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Relationships between seasonal thermal variations and cell proliferation in heterothermic vertebrates, as revealed by PCNA expression in the brain of adult *Podarcis sicula*

Vito Margotta

Dipartimento di Biologia animale e dell'Uomo, Università "La Sapienza" di Roma

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Abstract

In adult terrestrial heterothermic vertebrates, spontaneous or experimentally induced plasticity of the brain is widely demonstrated. This phenomenon is more pronounced in Amphibians than in lacertilians, the most investigated among the Reptiles. In the lizard it has been observed that the summer photoperiod and temperature exert a positive influence on the proliferative activity of cerebral putative stem cells, which then differentiate into glial or neuronal cells. In the present investigation, the behaviour of proliferating neural cells has been investigated by immunocytochemistry in the brain of normal adult *Podarcis sicula* caught in their habitat in summer. The results, qualitatively evaluated, were compared with those on normal lizards of the same species, caught from the wild in late spring and previously analysed by the same author. The comparison showed that summer environment stimulates cell proliferation, although to a limited extent. This response seems to involve the quiescent cells which mainly populate the ependymal layer of the forebrain, the telencephalic hemispheres being the best provided with these undifferentiated cells, while no substantial differences from spring values were found in more caudal cerebral portions. The comparison between present findings and previously reported ones indicates that summer environment stimulates the proliferation of putative neuronal stem cells but only in the forebrain; this proliferation might not be sufficient to support the regeneration upon partial removal of this portion of the encephalon, and previous findings of a much higher regenerative capacity in the lizard brain upon cerebral injury might depend on factors linked to injury itself or to the studied species and their habitat.

Key words

Season influence, stem cells, lizard, encephalon, ependyma.

Introduction

A large amount of observations demonstrate the outliving of peculiar stem cells in the brain of adult anamniotic and amniotic vertebrates (Kirsche, 1983; Margotta and Morelli, 1996; Margotta, 2007; Margotta et al., 2007). These cells can differentiate into nerve cells immediately or after proliferation (Kirsche, 1967, 1983) and are responsible for reparative processes, either spontaneous or experimentally induced. Therefore the nervous system should be defined as made of a "stable" rather than "perennial" tissue following the classification of Bizzozero (1894).

The putative neural stem cells are located in the ependymal epithelium, in the periventricular grey matter and sometimes within other encephalic areas. They may appear scattered (“matrix cells”) or clustered to form “matrix areas”, classically named “Matrixzonen”.

The site and presence of these progenitors differ among the animal groups and their number is higher in young than in older individuals and in lower than in higher vertebrates. In particular, in the Osteichthyes Teleosts a supply of undifferentiated cells survives for longer time than in more advanced vertebrates and their mitotic activity continues after cessation of body growth, which leads to only late determination of the number of nerve cells.

Among heterothermic terrestrial vertebrates, Amphibians are the best provided with such neural progenitors and therefore have been the most investigated upon, while lacertilians are almost the only group studied among Reptiles.

Kirsche (1967) carried out an exhaustive and enlightening study on the physiological proliferative activity in the adult brain of Teleosts, Urodelan and Anuran Amphibians, lacertilian Reptiles and Birds. In *Lacerta agilis agilis* he discovered, in the telencephalic regions, thickenings of these neural proliferating cells which he named *zonae germinativae dorsales* and *zonae germinativae ventrales*. The survival of such matrix areas was confirmed for the same species by Schulz (1969). Del Grande and Minelli (1980) identified a *pars medialis* and a *pars lateralis* in each *zona germinativa dorsalis* of *L. viridis*. Kirsche (1967) found other putative stem cells in various diencephalic areas in *L. agilis agilis*, similar to what Fleischhauer (1957) had reported in *Testudo graeca*. Moreover, Del Grande et al. (1981) observed some quiescent cells spread in the innermost tectal layers of the midbrain of *L. viridis*.

Several literature data indicate that in adult Anamnia and Amniota both the thermal cyclic changes which are linked to seasonal variations in photoperiod and the experimental exposure to a sudden transient drop in body temperature can induce fluctuations in the proliferative activity of putative neuroblasts.

The differential influence of photoperiod and/or temperature on adult neurogenesis has been observed first in Anuran Amphibians (Minelli et al., 1982a; Bernocchi et al., 1990; Chetverukhin and Polenov, 1993; Polenov and Chetverukhin, 1993; Chieffi Baccari et al., 1994) and later in lacertilian Reptiles (Ramirez et al., 1997), in other poikilothermal vertebrates living in fresh or sea water, like the teleostean *Tinca tinca* (Velasco et al., 2001), and in the lamprey *Petromyzon marinus* (Vidal Pizarro et al., 2004).

A peak of mitotic activity has also been described in cells of the frog eye (Rothstein et al., 1975) and of the salamander chemosensory epithelium (Dawley et al., 2000) caught in their habitat in late spring and at beginning of summer, respectively.

The photoperiod has an impact on sexual hormone secretion and therefore on the reproductive cycle-related differentiation events in encephalic centres involved in reproduction-related behaviour, in particular those which control song in male canaries (for more details about such interrelationships, see Margotta et al., 2005a; Margotta and Caronti, 2005).

Ramirez et al. (1997) in *Podarcis hispanica*, after ablation of an encephalic area, found signs of neuronal regeneration by autoradiography and immunocytochemistry. These authors also studied the effects of the annual light/temperature cycle on postnatal neurogenesis in this lizard and demonstrated that summer environmental condition stimulate the mitotic activity and differentiation of cells in the ependymal

epithelium, while a drop in cell proliferation and in the migration of newly generated neurons to definitive sites was observed in specimens caught during hibernation.

In adult vertebrates, in normal and experimental conditions, a role in directing the migration of new cells within the brain seems to be played by radial glial cells. Possible mutual relationships between normal or post-traumatic proliferative activity and persistence of these neurepithelial cells is supported by some reports taking advantage of autoradiography or immunocytochemistry (reviewed by Margotta and Morelli, 1997, and Alvarez-Buylla et al., 2002). It has been proposed that such cells favour both the generation of neuroblasts from cells in stand-by and the migration of the generated cells. It is noteworthy that the body of radial glial cells co-localizes with *zonae germinativae* (Kirsche, 1967).

A recent immunocytochemical study and a reappraisal of previous ones on the natural proliferative power in adult vertebrate encephalon (Margotta, 2012) - following the observations of Minelli et al. (1982a) on *R. esculenta* (synonymous of *R. bergeri*, Capula, 2000) subjected to cerebral ablation - have given indications that autumnal environmental conditions exert a widespread inhibition on cell proliferation.

In view of the scenario outlined by Ramirez et al. (1997) and our own results in *P. sicula* caught from the wild in late spring (Margotta et al., 1999a, 2005b), I have now addressed, in the latter species, the impact of summer environmental conditions on the natural proliferative potential of putative encephalic neuronal progenitors.

The present, qualitative observations have been carried out on samples of *P. sicula* caught in the same period of the year as Ramirez et al. (1997), using the Proliferating Cell Nuclear Antigen (PCNA; Miyachi et al., 1978) to identify cycling cells since this antigen, which belongs to the cyclin family of proteins, is expressed during DNA synthesis. This method does not require housing animals in the laboratory between catch and sacrifice, at variance with the methods based on the labelling of newly synthesized DNA, in turn it does not allow to trace the destiny of daughter cells.

Material and methods

Sexually mature specimens of *Podarcis sicula* (Capula, 2000), at times designated *L. viridis* Rafinesque (Tortonese and Lanza, 1968), of both sexes were caught from their natural habitat near Rome, Italy, at the end of July and immediately sacrificed under anaesthesia with tricaine methanesulfonate (MS 222 Sandoz, Switzerland; 1:1000). The head was cut off and after partial disarticulation of the cranial bones it was fixed in Bouin's fluid. It was then transferred in 80% ethyl alcohol, where the brain was removed under a stereomicroscope. The tissue was dehydrated in ethanol and embedded in paraffin under *vacuum*. Transverse serial sections 8 μm thick were cut in antero-posterior direction with a rotary microtome.

Upon removal of paraffin and hydration, the sections were rinsed in isotonic, 0.01 mol/litre phosphate buffered saline, pH 7.4 (PBS), incubated in 3% H_2O_2 in methanol for 30 min to block endogenous peroxidase, washed in PBS, incubated in 20% normal horse serum to block unspecific binding sites and incubated overnight at 4 °C in a monoclonal antibody against PCNA (PC10: mouse IgG, from Sigma, St. Louis, Missouri), diluted 1:1000 with PBS plus 1% normal horse serum. Negative control sections were incubated with non immune mouse IgG instead of the primary monoclonal.

The bound antibodies were detected using secondary horse anti-mouse biotinylated antibodies (Vector, Burlingame, California), diluted 1:100 with PBS plus 1% normal horse serum, for 1 h at room temperature, and avidin-biotin-peroxidase complex (ABC Kit, Vector), 30 min at room temperature. Peroxidase was detected with 3-3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) 1 mg/ml, plus 1% NiSO₄ and 0.017% H₂O₂ in 0.05% mol/litre Tris-HCl, pH 7.6. The slides were then dehydrated and mounted with Entellan (Merck, Germany).

The specificity of the immunostaining was tested by replacing the primary antibody with non-immune goat serum.

Results

The labelling for PCNA in the forebrain of normal adult *P. sicula* caught from their habitat at the end of July differed from that of lizards caught in late spring and previously reported upon (Margotta et al., 1999a, 2005b).

The proximal portions of the peduncles of the olfactory region showed a widespread PCNA immunoreaction both in the ependyma and peri-ependymal grey matter (Fig. 1a), as compared with a lighter labelling seen in the same region in specimens caught in late spring (Fig. 1b).

In each hemisphere, randomly arranged labelled cells were identifiable at end July in the epithelial walls of the ventricle, in the areas known as *zona germinativa dorsalis* (*pars lateralis* and *pars medialis*) and *zona germinativa ventralis*; the *pars lateralis* of

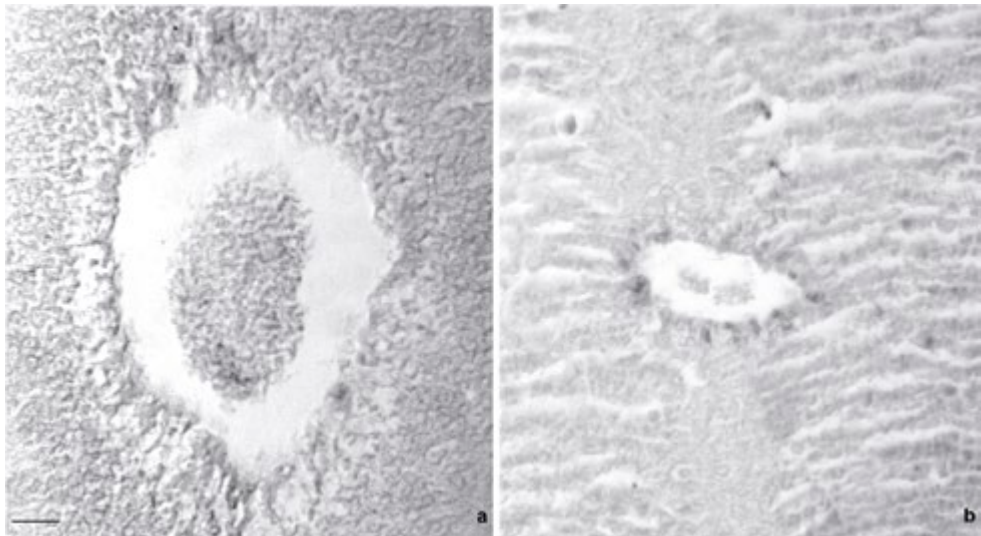


Figure 1 – Transverse section of the forebrain of normal adult lizards caught in their habitat at end July (a) and late spring (b). PCNA-positive cells are visible in the ependyma and the sub-ependymal grey matter in the proximal portion of the olfactory peduncle. PCNA immunocytochemistry, without nuclear counterstaining. Calibration bar = 20 μ m.

the *zona germinativa dorsalis* was located anterior to the *pars medialis* (Figs. 2a, 3a, 4a). Scanty labelled cells were also observed in other areas of the ventricular walls. A similar, but blurred immunoreactivity had been seen in individuals caught in late spring (Figs. 2b, 3b, 4b).

In more caudal cerebral districts no substantial differences were found between specimens caught at end July and those caught in late spring. In the diencephalon immunolabelled cells were found in clusters in habenular ganglia and sparse in the

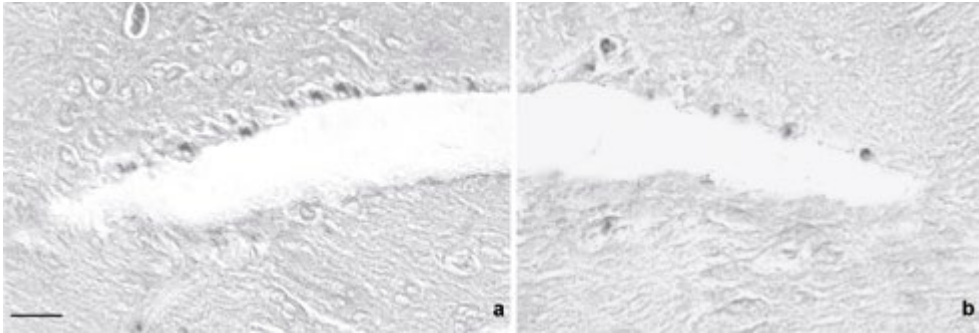


Figure 2 – Transverse section of the forebrain of normal adult lizards caught in their habitat at end July (a) and late spring (b). The falciform crescent shape of the ventricle profile is typical of anterior sections. PCNA-positive cells are visible in the *pars lateralis* of the *zona germinativa dorsalis* of a telencephalic hemisphere. PCNA immunocytochemistry, without nuclear counterstaining. Calibration bar = 20 μ m.

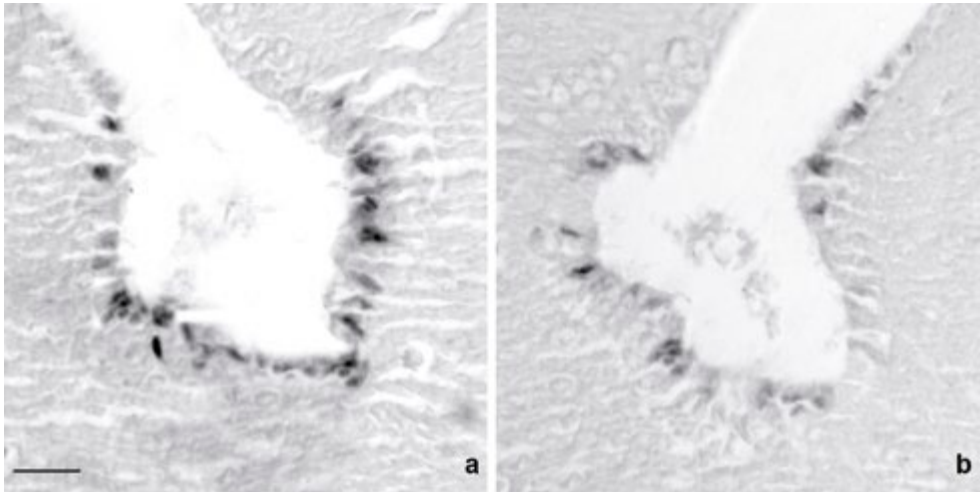


Figure 3 – Transverse section of the forebrain of normal adult lizards caught in their habitat at end July (a) and late spring (b). The ventral hollow shape of the ventricle profile is typical of intermediate sections. PCNA-positive cells are visible in the *zona germinativa ventralis* of a telencephalic hemisphere. PCNA immunocytochemistry, without nuclear counterstaining. Calibration bar = 20 μ m.

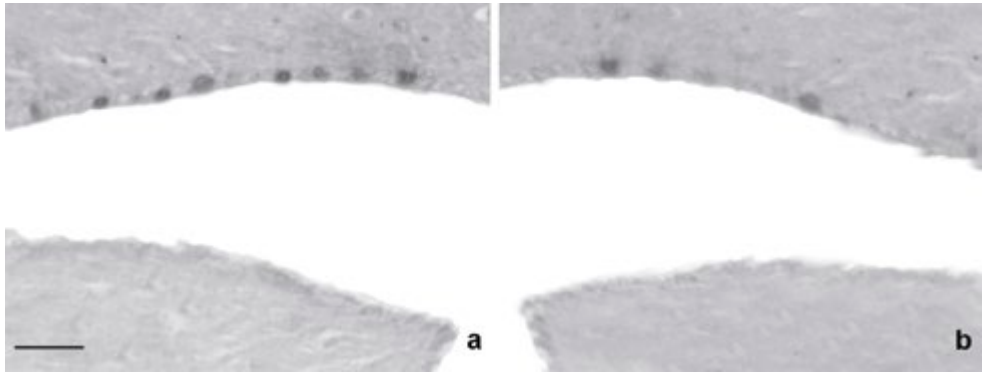


Figure 4 – Transverse section of the forebrain of normal adult lizards caught in their habitat at end July (a) and late spring (b). The T-shape of the ventricle profile is typical of posterior sections. PCNA-positive cells are visible in the *pars medialis* of the *zona germinativa dorsalis* of a telencephalic hemisphere. PCNA immunocytochemistry, without nuclear counterstaining. Calibration bar = 20 μ m.

ependyma of the III ventricle, including that of the hypothalamic preoptic and infundibular recesses. In the mesencephalon rare labelled cells appeared in narrow areas of the ventricular wall and, even more rare, deep in the optic tectum. No labelling was seen in the *cerebellum*. In the *medulla oblongata*, some PCNA-positive cells were visible ventro-laterally in the ependymal epithelium of the IV ventricle.

No staining was observed in negative control sections.

Discussion

Many studies to test the plasticity of the brain during adult life, carried out on vertebrates under normal and various experimental conditions, have shown that a change in environmental temperature, either spontaneous or experimental, gradual or sudden, induces the revival of the proliferative activity of undifferentiated cells in the central nervous system. These observations have mainly regarded poikilothermal vertebrates, especially Amphibians and to a lesser extent lacertilian Reptiles.

These topics have been investigated both addressing the effects of a short, experimental thermal shock and those of the annual cycle of photoperiod and of average temperature, which is co-ordinate with the photoperiod. A cold stimulus has been initially applied to *Triturus cristatus carnifex* deprived of a cerebral area (Del Grande and Minelli, 1971, Minelli and Del Grande, 1974a, b). Such stimulus was hypothesized to cause changes in the blood-brain barrier, which would re-acquire an embryonic-like condition in turn stimulating the proliferative activity of the putative stem cells. Similar studies were performed to assay the regenerative potential of the brain also in frogs and lizards deprived of telencephalic, diencephalic or mesencephalic areas and, rarely, in intact individuals. With regard to lacertilians, autoradiographic investigations were carried out in *L. viridis* (the same as *P. sicula*, according to Capula, 2000) by Minelli et al. (1978, 1982b) and Del Grande et al. (1981). In *P. hispanica* caught from the wild in summer and deprived of a telencephalic area, Ramirez et al.

(1997) found evidence that this season environment stimulated the mitotic activity in the undifferentiated cells which survive in the ependymal epithelium during post-natal life, promoting reparative or regenerative phenomena.

The author of the present study and his co-workers have previously reported on the physiologically proliferating cells in the brain of adult vertebrates of several species belonging to different taxonomic groups: the poikilothermal lizard (Margotta et al., 1999a, 2005b), newt (Margotta et al., 1999b, 2005b), frog (Margotta et al., 2000, 2005b), crucian (Margotta et al., 2001, 2002, 2004), cramp-fish (Margotta, 2007) and lamprey (Margotta et al., 2007) and the homeotherm canary (Margotta and Caronti, 2005; Margotta et al., 2005a).

In the lizard *P. sicula*, specimens caught in their habitat in late spring showed PCNA positive cells in the ependyma and the sub-ependymal layer of the olfactory peduncles, in the telencephalic ventricular epithelium (in higher amounts in hemispheric *zonae germinativae ventrales* than in the two parts of *zonae germinativae dorsales*: Margotta et al., 1999a) and in the habenular ganglia and the ependyma of the diencephalic ventricle; only scarce labelled cells were seen in the optic lobes and the rhombencephalic ventricular walls and none in the *cerebellum* (Margotta et al., 2005b).

The comparison between those findings (Margotta et al., 1999a, 2005b) and the present ones on specimens caught in their habitat at the end of July shows that in summer there are more labelled cells than in late spring. Therefore summer environment seems to stimulate the proliferation of putative neuronal stem cells, but only in the forebrain which is the encephalic portion apparently richest in proliferating cells at any time of the year.

The proliferative scenario found here seems inadequate to support the regeneration of a removed structure as compared with the results of Ramirez et al. (1997); the latter findings might depend on being the individuals subjected to cerebral injury (Ramirez et al., 1997), whereas they were normal in the present study, or on differences in the studied species and their habitat.

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