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Neural regeneration dynamics of *Xenopus laevis* olfactory epithelium after zinc sulfate-induced damage

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ABSTRACT

Neural stem cells (NSCs) of the olfactory epithelium (OE) are responsible for tissue maintenance and the neural regeneration after severe damage of the tissue. In the normal OE, NSCs are located in the basal layer, olfactory receptor neurons (ORNs) mainly in the middle layer, and sustentacular (SUS) cells in the most apical olfactory layer. In this work, we induced severe damage of the OE through treatment with a zinc sulfate ($ZnSO_4$) solution directly in the medium, which resulted in the loss of ORNs and SUS cells, but retention of the basal layer. During recovery following injury, the OE exhibited increased proliferation of NSCs and rapid neural regeneration. After 24 h of recovery, new ORNs and SUS cells were observed. Normal morphology and olfactory function were reached after 168 h (7 days) of recovery after $ZnSO_4$ treatment. Taken together, these data support the hypothesis that NSCs in the basal layer activate after OE injury and that these are sufficient for complete neural regeneration and olfactory function restoration. Our analysis provides histological and functional insights into the dynamics between olfactory neurogenesis and the neuronal integration into the neuronal circuitry of the olfactory bulb that restores the function of the olfactory system.

1. Introduction

The olfactory system processes sensory information vital for the interactions between individuals and the environment. Odor signals are transduced in different olfactory receptor neurons (ORNs), each of which expresses only one functional odorant receptor type (Mombaerts, 2004).

In vertebrates, ORNs are located in the olfactory epithelium (OE) along with glial-like sustentacular (SUS) and basal cells. The OE has the ability to be renewed because during neuronal turnover ORNs are continuously replaced, both in normal (physiological) conditions and in response to injury (Farbman, 1994; Brann and Firestein, 2014). During OE renewal, new neuronal cells differentiate from the basal epithelial area and migrate apically, differentiating into mature ORNs, which extend their axons through the basement membrane toward the olfactory bulb, to recover the olfactory function.

Two groups of neural progenitors or stem cells with different morphology and proliferative properties have been identified in the basal cells of the OE, namely the horizontal basal cells (HBCs) and the globose basal cells (GBCs). In the normal turnover process, GBCs are the main source of newly regenerated ORNs; however, under severe injury conditions, HBCs, which are normally very quiescent, become activated to regenerate ORNs and SUS cells (Duggan and Ngai, 2007; Leung et al., 2007; Brann and Firestein, 2014). In the last years, HBCs and GBCs have been well characterized in murine models; nevertheless, in *Xenopus laevis* the characterization of these neural progenitor cells has been limited to morphological aspects (Hassenklover et al., 2009).

Several studies have examined the effects of different chemicals on the olfactory system to characterize the ability of the OE to recover from damage. Some of these chemicals, such as zinc sulfate ($ZnSO_4$), methimazole and methyl bromide, induce the degeneration of all olfactory cell types (Schwob, 2002; Holbrook et al., 2014; Schnittke et al., 2015); others, such as the detergent Triton X-100 in zebrafish, are specific for neuronal degeneration (Iqbal and Byrd-Jacobs, 2010;

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White et al., 2015). Other methods involve mechanical procedures such as removal of the olfactory bulb (bulbectomy) and transaction of the olfactory nerve (axotomy), which induce only ORN degeneration and apoptosis (Graziadei and DeHan, 1973; Harding et al., 1977; Cancalon, 1987; Holcomb et al., 1995).

$ZnSO_4$ treatment has been previously used as a tool to induce severe injury in different models. It has been used to subject rodents to intranasal irrigation (Burd, 1993; Herzog and Otto, 1999), applied into the olfactory organs of catfish (Cancalon, 1982, 1983), and used to expose anuran larvae (Yovanovich et al., 2009). $ZnSO_4$ has been reported to cause necrotic death of all the cell types present in the olfactory tissue (Cancalon, 1982; Schwob, 2002).

Although OE regeneration after different degrees of injury has been studied in several species, no sequential analysis of the morphological and behavioral events occurring over time during the regeneration process in *X. laevis* has yet been conducted. Therefore, in the current study, we describe the regeneration dynamics of both neurons and glial cells of the NSCs located in the basal layer, after severe damage of the OE using $ZnSO_4$ in *X. laevis* larvae. Our results demonstrated that a single dose of $ZnSO_4$ results in significant but temporary damage of the OE followed by rapid regeneration of the structure and function of proliferation and differentiation of the olfactory basal cells. Moreover, during neural regeneration, as soon as 24 h after olfactory destruction, the OE began to recover E7 immunoreactivity (neuronal β-tubulin marker), which shows that both mature and immature neurons were present (Heer et al., 2008). In addition, new mature neurons identified with the Olfactory Marker Protein (OMP) as well as SUS cells identified with cytokeratin II (CytKII) were observed as soon as 24 h after the start of regeneration. Finally, at 168 h after the chemical lesion, all the different cell types were restored and a large portion of mature ORNs recovered. In addition, the behavioral test demonstrated that the olfactory function was recovered.

1.1. Material and methods

1.1.1. Animals

Wild-type *X. laevis* embryos were obtained by standard methods, staged according to Nieuwkoop and Faber (Gurdon, 1994) and cultured in 1X modified Ringer's solution until stage 46 (Wu and Gerhart, 1991). The animals were then raised at low density (7 tadpoles per liter) in tanks with chlorine-free water at $22 \pm 2^{\circ}$ C and fed *ad libitum* with spiruline. The breeding and care of the animals and all experiments were performed according to the Principles of Laboratory Animal Care of the Institutional Care and Use Committee of Facultad de Ciencias Exactas y Naturales, University of Buenos Aires, Argentina (Res CD: 140/00) and the National Institutes of Health Guide for the Care and Use of laboratory Animals.

1.2. $ZnSO_4$ treatment and OE regeneration

Larvae from stages 53–54 were exposed individually for 24 h to a $ZnSO_4$ solution (20 mg/l) prepared with dechlorinated water according to Yovanovich et al. (2009). Previously, we have described the effects of different $ZnSO_4$ concentrations and exposure periods on *Rhinella arenarum* tadpoles (Yovanovich et al., 2009), and tested and adjusted the parameters of the treatment for *X. laevis* larvae (Frontera et al., 2014). No morphological changes in the olfactory bulb were observed as a consequence of the exposure to $ZnSO_4$ at these conditions (Frontera et al., 2014). The control group was handled in the same way but exposed to dechlorinated tap water without $ZnSO_4$. The regeneration process of the olfactory tissue was analyzed at different periods of recovery after the treatment to determine the damage and the degree of recovery over time.

1.3. Immunohistochemistry

Animals were anesthetized by immersion in a 0.1% solution of tricaine methanesulfonate (M222, Sigma), fixed in Bouin's solution for 24 h at 4 °C, dehydrated, cleared in xylene and embedded in Histoplast (Biopack, Buenos Aires). Serial paraffin sections (5 μm thick) were cut and attached to charged glass slides, prepared for immunofluorescence or immunohistochemistry and counterstained using hematoxylin. General immunohistochemical procedures were followed as described in our previous reports (Pozzi et al., 2006; Jungblut et al., 2012). Staining was carried out using the following antibodies: mouse monoclonal anti-β-tubulin antibody (1:2000) (E7, Developmental Studies Hybridoma Bank), rabbit polyclonal anti-OMP antibody (1/1000) (Santa Cruz), mouse monoclonal anti-CytKII antibody (1:200) (1h5, Developmental studies Hybridoma Bank), biotinylated anti-rabbit secondary antibody (Millipore), anti-mouse IgG eFluor 660 (eBioscience), and Streptavidin-HRP (Dako), all used according to the manufacturer's instructions.

1.4. Bromodeoxyuridine (BrdU) labeling

5-bromo-2'-deoxyuridine (BrdU, Sigma) was dissolved in dechlorinated and filtered tap water at a concentration of 10 mM. Live tadpoles were individually placed in 100 ml of this solution for 1 h to label all the proliferative cells in controls and at different periods of recovery after $ZnSO_4$ treatment. Following BrdU incorporation, animals were anesthetized and fixed with Bouin's solution (Quick and Serrano, 2007; Frontera et al., 2014).

1.5. Image analysis

Slides containing comparable sections from each animal were randomly selected. Digital images of each section were taken with a Sony Cybershot DSC P-200 camera attached to a Leica Reichert Polyvar microscope. A rectangle that contained the medial region of the OE was defined, oriented, and superimposed on each photograph. Images were loaded into Image Pro Plus 4.1 software (Media Cybernetics) for measurement of the olfactory layer thickness and area determinations. For OMP quantification, the number of immunostained cells in the area, defined by the basement membrane and the upper limits of the epithelium, was counted manually. The ratio of this value to the perimeter of the basement membrane from this medial area selected was calculated and expressed as OMP+ cells/μm. The number of BrdU+ nuclei within the same medial region was counted manually, and the ratio of this value to the measured area in each photograph was calculated and expressed as BrdU+ cells/OE area.

To minimize the number of animals used, the values obtained from control larvae were taken at $t = 0$ h for all treatments.

1.6. Frequency of buccal pumping

The frequency of buccal pumping was used to assess the olfactory ability of the animals before and after the destruction of the OE with $ZnSO_4$ treatment. A spiruline solution of 2 mg/ml was used as the food stimulus. This concentration was reached from previous experiments. All behavioral tests were performed between 10 am and 2 pm, in a room conditioned for that purpose. Each experimental subject was starved for 4 days before the test and tested individually only once. The experimental arena was a glass cylinder 15 cm in diameter, containing 500 ml of