The Journal of Experimental Biology 206, 3085-3093

doi:10.1242/jeb.00515

Environment temperature affects cell proliferation in the spinal cord and brain of juvenile turtles

Milka Radmilovich[1], Anabel Fernández[2,3] and Omar Trujillo-Cenóz[2,3,*]

[1]*Departamento de Histología y Embriología, Facultad de Medicina, Montevideo, Uruguay ZC 11800,* [2]*Laboratorio de Neuroanatomía Comparada, Instituto de Investigaciones Biológicas Clemente Estable, Avda. Italia 3318, Montevideo, Uruguay ZC 11600* and [3]*Unidad Asociada a la Facultad de Ciencias, Montevideo, Uruguay ZC 11600*

*Author for correspondence (e-mail: omar@iibce.edu.uy)

Accepted 27 May 2003

Summary

The spinal cords and brains – comprising dorsal cortex (DC), medial cortex (MC) and diencephalon (Dien) – of juvenile turtles acclimated to warm temperature [27–30°C; warm-acclimated turtles (WATs)] revealed higher density values of bromodeoxyuridine-labeled cells (BrdU-LCs) than those acclimated to a cooler environment [5–14°C; cold-acclimated turtles (CATs)]. Both populations were under the influence of the seasonal daily light–dark rhythms. Pronounced differences between WATs and CATs (independent *t*-test; confidence level, $P<0.01$) were found in the central area of the spinal gray matter and in the ependymal epithelium lining the brain ventricles. Forebrain regions (DC, MC and Dien) also revealed significant differences between WATs and CATs (independent *t*-test; confidence level, $P<0.01$–0.05). Unexplored biological clocks that may be affecting cell proliferation were equalized by performing paired experiments involving one WAT and one CAT. Both animals were injected on the same day at the same time and both were sacrificed 24 h later. These experiments confirmed that a warm environment increased cell proliferation in the CNS of turtles. Double- and triple-labeling experiments involving anti-BrdU antibody together with anti-glial protein antibodies revealed that temperature modulates not only cell populations expressing glial markers but also other cells that do not express them. As expected, in the case of short post-injection (BrdU) surviving time points, no cells were found colabeling for BrdU and NeuN (neuronal marker). The probable direct effect of temperature on the cell division rate should be analyzed together with potential indirect effects involving increased motor activity and increased food intake. The fate of the increased BrdU-LCs (death, permanence as progenitor cells or differentiation following neuronal or glial lines) remains a matter for further investigation. Results are discussed in the light of current opinions concerned with post-natal neurogenesis in vertebrates.

Key words: neurogenesis, cell proliferation, temperature acclimation, brain, spinal cord, BrdU, turtle, *Chrysemys d'orbigny*.

Introduction

Since the pioneering studies by Altman and Kaplan (Altman, 1962, 1963; Altman and Das, 1965; Kaplan and Hinds, 1977; Kaplan, 1985), recent conceptions dealing with the organization of the vertebrate central nervous system (CNS) have subscribed to the idea that neurons and glial cells continue to be produced throughout life (Gross, 2000; Momma et al., 2000; Rakic, 2002a,b; Gould and Gross, 2002; Nottebohm, 2002). It is now well known that, at least in some regions of the CNS, there are not permanent neuronal assemblies but changing cell populations in which new neurons replace older ones (Carleton et al., 2003). Moreover, this novel conceptual framework has revealed additional complexities. Information is accumulating indicating that post-natal neurogenesis may be modulated by diverse factors including enriched environment living (Nilsson et al., 1999), stress (Gould et al., 1998; Jacobs et al., 2000; Tanapat et al., 2001) and hormones (Cameron and Gould, 1994; Cameron and McKay, 1999; review by Gould and Gross, 2002). In this context, findings demonstrating that seasonal variation modulates neurogenesis in the vocal centers of canaries constitute a paradigmatic landmark (Nottebohm et al., 1994; Barnea and Nottebohm, 1994; review by Nottebohm, 2002). Concomitantly, important advances have been made to explore experimentally the identity of the cells that retain post-natal neurogenic properties. To date, common views tend to indicate that primary neuronal precursors are cells '*contained in the neuroepithelium–radial-glia–astrocyte lineage*' (Alvarez-Buylla et al., 2001).

Despite important advances made in the field, information concerned with environmental factors influencing cell proliferation in the CNS of ectotherm vertebrates is still scarce. Since metabolic activity in these animals is largely dependent

on heat transfer from the environment (Prosser, 1952), temperature appeared to be a plausible external factor that could affect post-natal cell proliferation in the CNS. Confirming this hypothesis, Ramírez et al. (1997) and Peñafiel et al. (2001) have reported that temperature increases neurogenesis and neuroblast migration in the brain of adult lizards.

The purpose of the present paper is to demonstrate that environmental temperature modulates cell proliferation in the CNS, including the spinal cord, of juvenile turtles. Here, we employed bromodeoxyuridine (BrdU) to label proliferating cells and other immunostaining procedures to identify the temperature-affected cell population. Our studies have revealed that warm-acclimated turtles (WATs) showed a statistically significant increase in proliferating cells when compared with cold-acclimated turtles (CATs). Multiple-labeling experiments showed that an important percentage of the proliferating cells exhibited the morphological and immunostaining characteristics of glial cells, including typical radial glia (RG). Since unanimously accepted criteria for identifying stem/progenitor cells are still lacking (Scheffler et al., 1999; Seaberg and van der Kooy, 2003), we have preferred to use a purely descriptive term such as 'proliferating cells' to name the cell population that incorporated BrdU.

Materials and methods

Turtles (*Chrysemys d'orbigny* L.) were obtained from a local dealer following the guidelines established by the Ministerio de Agricultura y Pesca, Division Fauna de Uruguay. Juvenile specimens of *C. d'orbigny* (carapace length, 7–9 cm) were maintained according to protocols approved by the Institutional Animal Committee at the Instituto de Investigaciones Biológicas Clemente Estable (which conforms to NIH guidelines).

BrdU-labeling of proliferating cells

Twenty turtles were divided into two groups of 10 animals, and each group was maintained in separate aquaria at different temperatures. WATs were maintained in a warm, controlled environment (27–30°C), while CATs were maintained in an outdoor aquarium under the influence of the seasonal fluctuating temperature (5–14°C). Since recent investigations performed in mammals indicate the adverse influence of stress on post-natal neurogenesis (Gould et al., 1998), a fixed-temperature cold environment was avoided. On the other hand, the selected warm temperature range was revealed to be stimulating for turtles, increasing their motor activity, food intake and body mass. Both groups were under seasonal daily light–dark rhythms and were provided with abundant food (living *Tubifex* and small earthworms). In experiments performed with CATs, the mean temperature during measurements made six days before the injection time point was considered to be the environmental temperature for each of the experiments. Turtles from both groups received a single intraperitoneal dose of BrdU (100 mg kg^{-1}) and were perfused with the fixative solution 24 h later. It should be noted that, according to our test experiments (N=4), a dose as high as 800 mg kg^{-1} seems to be innocuous and does not induce labeling of non-mitotic cells.

We also performed 'paired experiments' (N=4) in which one WAT and one CAT received the BrdU pulse on the same day at the same time, and both animals were sacrificed 24 h later. These experiments were designed to rule out unexplored biological rhythms (Cermakian and Sassone-Corsi, 2001) that might be affecting cell proliferation in the CNS. To minimize potential variations during immunostaining, tissues from both turtles were processed together.

Fixation procedures were always performed in anesthetized animals unresponsive to nociceptive stimuli. To achieve complete anesthesia, 5 mg kg^{-1} of sodium methohexitone (Brietal, Lilly, Basingstoke, UK) were injected intraperitoneally. Saline used to wash the blood vessels as well as the fixative fluids were propelled into the vascular bed using a peristaltic pump. Brains and spinal cords were albumin–gelatin embedded and cross-sectioned using a vibrating microtome (each section was 60 μm thick). Sections were hydrolyzed (2 mol l^{-1} HCl for 1 h), passed through three washing buffered solutions and incubated overnight in a buffered solution containing 0.3% Triton X-100 and the anti-BrdU antibody (1:500 monoclonal; Dako A/S, Glostrup, Denmark). Detection of nuclei that had incorporated BrdU was achieved using horseradish peroxidase (HRP)-conjugated or fluorophore-conjugated secondary antibodies (anti-mouse made in goat; 1:500; Chemicon International, Inc., Temecula, CA, USA). The HRP was revealed using diaminobenzidine or the peroxidase substrate kit from Vector Labs (Burlingame, CA, USA).

Densities of BrdU-labeled nuclei (BrdU-LN) were calculated in the following spinal cord regions: lateral funiculus (LF), dorsal funiculus (DF), ventral funiculus (VF), dorsal horn (DH), ventral horn (VH), intermediate region (IR) and central region (CR). Densities of BrdU-labeled nuclei were also calculated in the dorsal cortex (DC) and medial cortex (MC) of the brain and in paraventricular zones of the diencephalon (Dien). In the case of the spinal cord, BrdU-LCs were counted within the limits of circles (radii, 50 μm) distributed to explore the main gray matter and white matter regions. In these counts, marked endothelial or blood cells were discarded. A similar procedure, but adapted to the geometry of the organ (using squares of the same area instead of circles), was employed to study different regions of the brain parenchyma. The circle or square, reproduced at the appropriate magnification on a transparent sheet, was overlaid onto the screen of a high-resolution monitor displaying images of the spinal cord or brain sections. Density (D) of labeled cells was calculated in each spinal cord region from counts made in samples of eight sections obtained at each segment from cervical to lumbar levels. Density of labeled cells in the brain was calculated from three samples (R1–R3) taken from the ependymal epithelium (EpE) towards the nervous parenchyma in each of the eight explored sections; homologous regions of

WATs and CATs were sampled. For the spinal cord, the algorithm was: $D=\sum N_L/(N_{SA}\times 32)$, in which N_L is the number of marked nuclei in each sample, N_{SA} is the number of sampled areas, and 32 is the number of sections (eight) multiplied by the four spinal cord segments explored (C1, C2, T and L). An analogous algorithm was used when dealing with the brain but the multiplier was 24 instead of 32, since eight sections from three zones (DC, MC and Dien) were explored (Fig. 1).

Multiple immunostaining

Using appropriated fluorophore/cromophore combinations, we obtained differential staining between nuclei that incorporated BrdU and the nuclear or cytoplasmic proteins characterizing neurons or glial cells. For these purposes, we employed eight turtles. Two basic criteria were established for proper identification of double-labeled (BrdU–glial/neuronal marker) and single-labeled (BrdU) cells in WATs and CATs: (1) close focus coincidence of BrdU-stained nuclei and the glial/neuronal-specific staining (cytoplasmic or nuclear) and (2) visualization of unstained cell compartments, alternating between epi-fluorescence and Nomarski illumination (Horner et al., 2000). For quantification studies, sections were processed for revealing BrdU-marked nuclei [we have selected sections from spinal cords and brains of WATs (N=4) and CATs (N=4)]. Sections processed for revealing BrdU (spinal cords and brains) were incubated in the following primary antibodies: rabbit anti-glial fibrillary acidic protein (GFAP; 1:500; Chemicon International, Inc.), rabbit anti-S100 proteins (S100; 1:200; Sigma–Aldrich, Inc., St Louis, MO, USA), mouse anti-oligodendrocyte (1:200; Chemicon International, Inc.), mouse anti-neuronal nuclei proteins (NeuN; 1:500; Chemicon International, Inc.). GFAP stains cytoskeleton proteins of supporting cells in the brain and spinal cord; S100 reacts with the S100 family of proteins present in glial and ependymal cells; and NeuN reacts with most neuronal cell types in the CNS of vertebrates (staining is primarily localized in the nucleus, extending in some cases into the cytoplasm). It should be noted that reliable results were not obtained with the anti-oligodendrocyte antibody. 50–100 nuclei were examined in the sections incubated in each antibody; single- and double-labeled cells were counted separately. The percentage of BrdU-LCs immunolabeled with a second antibody was defined as the double-labeling index (DLI) for that cell marker. When dealing with the spinal cord, we also made triple-labeling experiments in WATs (N=2) involving BrdU, GFAP and S100. After processing for BrdU detection, the sections were incubated in a solution containing GFAP and S100 primary antibodies. The sections were sequentially processed with two fluorophore-conjugated secondary antibodies emitting light at different wavelengths. In these preparations, we looked for single BrdU-LCs, alternating, as described, between epi-fluorescence and Nomarski illumination. In this particular case, quantification was expressed as the single-labeling index (SLI), representing the percentage of BrdU-LCs that do not express either S100 or GFAP. Control experiments were performed by omitting or replacing primary antibodies with normal serum. In these experiments, no detectable staining of cell structures was observed. Bright-field images were captured indistinctly with a photographic camera (using fine grain film) or with a CCD camera. In the latter, the images were processed with commercially available software.

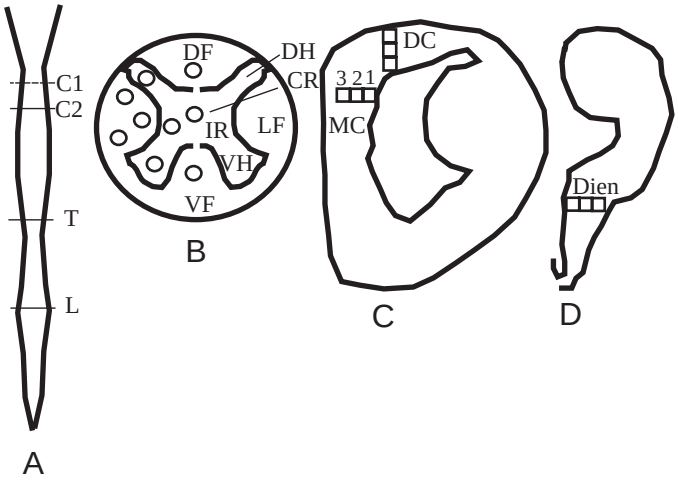


Fig. 1. Schematic drawings showing the regions of the central nervous system explored. (A) Spinal cord: cervical (C1, C2), thoracic (T) and lumbar (L) segments. (B) Sampled areas were circles (radius, 50 μm) distributed as shown in the cross section of the spinal cord. Bromodeoxyuridine-labeled cells were counted and densities were calculated for each of the following spinal cord regions: central region (CR), intermediate region (IR), dorsal funiculus (DF), ventral funiculus (VF), lateral funiculus (LF), ventral horn (VH) and dorsal horn (DH). (C,D) Forebrain regions: dorsal cortex (DC), medial cortex (MC), diencephalon (Dien); to better adapt to the geometry of the explored regions the circles were substituted by squares of the same area (1–3).

Results

Spinal cord

BrdU-labeled cells – density distribution

As reported in a previous paper (Fernández et al., 2002), injection of a single dose of BrdU resulted, 24 h later, in cell labeling throughout the gray matter and white matter of the spinal cord. Differences between mean densities of BrdU-LCs in WATs and CATs were easily perceived by examining the histological preparations (Fig. 2A–D). However, a proper evaluation needed topological quantitative studies. When mean density values of BrdU-LCs obtained from WATs and CATs were compared, WATs showed a significant increase in BrdU-LCs within the limits of the central and intermediate regions (CR–IR; independent t-test, $P<0.01$; Fig. 2E). Statistically significant density differences were also observed in other regions of the cord but these exhibited lower confidence levels. For example, the DF and LF of WATs were significantly different from the DF and LF of CATs with a confidence level of 95% ($P<0.05$). However, mean values from the VF, VH and

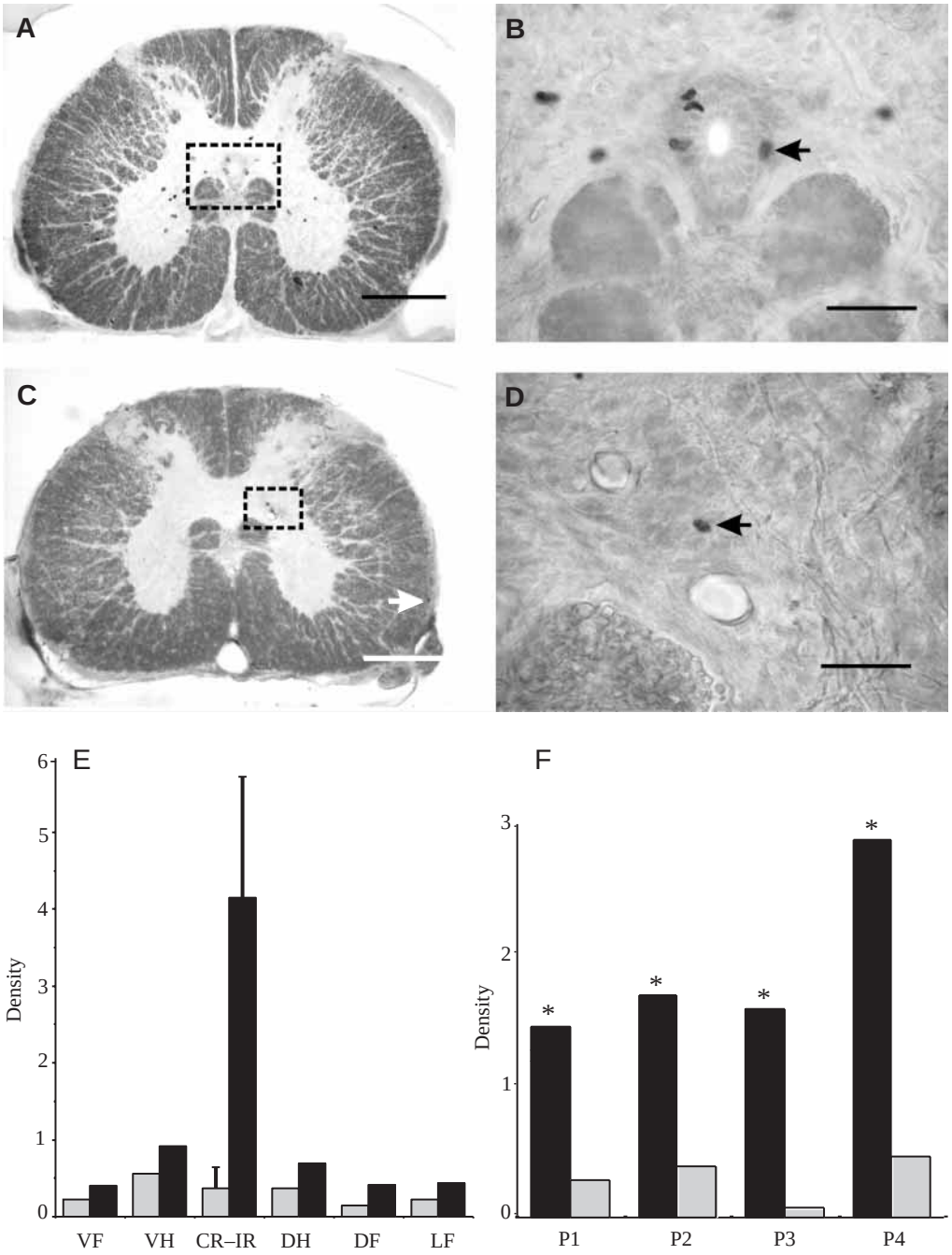


Fig. 2. (A) Low-power micrograph from a cross section through the spinal cord of a turtle acclimated to a warm environment (WAT). Note the presence of numerous bromodeoxyuridine-labeled cells (BrdU-LCs) mainly distributed in the gray matter. The area enclosed in the rectangle is shown in B at a higher magnification. The arrow indicates one of the BrdU-LCs. Scale bar, 200 μm (A) or 50 μm (B). (C) Low-power microphotograph from a cross section through the spinal cord of a turtle acclimated to a cooler environment (CAT). Only two nuclei appear in this section, one enclosed in the rectangular area and the other located in the white matter (arrow). (D) The rectangular area indicated in C is shown here at a higher magnification. One BrdU-stained nucleus is indicated by an arrow. Scale bar, 200 μm (C) or 50 μm (D). (E) Quantitative studies revealed that the difference of means of BrdU-LCs reached a maximum corresponding to the central gray matter (CR–IR). Density values of BrdU-LCs are indicated on the *y*-axis; the spinal cord regions are displayed on the *x*-axis: VF, ventral funiculus; VH, ventral horn; CR–IR, central and intermediate regions (averaged value); DH, dorsal horn; DF, dorsal funiculus; LF, lateral funiculus. Density values corresponding to the CR–IR are means ± 2 S.E.M. in both WATs (black bars; N=6) and CATs (gray bars; N=6). Independent *t*-test: $P<0.01$ for the CR–IR and $P<0.05$ for DF and LF. Differences between WATs and CATs for VF, VH and DH were statistically non-significant. (F) The effect of temperature was also evident when density values of BrdU-LCs corresponding to the central and intermediate regions (*y*-axis) were compared in four paired experiments (P1–P4). Black bars indicate values corresponding to WATs, while gray bars indicate values from CATs. Temperature values for each one of the paired experiments were as follows: P1, WAT 29°C/CAT 5°C; P2, WAT 29°C/CAT 10°C; P3, WAT 28.5°C/CAT 8°C; P4, WAT 29°C/CAT 11.4°C. Asterisks indicate where the WAT is significantly different from the corresponding CAT in each pair at $P<0.01$ (Fisher exact test).

DH obtained from WATs were not statistically different from values of homologous regions in CATs. It is worth noting that the CR–IR comprises the EpE lining of the central canal. Since the peri-ependymal cell mantle erases the limits between the CR and the IR in these animals, data from both regions were averaged and shown as a single value in the plots.

Data resulting from paired experiments (Fig. 2F) were of particular interest since, in these cases, the influence of unexplored biological rhythms should be ruled out. As already stated, in these circumstances tissues from both turtles were processed together to neutralize inherent variability of the immunostaining procedure. The obtained results also revealed significant differences (Fisher exact test, $P<0.01$) between density values of BrdU-LCs in members of each pair (Fig. 2E).

Multiple-labeling experiments

To determine the identity of the cell populations affected by temperature, we performed double-labeling experiments involving BrdU and specific cell markers. BrdU/S100-colabeled cells were found in different spinal cord regions including the EpE (Fig. 3A–D). For WATs, the DLI was 49% (N=100 nuclei), while for CATs the DLI was 11% (N=100). Close inspection revealed that colabeled cells found in the EpE were radial glia (RG) lining the central canal. These experiments also revealed BrdU-LCs that did not express S100 (Fig. 3E–H). Double-labeling experiments involving BrdU and GFAP also revealed BrdU/GFAP-colabeled cells coexisting with single BrdU-LCs (Fig. 3I–K). For WATs, the DLI was 22.5% (N=100 nuclei) and for CATs the DLI was 52% (N=100 nuclei). Triple-labeling experiments involving BrdU, S100 and GFAP also revealed cells showing single BrdU labeling (not shown; SLI=29%, N=50 nuclei). As expected, for short surviving time points after BrdU administration, BrdU/NeuN-colabeled cells were not found (Fernández et al., 2002; Cooper-Kuhn and Kuhn, 2002).

Forebrain

BrdU-labeled cells – density distribution

Similar to results obtained from studies in the spinal cord, the density of BrdU-LCs was significantly greater in animals maintained in a warmer than in a cooler environment. The difference was noticed in the histological preparations (Fig. 4A,B) and validated by the quantitative topological studies. For R1 (the sample area closest to the EpE), the mean density value of BrdU-LCs in WATs was significantly different (independent t-test, $P<0.01$) from the corresponding mean value of CATs. Most BrdU-LCs occurred within the limits of R1, but a few marked nuclei were found in the other sampled areas of the nervous parenchyma (R2–R3). However, in the latter regions, the differences between means were not statistically significant (Fig. 4C). Differences between WATs and CATs were also evident when comparing mean density values from particular brain zones such as the DC ($P<0.05$), MC ($P<0.01$) and Dien ($P<0.01$) (Fig. 5A). As occurred when studying the spinal cord, the paired experiments (Fig. 5B) confirmed that a warm environment increases cell proliferation in the brain of juvenile turtles (Fisher exact test, $P<0.01$).

Double-labeling experiments

In the EpE lining the brain cavities, immunostaining experiments involving both BrdU and S100 antibodies revealed cells colabeling for BrdU and S100 and also cells only stained for BrdU (Fig. 6A–C). For WATs, the DLI was 42% (N=100 nuclei) and for CATs the DLI was 12% (N=100 nuclei). The morphological characteristics of the double-labeled cells were coincident with those of typical RG (nuclei close to ventricle lumen, apical surface of the cells contacting the ventricle lumen and a fine radial process extending to the brain parenchyma). Double-labeling experiments also showed the occurrence of RG cells with BrdU-stained nuclei expressing GFAP (Fig. 6D–F). As in the double-labeling experiments involving S100, RG with BrdU-stained nuclei but not expressing GFAP were also found in the EpE (not shown). For WATs, the DLI was 74% (N=100 nuclei), while for CATs the DLI was 12% (N=50 nuclei).

Discussion

As reported in previous papers (García-Verdugo et al., 1986, 1989; López-García et al., 1988; Pérez-Cañellas and García-Verdugo, 1996; Pérez-Cañellas et al., 1997; Fernández et al., 2002), the CNS of lizards and turtles retains post-natal neurogenic and gliogenic potentialities. In lizards, the regenerative neurogenic activity of the MC is influenced by photoperiod temperature (Ramiréz et al., 1997). These authors found that '*Long (summer) photoperiods increased the number of proliferating neuroblasts in the ependymal neuroepithelium. Cold (winter) temperature prevents migration of the newly generated immature neurons*'. More recently, Peñafiel et al. (2001) reported, using BrdU-LCs, that in the telencephalon of the lizard *Psammodromus algirus* low temperature decreased the generation and migratory activity of new neurons. These results suggested that some CNS cells that appear to remain outside the cell cycle (in a somewhat dormant-like stage called G0) are induced by warm temperature to initiate mitotic activity.

Our studies on the CNS of turtles of the genus *Chrysemys* revealed a significant increase in the density of BrdU-LCs in turtles acclimated to 27–30°C when compared with turtles exposed to temperature fluctuating within the 5–14°C range. In the spinal cord, differences between WATs and CATs were particularly evident when exploring the central gray matter. These results suggest that the CR–IR contains the major cell population sensitive to the direct or indirect temperature effects. It has to be emphasized that within the limits of the CR–IR lies the central canal lined by the EpE. If the data obtained from the spinal cord are compared with those of the forebrain, we find that differences between WATs and CATs are significant at the level of R1 (the zone containing the EpE) and not significant in other regions of the nervous parenchyma. This also points to the cell-proliferative capacity of the EpE

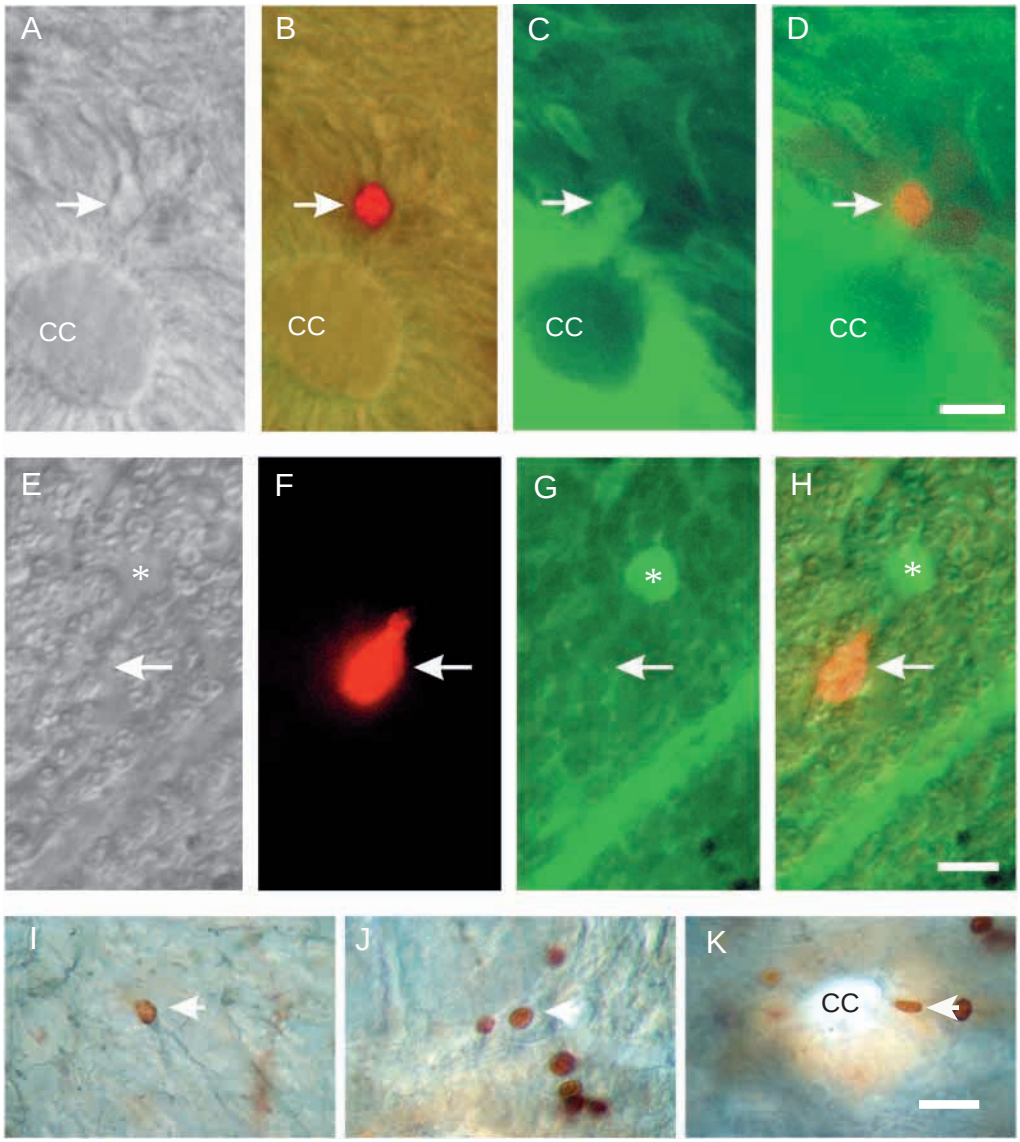
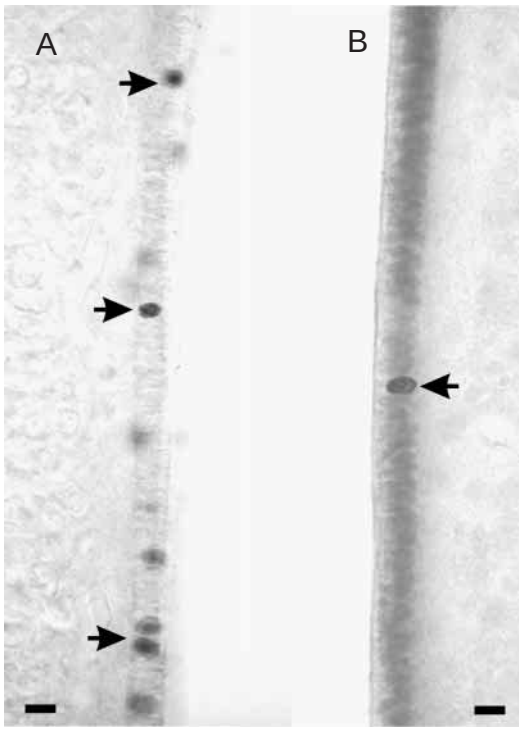


Fig. 3. (A–D) Double labeling with anti-bromodeoxyuridine (BrdU) and anti-S100 antibodies revealed the occurrence of colabeled cells forming part of the ependymal epithelium lining the central canal (CC) of the spinal cord. The nucleus of the cell marked by the arrow in A (Nomarski illumination) appears red in B due to BrdU labeling (arrow; Nomarski and epi-fluorescence). In C, the cell cytoplasm was immunostained in green with anti-S100 (arrow). The nuclear and the cytoplasmic images are shown merged in D. Scale bar, 10 μm. (E–H) BrdU-labeled cells that do not express S100 proteins were also found in the same preparations; the unstained cell compartments are shown in E (arrow and asterisk), one BrdU-labeled nucleus is shown in F (arrow) and the S100 immunoreactivity is shown in G [asterisk; the same panel shows the place occupied by the unstained nucleus (arrow)]. E, F and G are shown merged in H; the nucleus is indicated by an arrow and the S100-positive cell by an asterisk. Scale bar, 5 μm. (I–K) Double-labeling immunostaining with anti-BrdU and anti-GFAP (glial fibrillary acidic protein) antibodies also revealed double-stained (BrdU/GFAP; arrow in panel I) and single-stained (BrdU; arrows in panels J–K) cells. Scale bar, 20 μm. All micrographs were obtained from warm-acclimated turtles.

but does not exclude the presence of cells retaining mitotic activity in other regions of the CNS. These results were confirmed by paired experiments in which more elusive factors that might be affecting cell proliferation (Cermakian and Sassone Corsi, 2001) were equalized.

To identify the cell population affected by temperature, we combined BrdU-labeling with the labeling of glial and neuronal markers. Our results indicate that temperature mainly affected a population of GFAP-positive and S100-positive cells with the characteristics of pleomorphic neuroglia and typical RG [it should be noted that mammalian-like astrocytes are uncommon in reptiles (De Castro, 1920)]. The RGs reside in the EpE lining the brain cavities, including the central canal of the spinal cord. Recent studies indicate that GFAP-positive cells with the characteristics of astrocytes behave as neuronal precursors in the subventricular zone of rodents (Doetsch et al., 1999; Seri et al., 2001; Alvarez-Buylla and García-Verdugo, 2002). In addition, in the case of embryonic development,



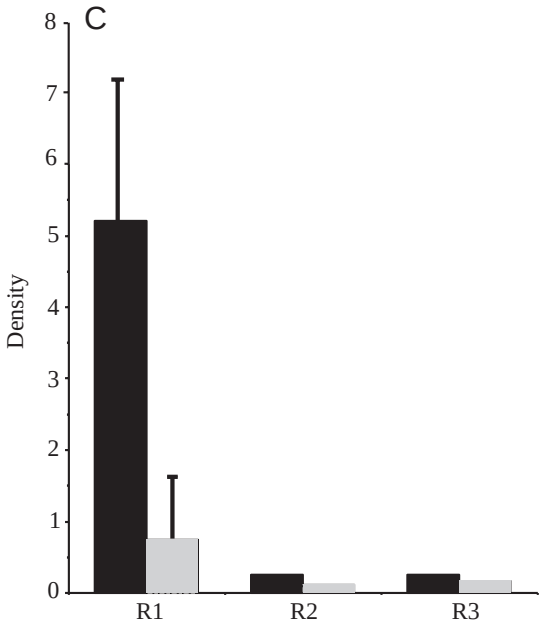


Fig. 4. Influence of temperature was also noticed in the histological preparations showing the distribution of bromodeoxyuridine-labeled cells (BrdU-LCs) in the forebrain regions. (A) The medial cortex of a turtle acclimated to a warm environment (WAT) contains a greater number of BrdU-LCs than (B) the homologous region of the cortex of a turtle acclimated to a cooler environment (CAT). Most BrdU-LCs (arrows) were seen distributed within the limits of the ependymal epithelium in both WATs and CATs. Scale bars, 10 μm. (C) Density values from WATs (N=6; black bars) are significantly different from density values from CATs (N=6; gray bars) when data from sampled region 1 (R1) were compared (independent t-test: P<0.01). The observed minor differences between other regions (R2 and R3) were not statistically significant.

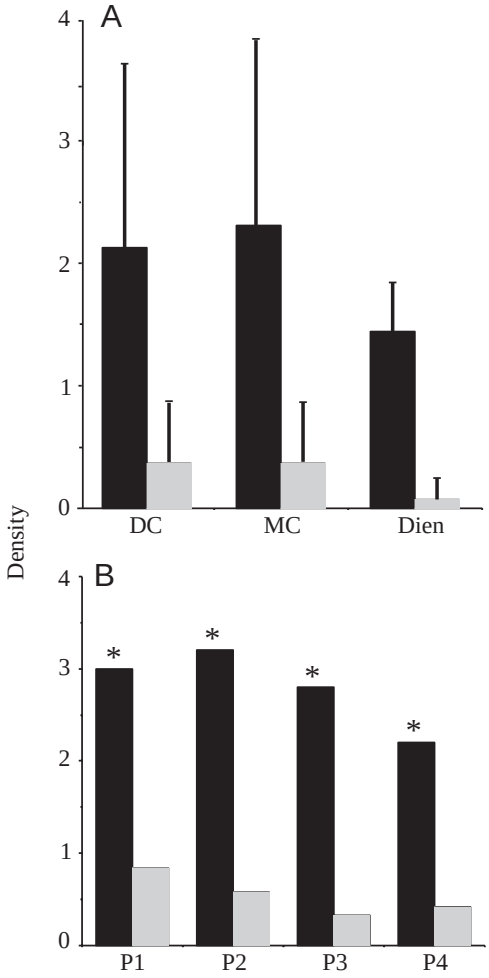


Fig. 5. (A) This plot shows mean density values of bromodeoxyuridine-labeled cells in the dorsal cortex (DC), medial cortex (MC) and diencephalon (Dien) samples of turtles acclimated to a warm environment (WATs; black bars) and turtles acclimated to a cooler environment (CATs; gray bars). Mean values ± 2 S.E.M. were significantly different in all the examined regions. Independent t-test: P<0.05 for the DC; P<0.01 for both MC and diencephalon (N=6). (B) The influence of temperature was also shown in data obtained from the paired experiments (P1–P4). Asterisks indicate where the WAT (black bars) is significantly different from the corresponding CAT (gray bars) in each pair at P<0.01 (Fisher exact test).

'*distinction between radial glial cells and neuronal progenitors has recently collapsed*' (Fishell and Kriegstein, 2003). The same line of thought is maintained by Noctor et al. (2001), suggesting that neurogenic potentialities of RG may be extended into post-natal periods. We have also found BrdU-LCs that do not express either GFAP or S100. Since our double-labeling experiments with NeuN have excluded the neuronal nature of these cells, it seems reasonable to consider them to be either oligodendrocytes or perhaps cells close to a more primitive undifferentiated lineage. (To test this hypothesis we tried oligodendrocyte markers, but available

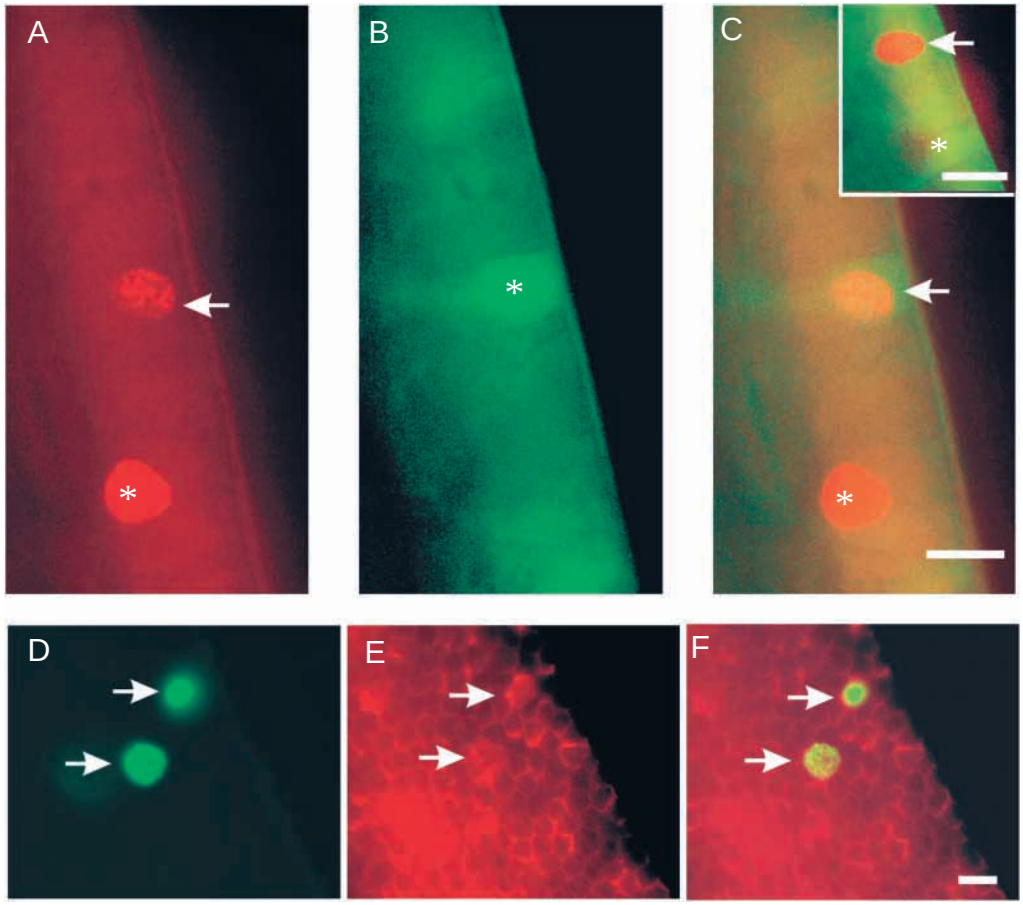


Fig. 6. (A–C) Double labeling with anti-bromodeoxyuridine (BrdU) and anti-S100 antibodies revealed the occurrence of colabeled cells forming part of the ependymal epithelium (EpE) lining the brain cavities. These cells showed the characteristics of radial glia (RG). Two BrdU-marked nuclei are shown by an arrow and an asterisk in A. The cell body stained with S100 is indicated by an asterisk in B. The two images are shown merged in C; the arrow indicates the BrdU/S100-colabeled cell, and the asterisk indicates the single BrdU-labeled nucleus shown in A. The inset shows a single BrdU-labeled cell (arrow) and a typical S100-stained RG without nuclear labeling (asterisk). Scale bars, 10 μm (main panels and inset). (D–F) In the EpE lining the brain cavities, there are numerous RG that express GFAP (glial fibrillary acidic protein) and that are able to incorporate BrdU. Two BrdU-stained nuclei are shown in D (arrows), two GFAP-positive cells that have incorporated BrdU are shown in E (arrows), and F shows the merged image of BrdU/GFAP colabeling (arrows). Microphotographs are from cross sections passing through the main axis of the RGs. Scale bar, 25 μm.

antibodies do not work properly when assayed in turtles.) Therefore, differences in the DLIs observed between WATs and CATs have to be considered as suggestive clues to be explored in detail in future work. Moreover, the current absence of modern glia cell descriptions in turtles contributes to the difficulties in assessing the identity of the BrdU-LCs not expressing glial markers. As mentioned above, we have not found BrdU-LCs expressing NeuN. This is consistent with our previous results in turtles (Fernández et al., 2002) and with the systematic studies performed by Cooper-Kuhn and Kuhn (2002) in rats. In both species, BrdU/NeuN-colabeled cells appear several days after BrdU administration.

Our results are in agreement with current views that emphasize the role played by glial cells in the process of cell proliferation and neural differentiation after birth. It can be concluded that temperature mainly affects cells that have to be considered as '*contained within the neuroepithelial–radial-glia–astrocyte lineage*' (Alvarez-Buylla et al., 2001). This is particularly evident when dealing with the RG lining the brain cavities and the central canal of the spinal cord.

There is little doubt that, within physiological limits, temperature could increase *per se* the cell metabolism and the mitotic rate ['*...the duration of the metaphase pause becomes shorter as the temperature is increased.*' (DuPraw, 1970)]. There are, however, indirect factors that may be affecting cell division. For example, WATs displayed an increased motor activity and were more voracious than CATs. Consequently, both behavioral (van Praag et al., 1999, 2000) and nutritional factors should be taken into account when dealing with possible mechanisms that could be operating in the temperature-induced increase of cell proliferation. With respect to the biological significance of this phenomenon, it is reasonable to relate it to the changing activity of turtles throughout the year. During winter, turtles of the genus *Chrysemys* have a reduced motor

activity and a reduced food intake. These behavioral patterns are dramatically increased in summertime. If, as suggested, cell proliferation may be reflected in an increased number of nerve cells (Fernández et al., 2002), the new neurons may facilitate the operation of circuits vinculated with more-demanding behavioral tasks such as prey capture and reproductive maneuvers. The differentiation fate of the increased number of BrdU-LCs remains a subject for further studies.

We thank Drs A. Caputi, D. Lorenzo and R. Russo for critical reading of the manuscript and useful suggestions. We also thank Mrs M. I. Rehermann for valuable technical assistance. This investigation was partially supported by TWAS Research Grant No. 98-078RG/BIO/LA and by Fondo C. Estable Project No. 7005.

References

Altman, J. (1962). Are new neurons formed in the brain of adult mammals? *Science* **135**, 1127-1128.

Altman, J. (1963). Autoradiographic investigation of cell proliferation in the brain of rats and cats. Postnatal growth and differentiation of the mammalian brain, with implication for a morphological theory of memory. *Anat. Rec.* **145**, 573-591.

Altman, J. and Das, G. D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.* **124**, 319-335.

Alvarez-Buylla, A. and García-Verdugo, J. M. (2002). Neurogenesis in adult subventricular zone. *J. Neurosci.* **22**, 629-634.

Alvarez-Buylla, A., García-Verdugo, J. M. and Tramontin, A. D. (2001). A unified hypothesis on the lineage of neural stem cells. *Nat. Rev. Neurosci.* **2**, 287-293.

Barnea, A. and Nottebohm, F. (1994). Seasonal recruitment of hippocampal neurons in adult free-ranging black-capped chickadees. *Proc. Natl. Acad. Sci. USA* **91**, 11217-11221.

Cameron, H. A. and Gould, E. (1994). Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. *Neuroscience* **61**, 203-209.

Cameron, H. A. and McKay, R. D. (1999). Restoring production of hippocampal neurons in old age. *Nat. Neurosci.* **2**, 894-897.

Carleton, A., Petrenau, T., Lansford, R., Alvarez-Buylla, A. and Lledo, P.-M. (2003). Becoming new neurons in the adult olfactory bulb. *Nat. Neurosci.* **6**, 507-518.

Cermakian, N. and Sassone-Corsi, P. (2001). Rhytmes biologiques: les secrets d'une horloge. *La Recherche* **338**, 38-42.

Cooper-Kuhn, C. M. and Kuhn, H. G. (2002). Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. *Dev. Brain Res.* **134**, 13-21.

De Castro, F. (1920). Algunas observaciones sobre la histogénesis de la neuroglía en el bulbo olfativo. *Trav. Lab. Invest. Biol. Madrid* **18**, 82-108.

Doetsch, F., Caille, I., Lin, D. A., García-Verdugo, J. M. and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97**, 703-716.

DuPraw, E. J. (1970). *Cell and Molecular Biology*. New York, London: Academic Press.

Fernández, A., Radmilovich, M. and Trujillo-Cenóz, O. (2002). Neurogenesis and gliogenesis in the spinal cord of turtles. *J. Comp. Neurol.* **453**, 131-144.

Fishell, G. and Kriegstein, A. R. (2003). Neurons from radial glia: the consequences of asymmetric inheritance. *Curr. Opin. Neurobiol.* **13**, 34-41.

García-Verdugo, J. M., Farinas, I., Molowny, A. and López-García, C. (1986). Ultrastructure of putative migrating cells in the cerebral cortex of *Lacerta galloti*. *J. Morphol.* **189**, 189-197.

García-Verdugo, J. M., Llahi, S., Ferrer, I. and López-García, C. (1989). Postnatal neurogenesis in the olfactory bulbs of a lizard. A tritiated thymidine autoradiographic study. *Neurosci. Lett.* **98**, 247-252.

Gould, E. and Gross, C. G. (2002). Neurogenesis in adult mammals: some progress and problems. *J. Neurosci.* **22**, 619-623.

Gould, E., Tanapat, P., McEwen, B. S., Fugge, G. and Fuchs, E. (1998). Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. *Proc. Natl. Acad. Sci. USA* **95**, 3168-3171.

Gross, C. G. (2000). Neurogenesis in the adult brain: death of the dogma. *Nat. Rev. Neurosci.* **1**, 67-73.

Horner, P. J., Power, A. E., Kempermann, G., Kuhn, H. G., Palmer, T. D., Winkler, J., Thal, L. J. and Gage, F. H. (2000). Proliferation and differentiation of progenitor cells throughout the intact adult rat spinal cord. *J. Neurosci.* **20**, 2218-2228.

Jacobs, B. L., van Praag, H. and Gage, F. H. (2000). Adult brain neurogenesis and psychiatry: a novel theory of depression. *Mol. Psych.* **5**, 262-269.

Kaplan, M. S. (1985). Formation and turnover of neurons in young and senescent animals: an electron microscope and morphometric analysis. *Ann. N.Y. Acad. Sci.* **457**, 173-192.

Kaplan, M. S. and Hinds, J. W. (1977). Neurogenesis in the adult rat: electron microscopy analysis of light radioautographs. *Science* **197**, 1092-1094.

Lopez-García, C., Molowny, A., García-Verdugo, J. M. and Ferrer, I. (1988). Delayed postnatal neurogenesis in the cerebral cortex of lizards. *Dev. Brain Res.* **471**, 167-174.

Momma, S., Johansson, C. B. and Frisén, J. (2000). Get to know your stem cells. *Curr. Opin. Neurobiol.* **10**, 45-49.

Nilsson, M., Perfilieva, E., Johansson, U., Orwar, O. and Eriksson, P. S. (1999). Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. *J. Neurobiol.* **39**, 569-578.

Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S. and Kriegstein, A. R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature* **409**, 714-720.

Nottebohm, F. (2002). Why are some neurons replaced in adult brain? *J. Neurosci.* **22**, 624-628.

Nottebohm, F., O'Loughlin, B., Gould, E., Yokay, K. and Alvarez-Buylla, A. (1994). The life span of new neurons in a song control nucleus of the adult canary brain depends on time of year when these cells are born. *Proc. Natl. Acad. Sci. USA* **91**, 7849-7853.

Peñafiel, A., Rivera, A., Gutiérrez, A., Trías, S. and De La Calle, A. (2001). Temperature affects adult neurogenesis in the lizard brain. *Int. J. Dev. Biol.* **45**, 83-84.

Pérez-Cañellas, M. M. and García-Verdugo, J. M. (1996). Adult neurogenesis in the telencephalon of a lizard: a (3H) thymidine autoradiographic and bromodeoxyuridine immunocytochemical study. *Brain Res. Dev. Brain Res.* **93**, 49-61.

Pérez-Cañellas, M. M., Font, E. and García-Verdugo, J. M. (1997). Postnatal neurogenesis in the telencephalon of turtles: evidence for non radial migration of new neurons from distant proliferative ventricular zones to the olfactory bulbs. *Brain Res. Dev. Brain Res.* **101**, 125-137.

Prosser, L. (1952). *Comparative Animal Physiology*. Philadelphia, London: Saunders.

Rakic, P. (2002a). Neurogenesis in adult primate neocortex: an evaluation of the evidence. *Nat. Rev. Neurosci.* **3**, 65-71.

Rakic, P. (2002b). Adult neurogenesis in mammals: an identity crisis. *J. Neurosci.* **22**, 614-618.

Ramírez, C., Nacher, J., Molowny, A., Sánchez-Sánchez, F., Irurzun, A. and López-García, C. (1997). Photoperiod-temperature and neuroblast proliferation-migration in the lizard cortex. *Neuroreport* **7**, 1257-1260.

Scheffler, B., Horn, M., Blumcke, I., Laywell, E. D., Coomes D., Kukekov, V. G. and Steindler, D. A. (1999). Marrow mindedness: a perspective on neuropoiesis. *Trends Neurosci.* **22**, 348-357.

Seaberg, R. M. and van der Kooy, D. (2003). Stem and progenitor cells: the premature desertion of rigorous definitions. *Trends Neurosci*. **26**, 125-131.

Seri, B., García-Verdugo, J. M., McEwen, B. S. and Alvarez-Buylla, A. (2001). Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J. Neurosci*. **21**, 7153-7160.

Tanapat, P., Hastings, N. B., Rydel, T. A., Galea, L. A. and Gould, E. (2001). Exposure to fox odor inhibits cell proliferation in the hippocampus of adult rats via an adrenal hormone-dependent mechanism. *J. Comp. Neurol.* **437**, 496-504.

van Praag, H., Kempermann, G. and Gage, F. H. (1999). Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat. Neurosci.* **2**, 266-270.

van Praag, H., Kempermann, G. and Gage, F. H. (2000). Neural consequences of environmental enrichment. *Nat. Rev. Neurosci.* **1**, 191-197.