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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 *Rana bergeri* (Günther, 1986)

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Summary

The cyclic variations of temperature with seasons, which are accompanied by variations in photoperiod, can activate proliferation in stem cells which survive in various organs and tissues of adult vertebrates, both poikilo- (aquatic and terrestrial) and homeothermic (male songbirds and Mammals). In the brain of these organisms such stem cells are mainly placed in the ependymal and/or sub-ependymal layers. To assess the influence of environmental temperature on the proliferative activity of those cells, an immunocytochemical investigation has been carried out on the brain of normal adult *Rana bergeri*, taken from the wild in late autumn and immediately submitted to observation. The results were compared with those of previous investigations on the same animal species, caught in their habitat in late autumn as in this study, but housed in standard laboratory environment for several days before beginning the experiments. I have now observed a widespread reduction in proliferation. This finding discordance is reasonably imputable to the former stay of the specimens in a thermostable environment during the previous investigations and appears in agreement with what is known from the literature for comparable experimental conditions, suggesting that a stay for days in a thermally stable, warm environment can counteract the anti-proliferative effect of exposition to the late autumn climate.

Key words

Seasonal influences; neural-like cells; *Rana*.

Introduction

In the past decades, a large amount of research, using different techniques, has been focused on adult vertebrates, both in normal and in various experimental conditions, with the aim of evaluating the persistence in the brain of a possible spontaneous or induced proliferative plasticity (see reviews: Kirsche, 1983; Margotta and Morelli, 1996).

A potential for plasticity is much greater in heterothermic than in homeothermic vertebrates; among the former, Amphibians benefit of a privilege, more pronounced for Urodeles than for Anurans. This is due to basophil, small neural-like cells, which

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live in a stand-by condition and appear as "matrix cells", if scattered, or "matrix areas", if clustered. Kirsche (1983) in a review specified that "… the term matrix zone… refers to neuroepithelium, subependymal layer, external granular layer of the cerebellum, and possibly pockets of undifferentiated cells capable of multiplying and /or differentiating into neuroblasts or glioblasts". The amount of such quiescent cells is variable in the different animal groups; in general they are much more numerous in lower than in higher vertebrates.

The literature reports that both in the adult Anamnia and Amniota, but especially in heterotherms, the cyclic thermal rhythm of the seasons and the correlated variations in photoperiod can activate spontaneous fluctuations of proliferation in these cells. This has been shown by researches carried out mainly in Amphibians and lacertilian Reptiles, and the *Rana esculenta* appears to be the most investigated species (Bernocchi et al., 1990; Chieffi Baccari et al., 1994). Similar relationships have been found in the brain of other Anamnia such as the Teleost *Tinca tinca* (Velasco et al., 2001) and the Petromyzontida *Petromyzon marinus* (Vidal Pizarro et al., 2004), and in the encephalon of homeothermic vertebrates, as songbirds, in particular in males (Margotta and Caronti, 2005; Margotta et al., 2005a). Besides the brain, it was noticed in cellular populations of the eye (Rothstein et al., 1975) and the spinal cord (Velasco et al., 2001) or in tissues like the chemosensory epithelium (Dawley et al., 2000).

Minelli et al. (1982) analysed by autoradiography adult *R. esculenta* frogs submitted to cerebral ablations, and found that environmental climatic factors and encephalic cell proliferation are strictly correlated. As a collateral finding the authors saw a sharp decline in proliferation in normal animals, collected in their wild habitat in late autumn.

We had previously addressed the proliferative activity in the brain of normal adult *R. esculenta* frogs collected from the wild in late autumn and housed in a thermally stable laboratory environment for several days before analysis (Margotta et al., 2000, 2005b). We did not observe the marked decrease in cell proliferation described by Minelli et al. (1982). Therefore I have addressed here the normal proliferative activity in the brain of the same frog, now better named by Capula (2000) as *R. bergeri* (Günther, 1986), synonymous of *R. esculenta* L. (Tortonese and Lanza, 1968), taken from the natural habitat in late autumn and submitted to observation without delay.

As in our previous studies, the actual investigation has been carried out using the Proliferating Cell Nuclear Antigen (PCNA) (Miyachi et al., 1978) as a marker of tissue proliferative activity.

Material and methods

Sexually mature specimens of *Rana bergeri* (Günther, 1986) of both sexes were taken from their natural habitat near Rome at the end of November and immediately sacrificed under anaesthesia with a solution (1:1000) of tricaine methanesulfonate (MS 222 Sandoz, Switzerland). The head was cut off and after partial disarticulation of the cranial bones it was fixed in Bouin's fluid. It was then transferred to 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 CH_3OH 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 commercially available monoclonal antibody anti PCNA, i.e. PC10 (mouse IgG from Sigma, St. Louis, Missouri), 1:1000 in 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), 1:100 in PBS plus 1% nomal horse serum, for 1h 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% $NiSO_4$ and 0.017 % H_2O_2 in 0.05 mol/litre Tris-HCl, pH 7.6. The slides were then dehydrated and mounted with using Entellan (Merck, Germany).

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

Results

In normal adult specimens of *R. bergeri* taken from the wild and immediately submitted to observation the results for each brain region were as follows.

In the olfactory bulbs, some immunoreactive cells were found in the ventricular epithelium, the periventricular grey matter and the internal granular layer (Fig. 1a).

In the anterior portion of each hemisphere, groups of PCNA labelled cells appeared in the ependyma and in proximity of it, in dorso-lateral position at more rostral levels and also ventrally, especially ventro-laterally, at more posterior levels. These symmetrical clusters of presumably undifferentiated cells are named *zonae ger-*

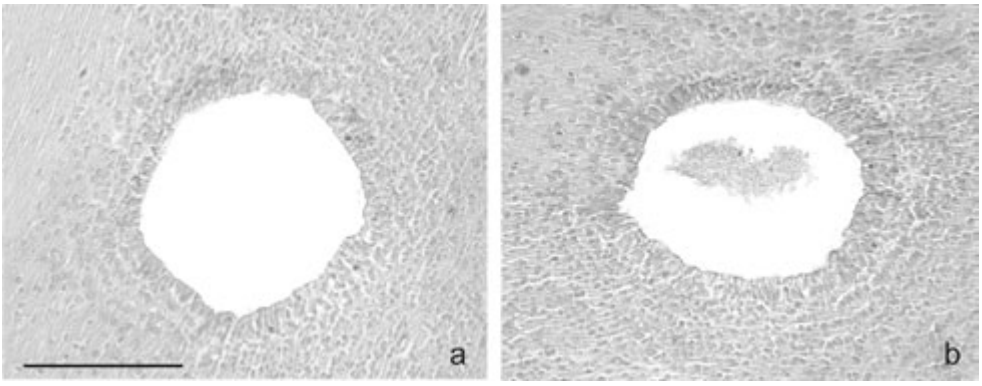

Fig. 1 – Ependymal epithelium, periventricular grey and internal granular layer of the olfactory bulbs of normal adult frogs caught from the wild in late autumn: a) immediately sacrificed; b) housed in thermally regulated environment before sacrifice (from Margotta et al., 2005b, modified, with permission). Transverse sections, PCNA immunocytochemistry, without nuclear counterstaining. Calibration bar = 200 µm.

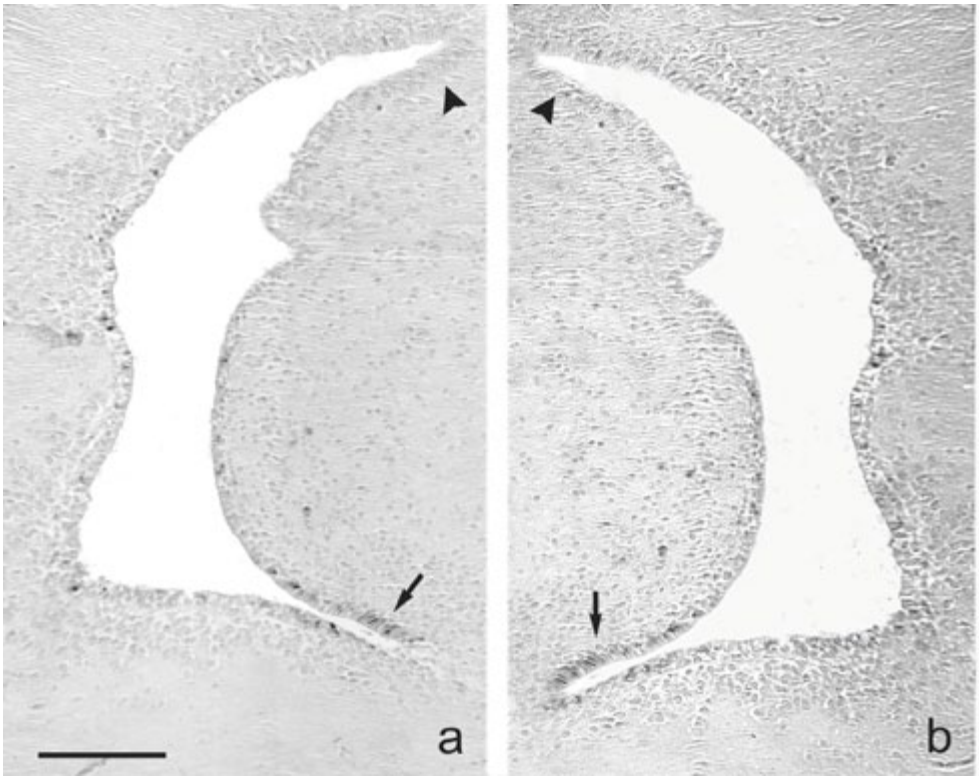

Fig. 2 – *Zonae germinativae dorsales* (arrowheads) and *ventrales* (arrows) in the hemispheres of normal adult frogs caught from the wild in late autumn: a) immediately sacrificed; b) housed in thermally regulated environment before sacrifice. Transverse sections, PCNA immunocytochemistry without nuclear counterstaining. Calibration bar = 100 µm.

minativae dorsales and *zonae germinativae ventrales* according to Kirsche (1967). In the intermediate hemispheric portion, labelled cells lined ventrally the walls and the bottom of the ventricles (*zona germinativa ventralis*)**.** In the posterior telencephalic region, except the caudal pole, labelled cells, although fewer than in more rostral regions, were also found in the lateral and medial ventricular walls as part of the *zona germinativa ventralis*. Labelled cells were also found in the periventricular grey matter. At the caudal pole there were few labelled cells both in dorsal and in ventral position. Therefore, along the whole telencephalon the *zonae germinativae ventrales* were much more extended than the *zonae germinativae dorsales* (Fig. 2a).

In the diencephalon, PCNA labelling was seen in the habenular ganglia, the ependyma and the sub-ependymal layer of the intermediate portions of the ventricular walls and in the ependymal epithelium of the pre-optic and infundibular recesses at the bottom of the III ventricle (Fig. 3a).

In the mesencephalon, PCNA positivity was occasionally visible in very few isolated cells of the intermediate layers of the optic tectum.

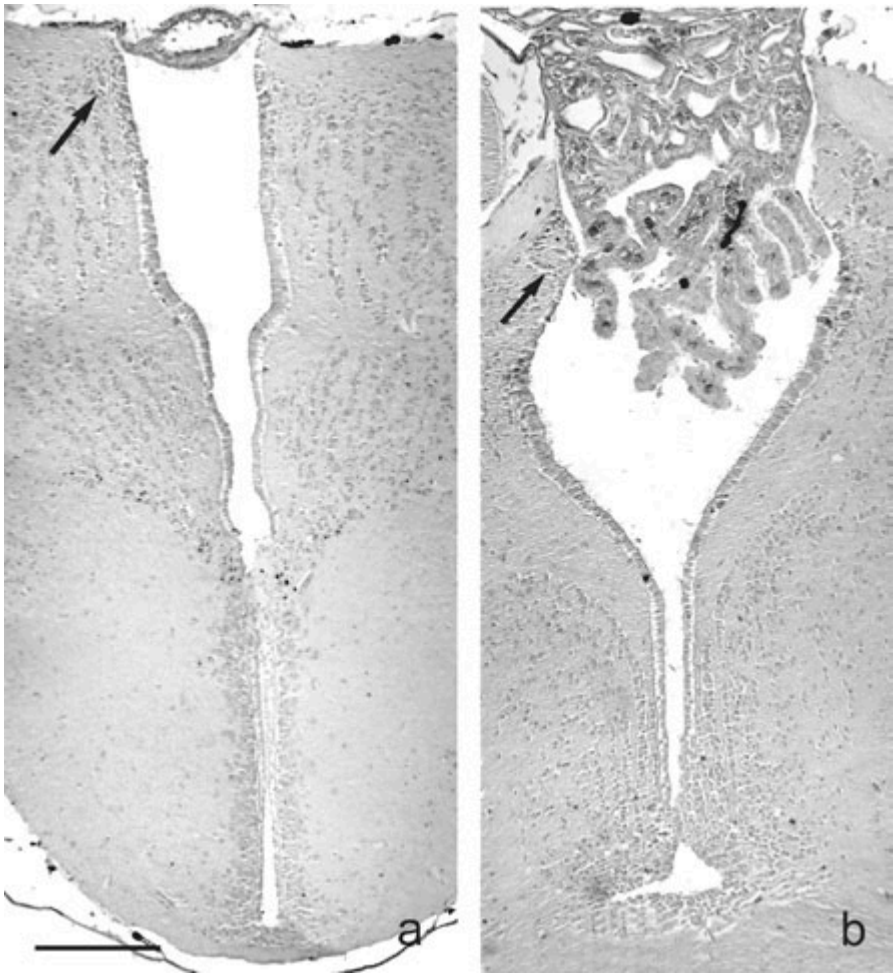

Fig. 3 – Third ventricle, habenular ganglia (arrows), ependymal and sub-ependymal layers, pre-optic recess of normal adult frogs caught from the wild in late autumn: a) immediately sacrificed; b) housed in thermally regulated environment before sacrifice (reprinted from Margotta et al., 2005b, with permission). Transverse sections, PCNA immunocytochemistry without nuclear counterstaining. Calibration bar = 100 µm.

The *cerebellum* did not present any clear cut immunoreactivity.

The *medulla oblongata* displayed a modest immunoreactivity in the floor and lateral edges of the IV ventricle (Fig. 4a).

By contrast, in our previous investigations (Margotta et al., 2000, 2005b) on normal individuals of this same species, but housed in a thermally stable environment before beginning the experiments, we had observed the following: more intense immunostaining in the olfactory bulb (Fig. 1b); more clear signs of proliferative activity in both the *zonae germinativae dorsales* and *ventrales*, and in both ependyma and peri-ependymal layers of the hemispheres (Fig. 2b); labelling in the diencephalon in the same areas as

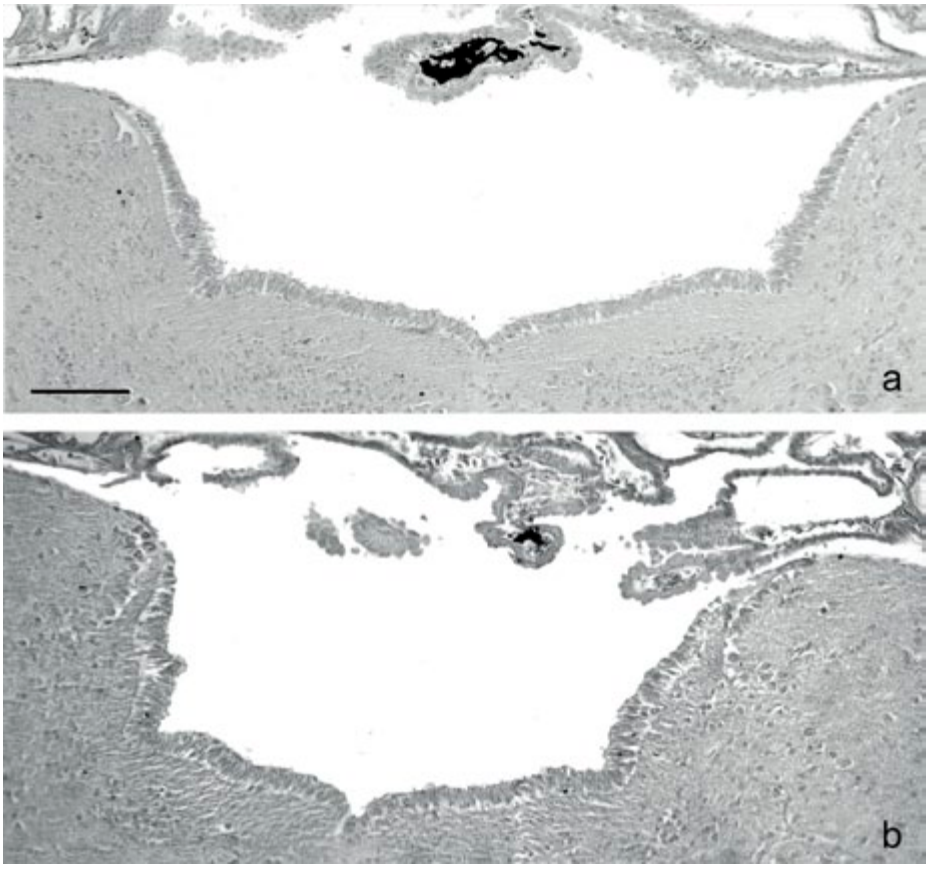

Fig. 4 – Floor and lateral edges of the IV ventricle of normal adult frogs caught from the wild in late autumn: a) immediately sacrificed; b) housed in thermally regulated environment before sacrifice. Transverse sections, PCNA immunocytochemistry without nuclear counterstaining. Calibration bar = 100 µm.

now, but in more cells than in the present research (Fig. 3b); much more labelled cells in the midbrain and more labelled cells in the *medulla oblongata* (Fig. 4b), in the same sites as in the current investigation; no difference from actual results for the *cerebellum*.

No staining was seen in negative control sections.

Discussion

The results of this study showed relevant differences from those of previous investigations on normal adult frogs, at that time named *R. esculenta*, caught in their habitat in late autumn and housed in a thermally regulated laboratory environment before beginning analyses (Margotta et al., 2000, 2005b).

The brain of adult heterothermic and homeothermic vertebrates is equipped with putative stem or precursor cells, which have been termed "matrix cells", and form

so-called "matrix areas" if clustered, which were first identified and described in the telencephalon of the Amphibians by Kirsche (1967). These cells are normally quiescent but remain capable to self-reproduce and to give rise to descendants which may differentiate into neurons or glia (Kirsche, 1967).

Matrix areas may be considered the vestiges of the germinative layers which give rise to the brain during morphogenesis; the sites hosting precursor cells are rather wide in some encephalic regions and narrow in others. As identifiable through markers of proliferating cells, the putative stem cells are almost exclusively found in the ependymal or subependymal layers, although a few are present deep in the cerebral tissue. These undifferentiated cells are much more numerous, and matrix areas are wider in younger than in older organisms (Kirsche, 1967). Some previous findings suggest that the final location of some neuron descendants of persisting precursor cells may depend on the persistence of radial glia (see reviews: Margotta and Morelli, 1997; Alvarez-Buylla et al., 2002).

A series of coordinated investigations has allowed to evaluate by immunocytochemistry the normal proliferative potential of the brain of adult vertebrates, from Petromymyzontidae (Margotta et al., 2007) through Chondrichthyes Elasmobranchs (Margotta, 2007), Osteichthyes Teleosts (Margotta et al., 2001, 2002, 2004), Urodele Amphibians (Margotta et al., 1999b, 2005b), Anuran Amphibians (Margotta et al., 2000, 2005b) and lacertilian Reptiles (Margotta et al., 1999a, 2005b), to songbirds (Margotta and Caronti, 2005; Margotta et al., 2005a),.

The cyclic, seasonal changes of temperature can affect the proliferation of progenitor cells in various tissues of adult heterotherms. In particular, Minelli et al. (1982) observed by autoradiography that seasons influence the trend and entity of spontaneous or induced proliferation in the brain of adult *R. esculenta*, either normal or subjected to partial cerebral ablation. Proliferation is low in specimens caught from the wild in May-June, much higher in September-October and intermediate in late November. Moreover, an artificial cold shock was capable of interfering with the seasonal pattern, producing effects opposite to those expected for the season (Minelli et al., 1982).

The proliferative potential of encephalic precursor cells has been evaluated here by tagging PCNA. It has been shown now that in *R. bergeri* caught in late November and immediately sacrificed, in comparison with previous investigations in this frog caught in the same period but housed in the laboratory before sacrifice (Margotta et al., 2000, 2005b), the ependymal and sub-ependymal PCNA expressing cells are reduced in number. The present results reinforce, with a different analytical method, those of Minelli et al. (1982) in similar experimental conditions as the present ones.

Therefore, I should conclude that the stay of animals in a thermally regulated, relatively warm environment for several days after the drawing and before the beginning of the experiments (Margotta et al., 2000, 2005b) is enough to counteract the effects of natural, late autumn environment and to stimulate an increase in the number of proliferating cells.

Acknowledgements

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