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Variable functional recovery and minor cell loss in the ganglion cell layer of the lizard *Gallotia galloti* after optic nerve axotomy

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ABSTRACT

The lizard *Gallotia galloti* shows spontaneous and slow axon regrowth through a permissive glial scar after optic nerve axotomy. Although much of the expression pattern of glial, neuronal and extracellular matrix markers have been analyzed by our group, an estimation of the cell loss in the ganglion cell layer (GCL) and the degree of visual function recovery remained unresolved. Thus, we performed a series of tests indicative of effective visual function (pupillary light reflex, accommodation, visually elicited behavior) in 18 lizards at 3, 6, 9 and 12 months post-axotomy which were then processed for immunohistochemistry for the neuronal markers SMI-31 (neurofilaments), Tuj1 (beta-III tubulin) and SV2 (synaptic vesicles) at the last timepoint. Separately, cell loss in the GCL was estimated by comparative quantitation of $DAPI^+$ nuclei in control and 12 months experimental lizards. Additionally, 15 lizards were processed for electron microscopy to monitor relevant ultrastructural changes in the GCL, optic nerve and optic tract throughout regeneration. Hypertrophy of RGCs was persistent, morphology of the regenerated nerves varied from narrow to neuroma-like features and larger regenerated axons underwent remyelination by 9 months. The estimated cell loss in the GCL was 27% and two-third of the animals recovered the pupillary light reflex which involves the pretectum. Strikingly, visually elicited behavior involving the tectum was only restored in two specimens, presumably due to the higher complexity of this pathway. These preliminary results indicate that limited functional regeneration occurs spontaneously in the severely injured visual system of the lacertid *G. galloti*.

1. Introduction

Retinal ganglion cells (RGCs) can regenerate their axons in fish, amphibians, and certain species of reptiles, but not in mammals. The axon regeneration process in the lizard *Gallotia galloti* occurs despite a mammalian-like environment represented by (1) persistent glial scar (Lang et al., 2002, 2008; Romero-Alemán et al., 2013), (2) upregulation of axon growth inhibitors such as the myelin-associated inhibitors (Lang et al., 1998), tenascin-R (Lang et al., 2008) and (3) the absence of proliferating cells in the control and experimental retina as a source of new-born neurons (Casañas et al., 2011; Romero-Alemán et al., 2013). In contrast, the regeneration process in anamniotes (e.g. fish and frogs) occurs in a highly supportive microenvironment represented by (1) transitory gliosis (Stafford et al., 1990), (2) continuous adult retinal neurogenesis, (3) lack, dysfunctionality or downregulation of axon growth inhibitors (Becker et al., 1999, 2004), (4) upregulation of axon growth promoters (e.g., neurotrophins, Caminos et al., 1999; myelin protein zero, Schweitzer et al., 2003; invading Schwann cells, Nona et al., 2000). These differences may account for the successful functional regeneration in fish and amphibians as compared to the limited functional recovery in the lizard *Ctenophorus ornatus*. Interestingly, visual training in *C. ornatus* restored vision in all animals, but to varying extents, and always less than in normal animals (Beazley et al., 2003).

To date, the agamid lizard *C. ornatus*, the lacertid *G. galloti*, and the snake *Vipera aspis* are the only reptilian species in which abundant RGC axons regrow after crush or transection reaching the superficial optic tectum and other visual areas (Rio et al., 1989; Beazley et al., 1997; Lang et al., 1998; Dunlop et al., 2000). However, in two Australian species of geckos and a turtle, axons show

Abbreviations: CNS, central nervous system; GCL, ganglion cell layer; NFL, nerve fiber layer; OCh, optic chiasm; OT, optic tectum; OTr, optic tract; Pia, piamater; RGCs, retinal ganglion cells; sfgs, stratum fibrosum et griseum superficialis; so, stratum opticum.

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minimal regrowth and/or take erroneous paths (e.g., ipsilateral optic tract, unlesioned optic nerve) and animals remained blind via the experimental eye (Dunlop et al., 2004). The expression pattern of glial and neuronal markers, axon growth promoters (e.g. laminin, fibronectin, neurotrophins), and the vascular structure named *conus papillaris* have been previously analyzed by our group (Lang et al., 1998, 2002, 2008; Santos et al., 2006, 2008, 2011; Romero-Alemán et al., 2010, 2012, 2013; Alfayate et al., 2011; Casañas et al., 2011). Nevertheless, two important aspects of this regeneration model were lacking: cell loss in the GCL (RGCs) and the visual function of the regenerated system.

Our aim was to determine if cell loss occurs in the GCL, as a previous study failed to demonstrate apoptotic cells throughout regeneration (Lang et al., 2002) and to assess whether *G. galloti* lizards undergo any functional recovery of the visual system after axon regeneration, to finally complement these data with macroscopic and immunohistochemical observations in the same specimens. We performed reflex/unconscious tests mediated by the pretectum which do not require any topographic organization (e.g., pupillary light reflex, and accommodation) and we also tested behaviorally relevant tasks mediated by the optic tectum, which require topographically organized projections (visually elicited behavior). The pupillary light reflex is driven by a small RGCs subpopulation in rat (ventral hemiretina) and birds (central and temporal retina) and involves the olivary pretectal nucleus and the area pretectalis, respectively (Gamlin et al., 1984; Trejo and Cicerone, 1984; Young and Lund, 1998). This pathway is also conserved in reptiles (Kozicz et al., 2011), and projections to pretectal nuclei have been demonstrated in several species of lizards, including *G. galloti* (de la Calle et al., 1986; Medina and Smeets, 1992; Casini et al., 1993). Visual accommodation occurs when the animal focus on a near object and it also involves pretectal areas (Kozicz et al., 2011). The main retinorecipient region in birds and reptiles is the superficial optic tectum, therefore visually elicited behavior relies mainly in this region. Finally, this study was completed with electron microscopy to monitor RGCs and their regenerating axons throughout the postlesion period.

A summary of the comparative time course of axon regeneration after both optic nerve transection in *G. galloti* and optic nerve crush in *C. ornatus* is represented in Table 3. The meningeal sheath remains intact as a conduit for regeneration in crush lesions, but it is cut in the transection injury. Therefore, the challenge of RGC regeneration is clearly greater after optic nerve transection, imitating the most harmful CNS traumatic injuries in mammals. In this context, the present study offers a complementary analysis of spontaneous CNS axonal regeneration capacity *in vivo*.

2. Materials and methods

2.1. Animals maintenance and unilateral optic nerve transection

We used 35 adult lizards indigenous to the Canary Islands, *G. galloti*. They were collected in the wild under license and maintained and treated in the laboratory according to European animal welfare legislation. Experimental lizards were lesioned according to Romero-Alemán et al. (2013). Briefly, adult lizards were anesthetized by intraperitoneal injection with diazepam 12.5 mg/kg and ketamine 250 mg/kg. Under a binocular microscope, an incision was made along the margin of the largest supraocular osteodermal plaque to expose the ON, which was transected at about 1 mm from the eye using iridectomy scissors. Next, the injury was covered with spongostan, the osteodermal plaque was put back into place and lizards recovered and behaved normally. Two animals were sacrificed at 4 days post-axotomy to observe early anatomical changes after surgery. Fifteen lizards were allowed to recover over periods between 0.5 and 12 months for electron microscopy studies and the remaining eighteen animals were allowed to recover 12 months for the functional tests and finally processed for immunohistochemistry. For the DAPI counts in the GCL we used sections of previous immunohistochemistry (Santos et al., 2011) corresponding to six unlesioned control lizards and six lesioned lizards. Lizards were maintained in large holding tanks fitted with heaters and overhead lighting (daily cycles of 12-h light/dark), with free access to water and a mixed diet of commercially available cat food and fruits. The temperature was maintained between 20 and 28 °C according to the average temperature in their natural environment.

2.2. Visual tests

Visual tests were designed according to Beazley et al. (1997), Dunlop et al. (2004) and Pinto and Enroth-Cugell (2000). Firstly, a T-maze test was used in control lizards to analyze lizard preference for dark/illuminated areas. However, our preliminary observations precluded the performance of this test in experimental lizards due to the heterogeneity of individuals' behavior response. Some animals chose rapidly dark areas (to hide) whereas others remained frozen or tried to climb the maze walls. Three other tests which proved adequate for *G. galloti* were performed in the 18 experimental lizards at 3, 6, 9 and 12 months postlesion, according to the slow process of axonal regrowth reported by our group (Lang et al., 1998). At each stage, tests were performed twice, with two independent observers in each trial (four observations per lizard and stage). Observers were blind to the previous results obtained for each of the specimens so that annotations were taken independently. Moreover, photos and videotapes were taken for each specimen to record and check our observations.

2.2.1. Pupillary light reflex

The first test examined the pupillary light reflex with the aid of a torch. It was tested in normal lizards and proved an adequate test to monitor visual function. Responses through the experimental eye were compared to those of the unaffected eye and were classified as robust/moderate/absent, taking into account both the degree of pupil constriction and the latency of the responses. Thus, robust was set as a clear and rapid contraction, moderate was set as protracted contraction confirmed in the images recorded, and absent refers to unequivocal unchanged pupil diameter. As in lizards RGC projections are fully crossed, no contribution of the opposite eye can control pupil mobility, contrary to rodents.

2.2.2. Visual accommodation

Secondly, visual accommodation for near vision (focus involving intraocular muscles) was tested by moving an object (mealworm hanging on a thread) at 2–6 cm from the eye. This response was clear and rapid in control eyes.

2.2.3. Visually guided behavior

Thirdly, behavioral head movements were analyzed by presenting the mealworm firstly to the affected eye of the lizards and then to the control unaffected eye. When the mealworm reached the visual field of the unaffected eye, lizards responded with head/body movements to avoid or to bite the prey. As a control, no responses were recorded via the lesioned eye during the first months postlesion when axons have not regrown to their targets.

2.3. Antibody characterization

Table 1 describes the primary antibodies used in this study.

Tuj1 (R&D Systems, Germany) is a mouse monoclonal antibody raised against rat microtubules. It recognizes the beta-III tubulin