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Research Report

Study of adult neurogenesis in the *gallotia galloti* lizard during different seasons☆

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ABSTRACT

In a previous study we found a seasonal distribution of cell proliferation (the first stage of adult neurogenesis) in the telencephalic ventricular walls of the adult *Gallotia galloti* lizard. The aim of the present work was to determine the influence of seasonality on the subsequent migration of the resulting immature neurons. We used wild animals injected with bromodeoxyuridine and kept in captivity within 30 days. To confirm the neuronal identity of these cells, we used double immunohistochemical 5-bromo-2′-deoxyuridine (BrdU) and doublecortin (DCX, an early neuronal marker) labeling, as well as autoradiography after the administration of methyl-[^{3}H] thymidine ([^{3}H]T). We found that: (1) the rate of cell division and/or migration from the ventricular walls varied with the season, especially in regions related with olfaction. (2) Immature neuron-like cells appeared to migrate in an apparently radial and tangential way towards different parts of the telencephalic parenchyma. (3) We did not observe ultrastructurally mature neurons until at least 90 days later, a period considerably greater than that reported for other species of vertebrates in similar studies.

1. Introduction

The production of new neurons in the adult vertebrate brain takes place by stages. New cells originate in specific brain sites, then migrate, and finally integrate into pre-existing circuits as mature neurons. Different factors have been found to affect each of these processes (Meerlo et al., 2008). These factors may be positive, such as enriched environments (Olson et al., 2006) or physical exercise (van Praag et al., 1999; Wu et al., 2008), or negative, such as stress (Gould et al., 1997, 1998). This suggests that the production and survival of new neurons play a role in adaptation to environmental demands and specific physiological states.

Lizards, as well as birds, show seasonal behavioral variations (courting and mating in Spring, egg-laying and hatching in Summer, etc.), but in lizards they have not be related to adult neurogenesis, except when photoperiod and/or temperature are

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manipulated (Ramirez et al., 1997). Interestingly, in songbirds, adult neurogenesis is regulated in a seasonal way (Alvarez-Buylla et al., 1990; Balthazart et al., 2008). In previous studies we found that adult *Gallotia galloti* lizards, like other lizard species, present cellular proliferation throughout the telencephalic ventricular walls or ventricular zone (VZ), described below in Experimental Procedure. However, in *G. galloti* this proliferation presents a particular regional distribution that differs from other reptiles (Delgado-Gonzalez et al., 2008; Perez-Canellas and Garcia-Verdugo, 1996; Perez-Canellas et al., 1997; Perez-Sanchez et al., 1989).

The aim of the present study was to determine the amount, identity and destination of new cells that migrate away from the VZ, and to what extent this process is influenced by seasonality.

2. Results

2.1. *Cell labeling with BrdU*

In Group C1 (day 1 of BrdU-injection), optical microscope observation revealed BrdU-positive cells, overwhelmingly in the VZ and very few in the parenchyma. Those in the VZ were rounded (Fig. 1A), or slightly elongated, in which case the long axis was perpendicular to the ventricular surface, except in the OT where they were parallel to the surface (Fig. 1B). In addition, they appeared singly, in pairs or in clusters. Labeled cells in the parenchyma were rounded and appeared as single entities.

In Group C30 (day 30 of BrdU-injection) we observed labeled cells in both the VZ (Fig. 1D) and in the parenchyma (Fig. 1E). The labeled cells in the VZ were very similar to those observed in Group C1. Those situated in the parenchyma invariably appeared as single cells. The great majority presented elongated nuclei, perpendicular to the surface of the VZ (Fig. 1F), while the rest had rounded nuclei.

Thirty days after BrdU administration, we found significant differences in the quantity of labeled cells in the VZ according to the different seasons ($F(3,8720) = 76.31$ $p = .000$). Labeled cell levels were low in Winter, peaked in Spring, decreased to below Winter levels in Summer, and increased again in Autumn to almost reach Spring levels (Fig. 1C, black bars).

Representing the data according to brain regions, we found that most regions presented a peak in Spring and/or Autumn, especially AON, LC and MC, thus following the pattern of

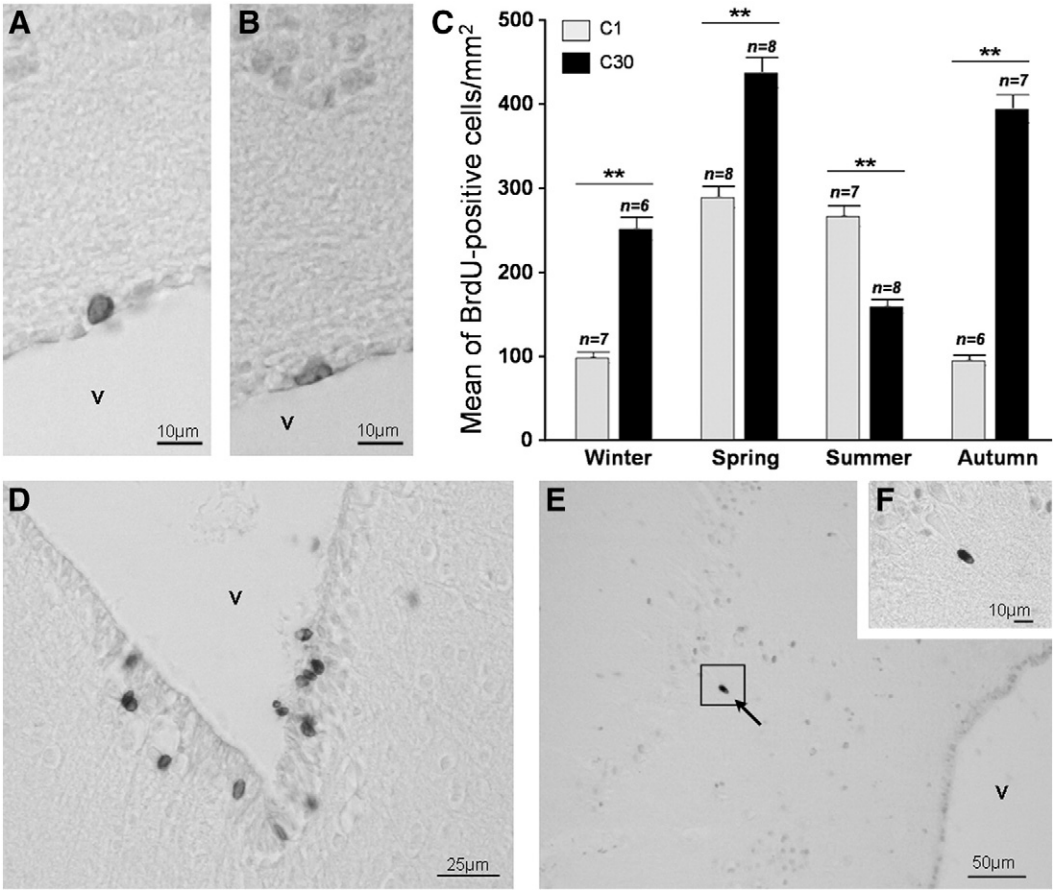

Fig. 1 – BrdU-positive cells observed in all seasons. The figure shows two examples of the types of cells found in the VZ of Group C1: rounded (A) and elongated, parallel to the VZ surface (B). Panel D shows labeled cells found in the VZ and panel E shows one labeled cell in the parenchyma (inset F), in Group C30. Panel C depicts the differences between the two groups in means and standard error of the mean (SEM) of the number of cells in the VZ according to season. Two asterisks indicate significant differences ($p = .000$).

Fig. 1C (black bars). The AON region showed the highest number of labeled cells in almost all seasons (Fig. 2A).

We compared the amount of BrdU-labeled cells in the VZ of groups C1 and C30. We observed a greater number of labeled cells in group C30 in Winter ($t(11) = -7.41$ $p = .000$), Spring ($t(14) = -8.48$ $p = .000$) and especially in Autumn ($t(11) = -16.73$ $p = .000$). In Summer there was a reduction in the number of labeled cells of many regions in the C30 vs. C1 ($t(13) = 6.11$ $p = .000$; Fig. 1C).

In group C30 the mean number of labeled cells in the parenchyma generally remained constant throughout the different seasons ($F(3,8470) = 0.654$ $p = 0.581$). However, we did find seasonal differences in certain regions ($F(3,3935) = 6.424$ $p = .000$): OB, OT, AON (Fig. 2), LC and MC (data not shown).

2.2. *DCX labeling and double BrdU–DCX labeling*

Using DCX as the neuronal marker, we observed cytoplasmic labeling of cells situated in the VZ (Fig. 3A–C) and in the parenchyma (Fig. 3D and E). In all the regions, the cells in the VZ were rounded – with cytoplasm surrounded by the nucleus and no long prolongations (Fig. 3A) – or elongated and perpendicular to the surface (Fig. 3B), except in the OT, where the elongated cells were parallel to the ventricular surface (Fig. 3C). The DCX-positive cells in the parenchyma were elongated, with the nucleus situated at one end (Fig. 3D), in all regions except in the DVR and NS where cells with more than two branches were observed (Fig. 3E).

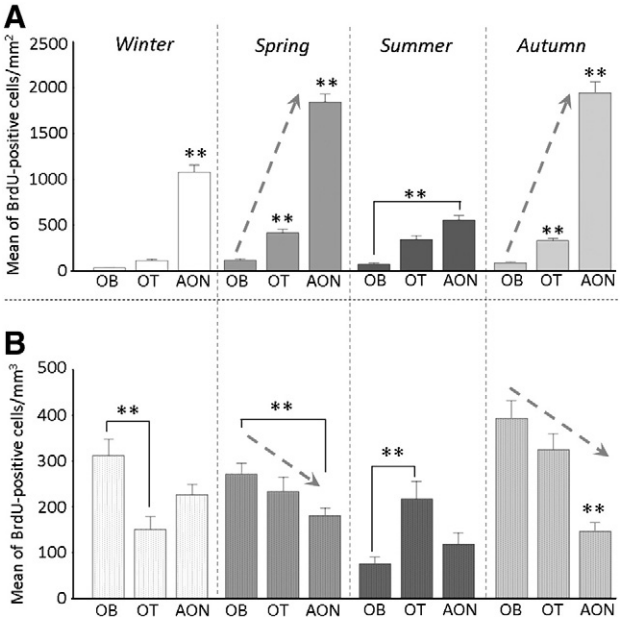

Fig. 2 – Seasonal differences of the number of BrdU-positive cells (mean ± SEM) after 30 days in: (A) the VZ and (B) the parenchyma of the three adjacent olfaction-related regions. The dotted arrow indicates the progression in amount of labeled cells. Strikingly, in A this progression occurs in a rostral-caudal direction and in B in the opposite direction, mainly in Spring and Autumn. Two asterisks indicate significant differences ($p = .000$).

After double labeling of group C30 samples, the VZ showed BrdU-positive and DCX-negative cells, as well as BrdU-positive and DCX-positive cells (Fig. 3F). However, the BrdU-positive cells out of the VZ were also DCX-positive (Fig. 3G) with rare exceptions (Fig. 3F, asterisk).

2.3. *Cell labeling with tritiated thymidine*

After 1 day of survival, most labeled cells were situated in the VZ, in contact with the ventricular cavity. They presented large nuclei with large invaginations, spongy chromatin, little cytoplasm and few organules. In one case we observed a single labeled cell at a distance from the VZ which presented the ultrastructural features of a migrating immature neuron, i.e. with irregular (elongated) cell form, little cytoplasm, few organules consisting mainly of mitochondria, nuclei with nuclear invaginations, and lax chromatin (Fig. 4A).

After 30 days of survival, we observed labeled cells in the VZ and the parenchyma (Fig. 4B and C). The labeled cells situated out of the VZ had ultrastructural characteristics of migrating immature neurons as previously described. In addition, they presented electrodense contacts with neighboring cells (Fig. 4. D). We observed no cases with these characteristics migrating in pairs or groups; they were always single entities (Fig. 4B and C).

In the OB we observed labeled cells of similar size to neighboring cells (mature neurons), but with darker chromatin.

Although scarce (less than 1% of the cases studied), we also found non-neuronal labeled cells, with ultrastructural features that appeared to correspond with those of microglial cells: a small nucleus 5 μm long, dense nuclear membrane (with associated chromatin) and prominent nucleole, associated to neuronal somas with lipofuscin granules.

On studying the ultrastructure of the labeled cells at 1 and 30 days of survival, we did not observe mature neuronal features. In lizards with greater survival times, the following findings were recorded:

After 60 days survival time, we observed a greater proportion of labeled cells in the main cell layers than after 1 or 30 days of survival time. In OB and OT, these labeled cells had abundant mitochondria, endoplasmic reticules, Golgi apparatus cisterns, vacuoles, polyribosomes, etc. However, no synaptic contacts were observed (Fig. 4E). In the other regions we observed immature neurons that appeared to be integrated into the main cell layers. They were more similar to neighboring cells with respect to cytoplasma. Their nuclei were now similar to those of neighboring cells but the state of chromatin aggregation and the presence of nuclear invaginations indicated incomplete maturation.

After 90 days survival time, most of the labeled cells were observed to be in the main layer of cells. The ultrastructure of mature telencephalic neurons in these animals is highly heterogeneous, which makes generalization difficult, but certain common features were observed: cells whose cytoplasm showed abundant organules (such as mitochondria, lysosomes etc.), dense lipofuscin granules and a highly developed system of endomembranes. The nuclear membrane rarely showed invaginations and the chromatin was usually pale and fine-grained. In the OB, OT and in MC (Fig. 4F)

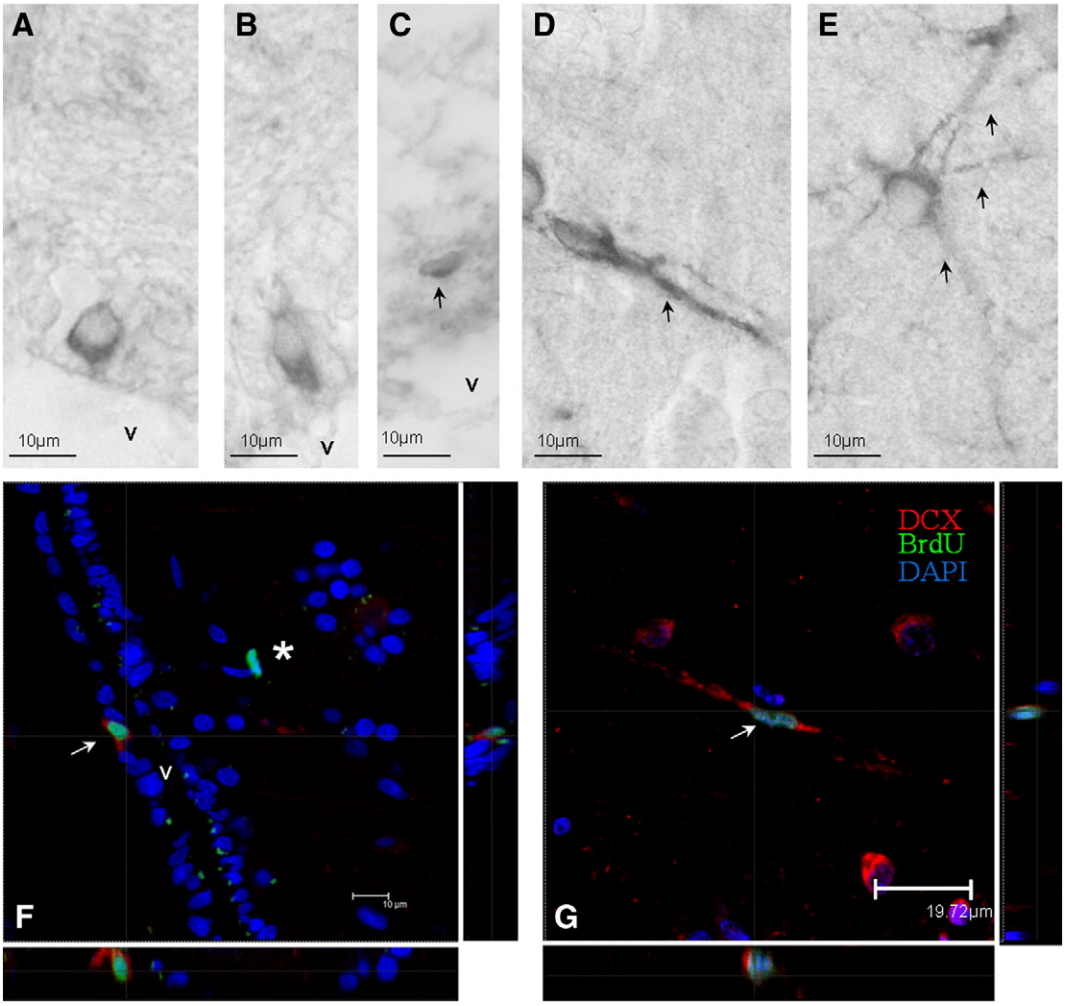

Fig. 3 – DCX-positive cells. The figure shows 5 morphological types of cells with DCX-labeled cytoplasm, observed in different telencephalic regions. In the VZ, we observed rounded cells (A), and elongated cells perpendicular to the surface (B). In the VZ of the OT (C) we also observed elongated cells, parallel to the ventricular surface (arrow). In the parenchyma, we observed elongated cells with one prolongation (D, arrow) and cells with more than two prolongations (E, arrows), normally found in the DVR and NS. Panels F and G show two microphotographs of BrdU-positive (green) and DCX-positive cells (red) situated in the VZ (F) and the parenchyma (G). In F the asterisk indicates a BrdU-postive cell and a DCX-negative cell, possibly an endothelial cell.

we mainly observed cells with these mature neuronal characteristics, but in the other regions the majority of the neurons observed appeared to be young.

In Fig. 5 we resume the cell type and the proportion of labeled cells at the survival times studied.

3. Discussion

3.1. *We observed immature neurons that originated in the VZ and migrated towards the parenchyma of different telencephalic regions*

One day after administering markers of cellular proliferation, the great majority of labeled cells were located in the VZ. Very few were observed in the telencephalic parenchyma. In these cases, they corresponded to: 1) microglial cells and blood vessel endothelial cells, whose number and mitotic activity were constant over time (we found the same frequency at all survival times) as described by other investigators working with the same species of lizard (Garcia-Verdugo et al., 1989; Lopez-Garcia et al., 1988) and with other species (Perez-Canellas et al., 1997; Perez-Sanchez et al., 1989) and 2) a few immature neurons, mainly situated in the internal plexiform layer of the MC (Fig. 4A). This suggests that a certain proportion of immature neurons may divide en route, away from the subventricular or ventricular zone. This coincides with reports of rapidly-dividing immature neurons (in less than 1 day) migrating towards the OB in adult mammals (Morshead and van der Kooy, 1992). It is feasible that a similar phenomenon occurs in lizards, which is a novel finding that needs to be confirmed using complementary techniques.

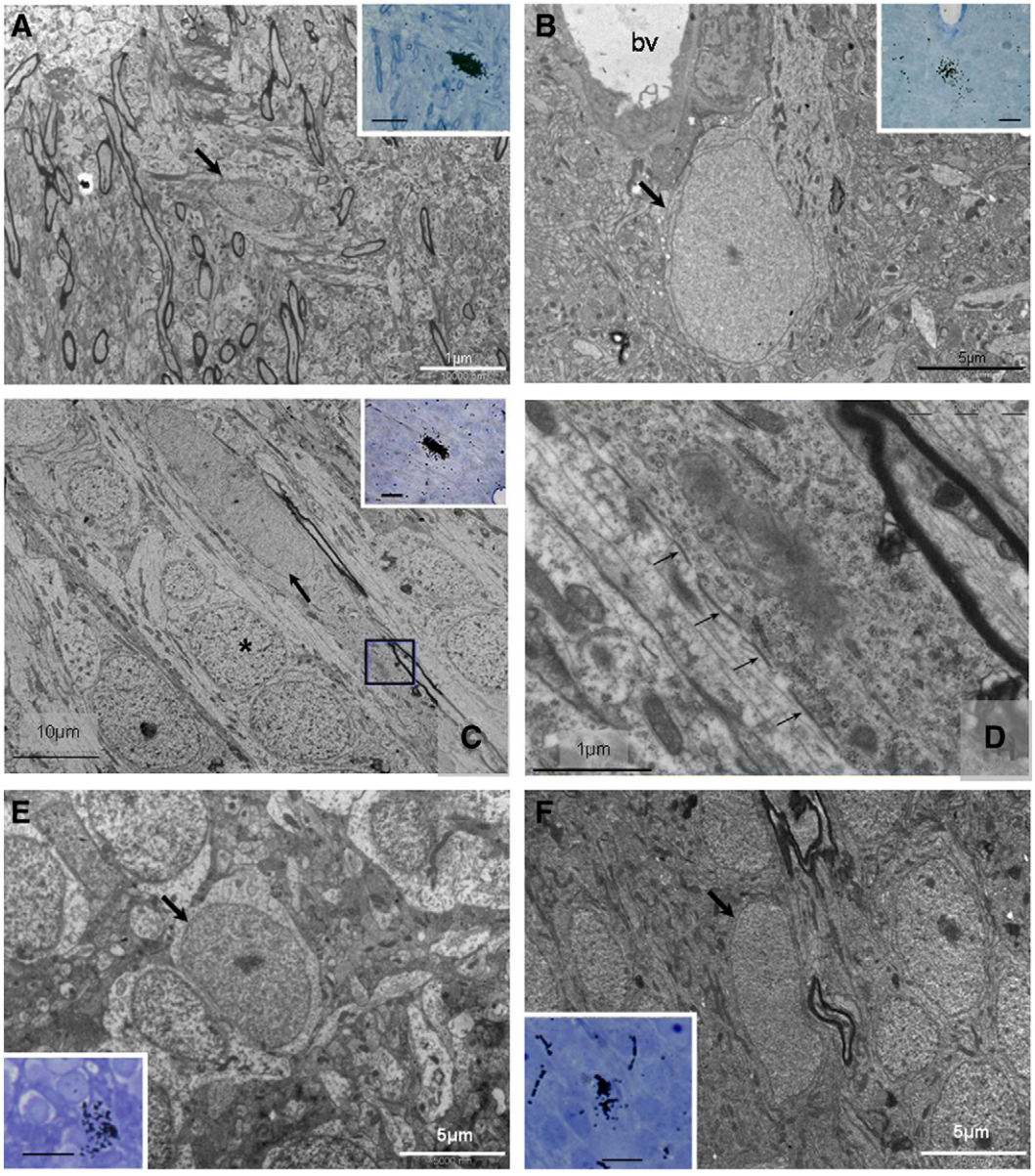

Fig. 4 – Cells labeled with tritiated thymidine (arrows) after 1 day (A), 30 (B–D), 60 (E) and 90 days (F). In A, a labeled cell can be seen in the internal plexiform layer of the MC, with ultrastructural features of a migrating immature neuron. Panel B shows a labeled cell in the VA, proximal to a blood vessel (bv), with ultrastructural features of immature migrating neurons (displaced cytoplasm with abundant mitochondria and elongated nucleus with lax chromatin). Panel C shows a cell with ultrastructural features of a migrating immature neuron: with elongated main axis, nucleus at one end, little cytoplasm and (D) electrodense contacts with neighboring cells (arrows). Panel E shows a labeled cell in the OT (arrow), without the typical morphology of migrating immature neurons (migration may have terminated), but with more lax chromatin than neighboring cells, and a prominent nucleole, which is a feature of immaturity. Panel F shows a labeled cell with ultrastructural features of neuronal maturity, situated in the MC. Inset scale bars: 10 µm.

The present study corroborates the findings of a previous work (Delgado-Gonzalez et al., 2008) where proliferation was observed in all telencephalic regions, especially in the AON, and proliferative rate fluctuated seasonally.

30 days after administering markers of cellular proliferation, we observed labeled cells in both the VZ and the cerebral parenchyma of all the telencephalic regions (Fig. 1D and E). On comparing proliferation in the VZ between goups C1 and C30 (Fig. 1C) we found a greater quantity of labeled cells in Winter, Spring and Autumn in group C30. This result may be explained by the increase of cell division and/or lower migration. In contrast, we observed a lower quantity in Summer in group C30 than in C1; this result could conceivably be explained by apoptosis occurring during Summer in the VZ. However, we did not observe any signs of apoptosis, nor has this been reported in previous studies on lizards under normal conditions (Font et al., 1997). We therefore believe that the most plausible explanation involves the migration of both daughter

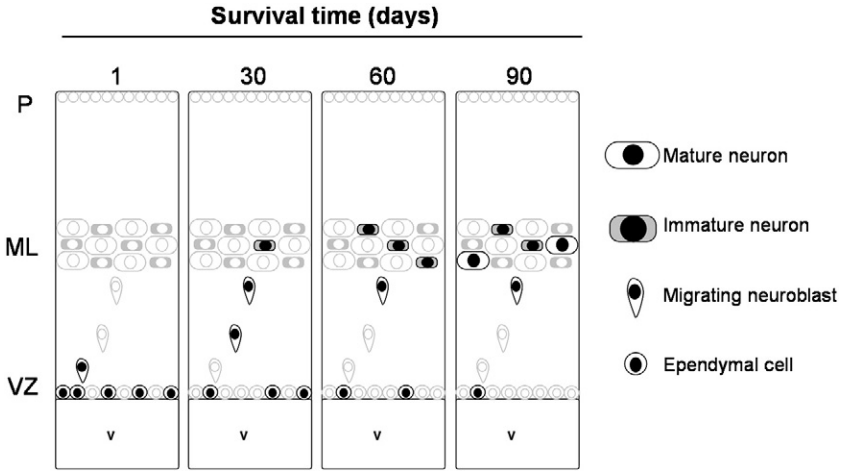

Fig. 5 – Schematic drawing summarizing the cell phenotype and the proportion of tritiated thymidine-labeled cells at 1, 30, 60 and 90 days of survival time. ML: main layer, P: pia, v: ventricle, VZ: ventricular zone.

cells after mitosis. These changes of VZ cell behavior depend on the season, implying interaction between different environmental cues and intrinsic physiological responses. This is supported by the known effect of photoperiod and temperature on reactive neurogenesis in the lesioned MC of adult lizards (Ramirez et al., 1997). However, further studies are required to determine other factors which regulate such VZ cell behavior.

Some of the VZ cells, BrdU-labeled after 30 days of survival, already began to manifest immature neuron characteristics and presented co-labeling with DCX (Fig. 3F). However, in the parenchyma, over 90% presented BrdU–DCX co-labeling (Fig. 3G), ultrastructure of immature neuron (irregular cytoplasm with few organules, nuclear invaginations and less dense chromatin than neighboring cells) and most appeared to be migrating. This clearly suggests that the great majority of parenchyma cells are in fact immature neurons that originated in the VZ and migrated towards the parenchyma, which supports the findings from studies on other species of reptiles (Garcia-Verdugo et al., 1989; Lopez-Garcia et al., 1988; Perez-Canellas et al., 1997; Perez-Sanchez et al., 1989).

3.2. *The only labeled immature neuron-like cells affected by seasonal changes were those related with olfactory regions*

A significant finding was that the total number of parenchyma cells showed no significant seasonal variation, which contrasts with seasonally fluctuating levels observed in the VZ. However, a detailed regional analysis showed large seasonal fluctuations in OB, OT, AON (Fig. 2B) and LC, while the remaining regions presented more or less stable levels throughout the year.

In *G. galloti*, the telencephalic region with the highest proliferative rate, in both groups and for all the seasons, was the VZ of the AON (Fig. 2A), and the only regions presenting seasonal fluctuations in the number of labeled immature neuron-like cells were also those related with olfaction (Fig. 2B). This contrasts with the finding in other species of lizards that the highest rates of proliferation and neurogenesis are found in the MC associated with memory and spatial navigation (Perez-Canellas and Garcia-Verdugo, 1996). Seasonal fluctuation in the number of labeled immature neuron-like cells in olfaction-related regions of *G. galloti* suggests that olfaction itself may also present seasonal fluctuations in effectiveness, sensitivity, reach etc. In fact, greater neuronal recruitment in these olfactory regions may modify smell discrimination (Gheusi et al., 2000) and olfactory memory (Rochefort et al., 2002).

Both our qualitative and quantitative results (Fig. 2) support the idea that olfactory neurons originate in the AON and migrate tangentially towards more rostral regions via the lateral ventricular walls, as postulated in previous studies performed with other reptiles (Penafiel et al., 1996; Perez-Canellas et al., 1997). Therefore, the rostral migratory stream demonstrated in mammals and birds (Doetsch and Scharff, 2001) may also occur in reptiles. In addition, we found that this migration varied according to season: the region with the highest number of labeled immature neuron-like cells was the OB, followed by the OT and AON, in all seasons except Summer (where OT>AON>OB). These results suggest that migration slows in Summer and the cells reach the OB in the other three seasons (Fig. 2B).

In addition to tangential migration, we also found qualitative evidence of radial migration throughout the telencephlon of cells oriented perpendicular to the VZ surface (Fig. 1E), occasionally showing electrodense contacts and white spaces (Fig. 4D), which have also been described in ultrastructural studies performed with the same species of lizard (Garcia-Verdugo et al., 1986).

Lastly, it is noteworthy that, in regions such as the DVR and the NS, we observed irregular-shaped DCX-positive cells with more than two branches (Fig. 3E). This morphology suggests neurons in the process of migration that are “testing” their surrounding environment.

3.3. *At 90 days of survival, we began to observe cells with ultrastructural features of mature neurons but without synaptic contacts*

After 60 days we found labeled cells presenting some features of mature neurons that appeared to have arrived at their

destination. It was not until 90 days of survival that we observed fully mature neurons (Fig. 4F). Nevertheless, we were unable to confirm the presence of synaptic contacts.

In other species of adult animals, less time is required to produce mature neurons; 20–40 days in canaries, (Alvarez-Buylla and Nottebohm, 1988), 8–11 days in zebra finch, (Kirn et al., 1999) and as from 7 days in the lizard *Podarcis hispanica* (Lopez-Garcia et al., 1990). The reasons for this relatively slow maturation in *G. galloti* may be related with the age of the specimens used in this study. Our study used lizards aged approximately 6 years, estimated according to a skeletochronological procedure for this species of lizard (Castanet and Baez, 1988). To our knowledge, other studies have not taken into consideration the age of the experimental reptiles, which may lead to erroneous comparisons. In this study, the delayed appearance of mature neurons (Fig. 5) may be due to (1) stem cells in the VZ continuously produced future neurons throughout the different survival times; (2) slow immature neuron-like cells migration; (3) slow maturational rate, or (4) any combination of these factors.

4. Experimental procedure

4.1. *Telencephalic subdivisions used in this study*

We defined 11 different telencephalic regions and their adjacent areas (Table 1) as described in a previous work (Delgado-Gonzalez et al., 2008), briefly as follows: olfactory bulb (OB), olfactory tract (OT), anterior olfactory nucleus (AON), lateral cortex (LC), dorsal cortex (DC), dorso-medial cortex (DMC), medial cortex (MC), septum (Sp), ventral area (VA), dorsal ventricular ridge (DVR) and nucleus sphericus (NS).

4.2. *Subject and groups*

Our experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All data were obtained using adult male *G. galloti* lizards, captured and kept in North Tenerife with a photoperiod of 10 h 35–55 min of light in Winter, 12 h 47 min of light in Spring, 13 h 33 min of light in Summer and 11 h 33 min in Autumn. The lizards studied were of similar weight (54.90 g ± 1.6) and size (11.97 cm ± 0.1). Therefore age was approximately 6 years old, according to studies previously performed on lizards of the same species (Castanet and Baez, 1991). Since seasonal change in Tenerife is slight, all lizards were captured in the middle of each season to avoid possible seasonal overlap effects which could produce erroneous results. Four 100 mg/kg doses of the cellular proliferation marker 5-bromo-2′-deoxydeuridine –BrdU (Sigma) — were injected at two-hourly intervals, with the first injection at 8:30 a.m. In order to study the seasonal distribution of ventricular proliferation and the labeled cells situated in the parenchyma during the different seasons, we established two groups of lizards in each season: C1 and C30. We used a total of 57 lizards (with a mínimum of 6 per group). Group C1 was captured each season in their natural habitat and kept in captivity for 29 days, then injected with BrdU and killed 1 day after injection. Group C30 was captured, injected with BrdU 1 day after capture, kept in captivity for 29 days and killed 30 days after injection.

For the purpose of autoradiographic studies, we used 3 animals with a survival time of 1 day, 3 of 30 days, 2 of 60 days, and 2 with a survival time of 90 days. These animals were injected with tritiated thymidine (5 μCi/g b wt.).

Table 1 – Telencephalic regions of adult lizard *Gallotia galloti*.

OB	olfactory bulb
OT	olfactory tract
AON	anterior olfactory nucleus
LC	lateral cortex
DC	dorsal cortex
DMC	dorso-medial cortex
MC	medial cortex
Sp	septum
VA	ventral area
DVR	dorsal ventricular ridge
NS	nucleus sphericus

4.3. *Captivity conditions*

After capture, each animal was singly housed in a well ventilated box of 70 × 40 × 30 cm, made of transparent plastic material, isolated from companions. These boxes were located outside, i.e. in natural light–dark photoperiod and temperature conditions. Each animal had ad libitum access to biological food prepared with fruit and/or vegetables, as well as protein (HiPP GmbH and Co.) and water.

4.4. *Perfusion and histology*

After receiving an overdose of chloral hydrate (Sigma) to minimise pain or discomfort, the lizards were intracardially perfused with 0.9% saline followed by 2% paraformaldehyde (Sigma). The brains were removed, washed in phosphate buffer and kept in 70° alcohol for 3 days, then dehydrated and embedded in paraffin. Transversal 10 μm sections were treated for BrdU immunohistochemistry (anti-BrdU, 1:75, Hybridoma Bank, Iowa, USA) as previously described (Choi et al., 2003).

4.5. *Quantification of results*

Cell counts were done on sections as previously described (Delgado-Gonzalez et al., 2008) The number of marked cells by area is expressed in mm2. The number of marked cells by volume is expressed in mm3. We worked with a 4 × 2 factor design. The first independent variable was “Season” with 4 levels. The second independent variable was “post-injection survival time” with 2 levels, C1 and C30. Thus we had 8 experimental conditions, with different subjects in each, totalling 57 subjects. Measures of the dependent variable (no. of marked cells) were performed in different brains in each case. Results were compared using multivariate ANOVA. We used Tukey test for post hoc comparisons between results obtained under the 8 different experimental conditions.

4.6. *DCX–BrdU immunofluorescence procedure*

Hidrated paraffin sections were incubated with goat-anti DCX antibody diluted 1:200 (DAKO), with Donkey anti-Goat

Texas Red diluted 1:400 (Jackson Inmuno-Research), with a mouse monoclonal antibody against BrdU diluted 1:75 (DAKO) and with goat anti-mouse FITC diluted 1:400 (Jackson Inmuno-Research) as previously described (Brown et al., 2003). We used 6′-diamin-2-phenylindol (DAPI) diluted 1:1000 to ensure that the BrdU labeling corresponded to cellular nuclei and not to artifacts. The samples were observed using a confocal microscope (Leica SP2-AOBS).

4.7. *Western-blot procedure*

The telencephalon from adult lizards and 1-month-old male laboratory white mice (control) were homogenized in TENT 1% buffer (with the protease inhibitors COMPLEATE 1x, Roche) and centrifuged at room temperature for 10 min at 15,000 rpm to remove the insoluble debris. The protein concentration in the supernatant was determined using Bicinconinic acid method. The samples (14.25 μg/μl) were electrophoresed in a 12% polyacrylamide SDS-polyacrylamide gel and transferred onto a PVDF (Polyvinylidene Difluoride) membrane, then placed into blocking buffer (Carnation Nonfat Dry Milk 5% in TBS–Tx buffer) for 1 h at room temperature. The same buffer composition served for antibody dilutions, as well as for washes. For detection of DCX, we used goat anti-DCX C-18 (1:500, Santa Cruz Labs, Santa Cruz, CA). The blots were incubated overnight in primary antibody solutions at 4 °C on a shaking table. The following day, the blots were washed and further incubated with peroxidase-conjugated species-specific secondary antibody for 2 h at room temperature (Donkey anti-goat peroxidase 1/4000; Jackson Immunoresearch). Blots were washed and the immune complex was detected by using the detection kit Western Blotting ECL plus (Amersham, UK), according to the manufacturer's protocol.

The result is a band measuring approximately 40 kDa, indicating that the antibody specifically detected DCX, in agreement with findings from previous studies (Brown et al., 2003).

4.8. *Autoradiography procedure*

The phenotype of newly generated cells was determined by combining tritiated thymidine ([^{3}H]T) autorradiography and electron microscopy. Lizards received one subcutaneous injection of [^{3}H]T (Amersham; specific activity 25 Ci/nmol) in one day up to a total dose of 5 μl per gram of animal. Following survival times of 1, 30, 60 and 90 days after the first [^{3}H]T injection, the lizards were deeply anesthetized with chloral hydrate (Sigma) and perfused with a fixative consisting of 2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed from the braincase and immersed in the same fixative for 12 h. Transverse sections were then cut at 200 μm using a tissue chopper, postfixed in 1% osmium tetroxide for 2 h, rinsed, dehydrated and embedded in araldite. Semithin sections were cut at 1.5 μm and mounted on slides, which were dipped in NTB-3 emulsion, exposed in the dark for four weeks, developed in Kodak D-19, and counterstained with 1% Toluidine Blue.

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