evaginate to generate the optic vesicles (Rembold et al., 2006). In zebrafish optic vesicle evagination begins at 2–3 somite stage (ss), when the medially located eye field acquires a bi-lobed shape. At this stage of development, the eye field is delimited anteriorly by the telencephalon and posteriorly by the hypothalamus anlage (England et al., 2006), organization that is partially inverted at later stages. Just 4 h later (at 10 ss), two optic primordia are distinguishable in the lateral parts of the embryo. The exact mechanism by which the eye field splits in two domains and the optic vesicles evaginate remains unclear. Some studies suggest that retinal progenitors actively migrate toward lateral regions during eye morphogenesis and that this process, regulated by the transcription factor *rx3*, provides the driving force for optic vesicle evagination (Rembold et al., 2006). Other studies instead suggest that the cells within the eye field do not actively migrate, but follow the morphogenetic reorganizations promoting forebrain morphogenesis (England et al., 2006).

Many transcription factors are involved in the specification of the eye field, including *otx2*, *pax6*, *rx3*, *six3* and *zic2* among others (review in Bailey et al., 2004; Zaghloul and Moody, 2007). These transcription factors are coincidently expressed in the eye field, and their combined activity is sufficient to induce eye fate. Indeed, ectopic eyes are induced when a cocktail of these factors is ectopically expressed outside of the neural plate (Zuber et al., 2003). The molecular mechanisms involved in the morphogenesis of the eye field are not so well understood, but some reports suggest that the same genes that control eye field specification subsequently control its morphogenesis. For example, the absence of *rx3* leads to a failure in the splitting of the eye field and results in complete absence of the optic vesicles, a phenotype known as anophthalmia (Mathers et al., 1997; Winkler et al., 2000; Kennedy et al., 2004). Mutations on *six3* or *zic2* lead to holoprosencephaly and cyclopia

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Abbreviations: hpf, hours post-fertilization; ISH, *in situ* hybridization; MET, mesenchymal–epithelial transition; MHB, midbrain–hindbrain boundary; *oep*, *one-eye pinhead*; qRT-PCR, quantitative real-time polymerase chain reaction; SEM, standard error of mean; ss, somites stage; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; ZO-1, zonula-occludens-1.

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(partially fused optic vesicles) in humans (Brown et al., 1998; Pasquier et al., 2000), also suggesting a role of these genes in the morphogenetic reorganization underlying optic vesicle evagination.

In addition to genetic factors, drugs like cyclopamine, forskolin or ethanol can also result in micro/anophthalmic and cyclopic phenotypes (Arenzana et al., 2006; Loucks et al., 2007; Santos-Ledo et al., 2011). The aim of this work is the analysis of the molecular and cellular mechanisms underlying ethanol-induced cyclopia. This teratogenic substance induces a constellation of problems during development such as delayed differentiation, increased apoptosis or migration failures, among others (Blader and Strähle, 1998; Loucks et al., 2007). The developing visual system is very sensitive to exposure to ethanol (Kashyap et al., 2007; Santos-Ledo et al., 2011) but there is no agreement about how this drug induces cyclopic phenotypes. The most prevalent model states that ethanol disrupts the collective migration of prechordal plate progenitors to the anterior part of the embryo, leading to cyclopia (Blader and Strähle, 1998). On the other hand, some studies have shown a rescue of the cyclopic phenotype by exposing zebrafish embryos to substances such as Shh (Loucks and Ahlgren, 2009) or retinoic acid (Marrs et al., 2010). However, the behavior of eye field cells after exposure to ethanol has not been analyzed.

In this study, we have analyzed the expression pattern of genes known to be involved in eye field specification and morphogenesis (*otx2*, *zic2*, *pax6*, *six3*, *rx3* and *rx1*) after exposure to ethanol. We have also analyzed the cytoarchitecture of the eye field during the early stages of eye morphogenesis and the distribution and expression levels of zonula-occludens-1 (ZO-1), a protein involved in tight junction formation and apico-basal cell polarization. Our results suggest that ethanol not only alters the expression patterns of some of the genes important for eye formation, but also prevents the cellular rearrangements that normally occur during optic vesicle evagination. Since up to now the effect of ethanol on cell morphology had only been studied in cell culture (Guasch et al., 2007; Martínez et al., 2007), our results expand our understanding of ethylic teratology *in vivo*, and suggest that ethanol-induced phenotypes result from a combination of molecular defects on both gene function and cell morphology.

EXPERIMENTAL PROCEDURES

Specimens and ethanol treatment

AB zebrafish strain embryos were used in all the experiments. They were obtained by natural pair-wise mating and staged and reared according to standard procedures (Westerfield, 1995). A previous work in our lab showed that this strain is sensitive to ethanol and a concentration of 1.5% is enough to consistently produce cyclopia (Arenzana et al., 2006).

All of the following procedures were carried out in untreated control embryos and embryos exposed to different concentrations of ethanol: 0.5%, 1%, 1.5%, 2% and 2.4%. Embryos were exposed from dome/30% epiboly [4.3 hours post-fertilization (hpf)] to tailbud stage (10 hpf), then they were washed out of the ethanol and developed to the desired stage. Embryos were collected at 3 ss (11 hpf, prior to evagination), 6 ss (12 hpf, mid-evagination), 10 ss (14 hpf, two optic primordial are distinguishable) and 18 ss (18 hpf, optic cup formation). This protocol is similar to others previously described where cyclopic phenotypes were analyzed and a dose-dependent response of ethanol was assessed (Arenzana et al., 2006; Loucks et al., 2007).

The mutant lines *cyclops*, *one-eyed pinhead* and *trilobite* were obtained from the zebrafish Stock Centre at UCL and *silberblick* mutants were a generous gift from Dr. Masazumi Tada.

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.

Semi-thin sections and electron microscopy

Semi-thin sections were obtained as previously reported (Santos-Ledo et al., 2011). Briefly, embryos were fixed by immersion in 2% paraformaldehyde and 2% 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 (Electron Microscopy Science, Fort Washington, PA, EE.UU). Coronal 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.

The same blocks were used to obtain ultra-thin sections for electron microscopy. 70-nm-thickness sections were cut in the ultramicrotome. Sections were counter-stained with 2% of uranil acetate during 15 min in darkness at room temperature and with lead citrate during 10 min at room temperature and without CO_2. Sections were washed with distilled water and dried before observation in the electron microscope.

In situ hybridization (ISH) and immunohistochemistry

ISH was performed by using published protocols (Thisse and Thisse, 2008) in embryos at 3, 6, 10 and 18 ss. Digoxigenin-labeled RNA probes were synthesized using a DIG labeling kit (Roche, Barcelona, Spain) and probes were detected with anti-DIG-AP antibody (1:5000, Roche, Barcelona, Spain) and NBT/BCIP substrate.

Whole-mount immunohistochemistry was performed as previously described (Wilson et al., 1990) using an anti-ZO-1 antibody (1:250, Invitrogen, Carlsbad, CA, U.S.A.) that has been previously tested in zebrafish (Zhang et al., 2010). Nuclei were counterstained using sytox Orange (1:10,000, Invitrogen, Carlsbad, CA, U.S.A.) or DAPI (1:10,000, Sigma, St. Louis, MO, EE.UU).