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NEURAL REGENERATION DYNAMICS OF *XENOPUS LAEVIS* OLFACTORY EPITHELIUM AFTER ZINC SULFATE-INDUCED DAMAGE

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Highlights

According with the requirements of the Journal of Chemical Neuroanatomy.

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Title: NEURAL REGENERATION DYNAMICS OF XENOPUS LAEVIS OLFACTORY EPITHELIUM AFTER ZINC SULFATE INDUCED-DAMAGE

- Zinc sulfate induces glial and neuronal degeneration s of *Xenopus laevis* olfactory epithelium.
- Neural progenitors in the basal layer of the olfactory epithelium regenerate both neuronal and glial cells.
- Mature olfactory neurons and sustentacular cells were restored after seven days of severe damage.
- Seven days after massive destruction the olfactory epithelium recovers its normal function.

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.

Keywords: Neurogenesis, Olfactory Receptor Neurons, Olfactory injury, Neural stem cells, Olfaction

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; 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 *Xenopus 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 *Xenopus 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.

Material and methods

Animals

Wild-type *Xenopus 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 ± 2° 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.

$ZnSO_4$ 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.

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.

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).

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.

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 dechlorinated water at 21° C. At the time of testing, the tadpoles were transferred to the experimental arena. After a 10-min period for acclimation, the times at which buccal pumping occurred were recorded. Each trial lasted 8 min, and consisted of a 4-min pre-stimulus period and a 4-min post-stimulus period. Then, 400 µl of spiruline or H_2O (dechlorinated water) was added to the experimental or the control group, respectively, at approximately 100 µl /sec. The control group (Ct_{H2O}, n = 4) was used to compare the different groups. The variable "change in frequency pumping" was calculated as the ratio of (buccal pumping frequency of post-stimulus / buccal pumping frequency of pre-stimulus). The number of animals in each experimental group was as follows: Control group (Ct, $ZnSO_4$ untreated animals, n = 4), 0 h (n = 4), 24 h (n = 4), 120 h (n = 6), 168 h of recovery (n = 5).

Statistics

Data were analyzed by using a one-way analysis of variance (ANOVA) (with different levels corresponding to the different recovery periods), with a significance level of $P<0.05$. Means within groups were tested by using Tukey multiple comparisons. Results were considered significantly different when $P<0.05$.

Results

Chemical lesion of the OE by $ZnSO_4$ treatment

In *Xenopus laevis* larvae from stages 53-54 (Fig. 1A), the OE presents an organized neuroepithelium in which the layers containing somas of the different cell types can be distinguished easily (Fig. 1B). The SUS cells are localized in the apical layer, the somas of the immature and mature ORNs in the middle layer, and the NSCs and neuronal precursors within the basal layer. To analyze the regenerative capacity of the OE, olfactory degeneration was induced by $ZnSO_4$ treatment (Yovanovich et al., 2009). *Xenopus laevis* tadpoles exposed to $ZnSO_4$ (20 mg/l) for 24 h showed extensive destruction of the OE in the apical and middle layers, but with the basal cell layer as the main retaining OE component (Fig. 1C). $ZnSO_4$ treatment was sublethal at this concentration and length of exposure.

Regeneration of the OE after $ZnSO_4$ treatment

Tadpoles treated with $ZnSO_4$ for 24 h were then returned to individual containers containing untreated dechlorinated water to recover from the damage incurred. Regeneration was analyzed at: 12 h, 24 h, 72 h, 120 h, and 168 h of recovery time.

Although the tadpoles exhibited some variability in their regenerative potential, the OE began to replenish both the medial and apical layers after 24 h of recovery, increasing the layer thickness (length from the basal lamina to the lumen) (Fig. 1). After 72 h of regeneration, the thickness of the apical layer increased significantly and then remained constant throughout the entire 7-day (168-h) recovery period. Although there was a rapid initial regeneration of the apical and medial layers, the total thickness of the OE did not reach the thickness of the control group during the 7 days following the chemical olfactory destruction.

The normal OE showed proliferating cells labeled with BrdU (BrdU+), mainly proliferating basal cells corresponding to NSCs / progenitors and neuronal precursors, and a few medial and apical cells. Moreover, after 24 h of recovery, the number of BrdU+ cells increased in the OE (Fig. 2). These results demonstrate that under normal physiological conditions there is a constant replenishment of the cells from these basal progenitors adjacent to the basement membrane, and that severe injury of the OE significantly promotes proliferation of these neural progenitors to replenish all the cell types.

Regeneration of ORNs

$ZnSO_4$ treatment causes a non-specific destruction of the OE, affecting both SUS cells and mature and immature ORNs. Immunostaining of β-tubulin-specific neurons (E7) showed the distribution of neurons in the apical half of the normal OE (Fig. 3A), with their axonal projections grouped into the base, forming fascicles of nerves that converged to connect to the olfactory bulb. Furthermore, the ORNs projected their dendrites toward the lumen of the principal cavity to contact with the external environment and capture odorant molecules. After $ZnSO_4$ treatment, the OE lost this organization. Only a few cell layers were retained and cell debris of the tissue remained in the lumen of the principal cavity (Fig. 3B). At 12 h of recovery, a reorganization of the retained cells was observed, although without replenishment of the cell layers (Fig. 3C). After 24 h of regeneration, an increase in the E7+ layer was observed, suggesting that new ORNs were located in the apical region of the OE (Fig. 3D). After 120 h, renewal of ORNs was increased, with cells that had longer dendritic projections, and new neuronal cell layers were present, resembling the control (Fig. 3E). After 7 days of recovery, the OE showed a homogeneous distribution of the ORNs throughout the epithelium, and the cell layers had been restored (Fig. 3F).

Replenishment of Mature ORNs

Mature ORNs are located in the middle layer and are distinguished from the immature neurons by the expression of the OMP, referred to as OMP+ (Fig. 4). $ZnSO_4$ treatment caused destruction of mature ORNs, as shown by the decrease in OMP immunodetection. Immediately after $ZnSO_4$ exposure (0 h of recovery), only a few resistant OMP+ cells were observed. After 12 h of recovery, about a third of OMP+ cells were detected compared to the control group. During the first 24 h of recovery, the OE began to recover the number of mature ORNs, although it still remained without the normal cell organization. After 120 h, the organization of the different cell layers was recovered. Finally, at 7 days (168 h) of recovery, a significant increase in the number of OMP+ cells corresponding to mature ORNs was observed in the middle layer of the OE, similar to control animals (Fig. 4G). This suggests that after a recovery period of 7 days, the neuroepithelium completes tissue regeneration and returns to its original structure and organization.

Regeneration of SUS cells

SUS cells are supporting cells that are anchored at the base of the OE and whose cell bodies are located in the apical layer (Fig. 5A). Dendritic projections of the ORNs are located between them. After the chemical destruction, the middle and apical layers, corresponding to ORNs and SUS cells, respectively, were lost, leaving cell debris dislodged into the lumen of the principal cavity. However, immunostaining was observed in the retained basal layer due to the fact that all cells are in contact with the basement membrane in this pseudostratified neuroepithelium. During regeneration, the SUS cells that began to differentiate appeared in the apical region as epithelial cell layers were recovered. At 24 h of recovery, some cells were

observed with their soma in the apical position, but in a disorganized manner. The OE continued replenishing its cells, and at 5 days (120 h) of recovery, almost complete recovery of the cell types was observed. The SUS cells were distributed throughout the OE, primarily in apical position. However, it was at 7 days (168 h) of recovery that the cellular distribution and morphology were similar to those of the control animals (Fig. 5A-F).

Together, these results indicate that neural regeneration occurs in the OE due to proliferation and differentiation of basal cells. At 24 h post-treatment, the OE is in the middle stage of proliferation and cell differentiation process, and then new SUS cells appear in the apical area and ORNs in the middle layer, which then mature at 7 days after the partial destruction of the OE.

Recovery of the olfactory function

To analyze the correlation between the OE histological structure and function of the olfactory system, a behavioral test that assessed the ability of the larvae to smell was conducted. Because these animals are obligate suspension-feeders, they take in water to the oral cavity and then to the olfactory cavity and gills (Deban and Olson, 2002; Ryerson and Deban, 2010). After evaluating several odor alternatives and experimental arenas, we noted that the frequency of buccal pumping could be a good indicator of the olfactory ability in larvae of *X. laevis* when they perceive the food odor stimulus.

Animals under normal physiological conditions (control) and at different periods of recovery after treatment with $ZnSO_4$ (0 h, 24 h, 120 h and 168 h) were analyzed. The control animals exhibited a more than two-fold higher frequency of buccal pumping when presented with the food odor stimulus (spiruline), indicating that the stimulus was perceived (Fig. 6).

At 0 h and 24 h of recovery, larvae showed no changes in the frequency of pumping in the presence of the food stimulus. At 5 days (120 h), larvae showed an increase in the buccal pumping and, finally at 7 days of recovery, larvae showed a statistically significant increase in the pumping frequency compared to non-responding tadpoles and similar to the control group, suggesting a complete restoration of the olfactory function (Fig. 6).

Discussion

Pathogens and noxious agents constantly affect the ORNs in the nasal cavity. These environmental agents may damage neurons, which ultimately may result in the loss of the sense of smell. However, the olfactory system has the unique ability to generate new neurons throughout the life of an organism. Olfactory neural stem cells in the basal part of the OE give rise to new sensory neurons and extend their axons to the olfactory bulb, and are integrated into existing circuits continuously. Therefore, the olfactory system provides a unique opportunity to study axonal guidance, formation of synaptic connections and the molecular mechanisms involved in neuronal differentiation and neural specification. Currently, the cellular and molecular mechanisms that control the formation and maintenance of the olfactory system are poorly understood. Therefore, our work focused on the analysis of the regeneration dynamics of the different cell types after severe damage to the *X. laevis* OE after $ZnSO_4$ treatment.

Self-renewal and regenerative capacity of the OE

To study the self-renewal of ORNs and SUS cells in the damaged OE, we used pre-metamorphic *X. laevis* larvae at stages 53-54. In these stages, the larvae have a developed principal cavity with its different cell layers, and this cavity continues to function in aquatic olfaction (Dittrich et al., 2015). The fact that the principal cavity is in direct contact with water has allowed us to expose tadpoles to $ZnSO_4$ dissolved in water, which causes degeneration and destruction of the OE, without damaging the respiratory system of the larvae, which is comprised of the gills during the larval stage (Yovanovich et al., 2009).

Other studies have used different techniques to induce injuries of varying degrees of severity. For example, bulbectomy and axotomy, where the olfactory bulb is removed or the olfactory nerve is cut, generate a slight injury and only the ORNs are ablated (Cancalon, 1987; Leung et al., 2007; Iwai et al., 2008). Other treatments, such as exposure to methyl bromide gas or $ZnSO_4$, generate severe damage, causing the degeneration of both ORNs and SUS cells (Herzog and Otto, 1999; Jang et al., 2003; Yovanovich et al., 2009). Therefore, bulbectomy and axotomy provide opportunities to study different aspects of neuronal regeneration, whereas treatment with $ZnSO_4$ allows the analysis of neural differentiation, both in the neuronal lineage and in glial cells.

In the present work, we used $ZnSO_4$ treatment optimized for *X. laevis* stages 53-54, and found that a sublethal concentration of $ZnSO_4$ (20 mg/l) causes severe damage to the OE, apparently without affecting the morphology of other tissues and organs. These observations are consistent with previous studies on the effects of $ZnSO_4$ on the OE of another anuran, *Rhinella arenarum* (Yovanovich et al., 2009). The destruction of the OE by this treatment was evidenced by the morphological rearrangement of the OE, including loss of ORNs and SUS cells but not of basal cells. This massive destruction of the OE was accompanied by loss of the olfactory function, since animals treated with $ZnSO_4$ showed no behavioral response to an

odorous stimulus, which would be the result of the significant decrease in the number of mature ORNs.

Our results demonstrate that, under normal physiological conditions, a homeostatic steady state involves a constant renewal of OE cells, mainly through basal cell proliferation, as evidenced by BrdU incorporation during cell division (Fig. 2). A small number of dividing cells were localized in the apical layer corresponding to SUS cells, which is consistent with that observed in mammals, where some SUS cells are capable of replicating (Graziadei and Graziadei, 1979; Calof and Chikaraishi, 1989). However, the neural progenitor cells are located in the basal layer. Basal cells include NSCs (HBCs and GBCs) and neuronal precursors, and may include most immature stages of new differentiating ORNs (Calof et al., 2002; Beites et al., 2005). We observed the constant renewal capacity of the OE under normal physiological conditions as well as the increased proliferation rate of basal cells of the OE following the destruction with $ZnSO_4$. This increase in proliferation may be a consequence of trophic factors and molecular mechanisms activated and / or increased, which promote rapid renewal of the ORNs and SUS cells that restore the function of the olfactory system (Frontera et al., 2014). Certainly, neurogenesis in the OE either for self-renewal during normal physiological conditions or for the regeneration of the OE after injury involves different factors and biological processes (Brann and Firestein, 2014). In mice, GBCs have a high rate of cell division and incorporation of BrdU (Huard and Schwob, 1995) and are considered the neural progenitors for self-renewal in normal conditions and during regeneration after slight damage. In contrast, HBCs have a low rate of division and are considered to be quiescent NSCs. HBCs respond to severe injury of the OE (Wang et al., 2011; Brann and Firestein, 2014; Joiner et al., 2015; Schnittke et al., 2015). In *X. laevis*, the existence of HBCs and GBCs has not been determined precisely, although OE basal cells have been described based

on morphological features that differentiate two different cell types in the basal layer (Hassenklover et al., 2009).

In *X. laevis* larvae from the same stage, the extent of damage generated by $ZnSO_4$ treatment differed among animals, generating some variability in the regeneration kinetics. However, we observed that, after injury, the increased number of proliferating basal cells was accompanied by the gradual restoration of different cell markers. At 12 h of recovery, the OE retained only the basal cell layer. At 24 h, we observed an increased number of cell layers along with a significant increase in mature ORNs (Figs. 1 and 4). New neurons are distinguished by neuronal β-tubulin located in the middle layer, with their axonal projections to the basal region, and dendritic projections with their cilia towards the lumen, whereas SUS cells begin to extend their somas to the apical layer of the OE. At 24 h of recovery, we found a significant increase in the proliferation rate of basal cells and a significant increase in mature ORNs as compared to 0 h of recovery, although even still significantly lower than in control animals. We considered this recovery period as the time window in which the OE has been largely in the regeneration process and activation of neuronal differentiation.

At 72 h of recovery, none of the cell types and the respective cell layers differed from that observed at 24 h. Therefore, this time period would not be sufficient to fully recover the natural organization and morphology of the OE. At 120 h, the organization of the OE resembled that of the control group; however, the number of mature ORNs was lower. The OE seemed to have partially recovered the olfactory function, although the response of olfaction remained lower than in control animals. At 7 days of recovery, the OE reached a morphology similar to that of the control, the number of mature ORNs was recovered and animals restored the response to the odorant stimulus. These results suggest that 168 h of recovery after severe injury of the OE is sufficient to complete regeneration and re-innervations of new ORNs to the olfactory bulb. Therefore, *X. laevis* larvae seem to have a

greater regenerative capacity than adult rodents, where the recovery of the OE takes three weeks after the intranasal infusion of $ZnSO_4$ (Herzog and Otto, 1999; Schwob, 2002; Williams et al., 2004). This difference could be due to the fact that cell proliferation and differentiation processes are faster during development than in adult life.

In conclusion, this study describes the regenerative capacity of the *X. laevis* olfactory epithelia after massive chemical destruction. Our results demonstrate that after severe damage of the OE, rapid regeneration occurs from multipotent stem cells (NSCs) present in the basal layer. The regenerating mechanisms activated allow the recovery of the olfactory morphology and physiology, including glomerular innervation and restoration of odor detection, 7 days after the treatment. Thus, the olfactory system of *X. laevis* tadpoles is a valuable model to study neural regeneration after severe damage due to the remarkable plasticity of the olfactory system, as recently demonstrated in fish species (White et al., 2015).

Figures

Fig. 1. Olfactory epithelium of *Xenopus laevis* larvae, chemical destruction and subsequent regeneration. (A) dorsal view of an *X. laevis* larva (stage 54) olfactory epithelium (OE), olfactory nerve (ON), olfactory bulb (OB); (B) longitudinal section of normal OE at the principal cavity (PC), basal layer (BL), medial layer (ML), apical layer (AL), scale bar: 50 µm; (C) OE after severe injury with zinc sulfate treatment (0 h); (D) quantification of the average thickness (µm) ± standard error of the OE layers at different periods of olfactory recovery. Different superscripts mean statistically significant difference between groups, for each layer. n = 6 for each group, ANOVA and Tukey test, $p < 0.05$.

Fig. 2. Proliferating cells labeled with BrdU (BrdU+) in normal and regenerating OE. (A) OE under normal physiological conditions; (B) OE after treatment with zinc sulfate without recovery (0h); (C) 24 h of recovery after $ZnSO_4$ treatment (24h); (D) Quantification of BrdU+ cells per area of the OE in control (Ct) and $ZnSO_4$-treated animals without recovery (0h) and after 24 h of recovery. Asterisks indicate significant differences between groups, ANOVA and Tukey test, ***p <0.0001.

Fig. 3. Organization of olfactory receptor neurons (ORNs) and dynamic regeneration of the OE after severe damage by $ZnSO_4$ treatment. Confocal analysis of neuronal β-tubulin (E7) immunodetection used as an ORN marker. (A) OE under normal physiological conditions; (B) OE after treatment with $ZnSO_4$ without recovery (0 h); (C) 12 h; (D) 24 h; (E) 120 h; and (F) 168 h of recovery, showing the reorganization of ORN distribution. Dotted lines indicate the extent of the OE from the basement membrane to upper limits next to the lumen. Arrows indicate cell debris in the lumen of the principal cavity, and head arrows mark the nerve fascicles. Scale bar: 50 µm.

Fig. 4. Location and regenerative dynamics of mature ORNs in the OE of *X. laevis*. Immunostaining of mature ORNs with the specific marker OMP. (A) OE under normal physiological conditions; (B) OE after severe injury with $ZnSO_4$ treatment (0 h); (C) 12 h; (D) 24 h; (E) 120 h; and (F) 168 h of recovery. Scale bar: 50 µm. (G) ORNs OMP+ per µm of OE perimeter, in control animals (Ct) and different periods of recovery. Statistically significant

differences between groups are denoted by different superscripts, n = 6 for each group, ANOVA and Tukey test, $p < 0.05$.

Fig. 5. Organization and regeneration of sustentacular (SUS) cells in the OE of *X. laevis* larvae after severe injury with $ZnSO_4$ treatment. Confocal microscopy images, immunodetection of SUS cells by the specific marker cytokeratin II (CytKII). (A) SUS cells in the normal OE; (B) OE after severe chemical injury without recovery period (0 h); (C) OE after 12 h of regeneration; (D) 24 h of regeneration; (E) 120 h of regeneration; (F) 168 h of regeneration. Dotted lines indicate the extent of the OE from the basement membrane to upper limits next to the lumen; and arrows indicate cell debris in the lumen of the principal cavity. Scale bar: 50 µm.

Fig. 6. Perception of chemical stimulus through olfaction. Change of buccal pumping frequency was analyzed in the presence of a food stimulus (spiruline) in water (frequency post-stimulus / pre-stimulus) (see Materials and methods). In control animals (Ct) and at different periods of recovery after destruction of the OE (0 h, 24 h, 120 h and 168 h). Different letters on the bars indicate significant differences between groups, ANOVA and Tukey test, $p < 0.05$.

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Figures