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Relationships between seasonal (spring or autumnal) 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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Abstract

The question has arisen if the seasonal cycle, made of temperature and photoperiod variations, might activate proliferation of quiescent stem cells still present in adult brain of some living in fresh water, earth-dwelling Anamnia and heterothermic Amniota. Previously, some authors performed seminal autoradiographic, quantitative observations focused on a handful of adult *Rana esculenta* specimens which, once caught in nature in spring and autumn, were submitted to temporary artificial hibernation and compared with untreated controls. In not-brain-injured and not-cold-shocked samples the encephalic proliferation appeared lower in frogs caught in spring than in those caught in autumn. At the light of these data, an immunohistochemical investigation has been carried on not-brain-injured, not-cold-stressed adult *Rana bergeri*, captured in their habitat in spring and in autumn. The labelling was observed mainly in the forebrain, where it was more pronounced in the specimens caught in autumn than in those captured in spring. This pattern confirms and reinforces the findings of past authors in the same species and under similar experimental conditions.

Key words

"spring" / "autumnal" influence, neural-like cells, *Rana*.

Introduction

The persistence of proliferative potentialities and therefore of reparative and even regenerative processes in the adult brain has been mainly ascertained in some fresh water (like Teleosts), earth-dwelling Anamnia (like urodelan and anuran Amphibia) and heterothermic Amniota (like lacertilian Reptiles).

This awareness was reached by means of various techniques: at first classical histology, then autoradiography, seldom electron microscopy and immunohistochemistry, the last method being applied to target proliferation-related enzyme activity.

Proliferative plasticity is due to the survival in adult brain of small and basophilic neural-like cells, remnants of the embryonal neural layer (Kahle, 1951; Fujita, 1963; Kirsche, 1967). The number of these undifferentiated cells decreases during the embryonic phase, the subsequent larval stages and then aging from younger to advanced

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life. Furthermore, the number of these sleeping cells can vary among the various animal groups; generally speaking they are much more in lower than higher vertebrates.

These putative precursor or stem cell are normally silent, but capable of self-reproduction and can start cycling again and give rise to descendants that undergo late differentiation and evolve into neurons or glia (Kirsche, 1967, 1983).

A high amount of investigations on this issue in adult animals have showed, besides diffuse immunohistochemical signs of potential proliferation on the walls (ependyma and sub-ependyma) of the cerebral ventricles in lower vertebrates (Petromyzontidae: Margotta et al., 2007; Selacians: Margotta, 2007), that stem cells in standby can appear as scattered "matrix cells" in the ependyma and sub-ependymal layer of the olfactory district, in the deep cerebellar tissue (in Teleosts alone), in the ependymal epithelium and periventricular grey matter of the *medulla oblongata* in some Anamnia living in fresh water and earth-dwelling, and in some heterothermic Amniota. In several of those studies, focused on the telencephalic district of Teleosts, Amphibia, poikilothermal (like lacertilian Reptiles) and homothermic (like Birds) Amniota, these quiescent cells were found clustered in "matrix areas" (once *Matrixzonen*: Kirsche, 1967) sites at the dorsal and ventral edges of each lateral ventricular surface making up the *zonae germinativae dorsales* and *ventrales*, both extended anteroposteriorly. The latter areas are generally wider and more populated in cells which are exploited less quickly than those of *zonae germinativae dorsales* (Kirsche, 1967). Furthermore, another pair of matrix areas, the *zonae germinativae caudales* exist in the midbrain of Teleosts. Other silent cells appear grouped as "hot spots" in male songbirds and as "matrix tissue" in some Mammals.

The above summarized knowledge has been acquired mainly by submitting specimens to surgical ablation of encephalic plugs or areas, at times submitting them to heterotopic hetero-, more rarely homo-transplants of encephalic portions (Kirsche, 1983; Margotta and Morelli, 1996).

Investigations have been carried out in these same vertebrates to address various questions, including if season cycle, made of temperature and photoperiod variations, or a thermal stress (environmental or experimentally applied), alone or coupled with a cerebral surgical intervention might activate encephalic latent spontaneous proliferation, and consequently reparative and even regenerative potential, due to an otherwise hidden mitotic capacity of such sleeping cells still present in their brain. On such issues only a handful of normal specimens was object of observation.

The first issue was investigated by Minelli et al. (1982), evaluating the capacity to take up 6-H^3 thymidine in normal and regenerating brain of adult *Rana esculenta* (caught in nature in different seasons), under the influence of surgery and thermal variations (natural/seasonal alone or coupled to an experimental change). The study was planned as a main set of observations and a seminal, subordinate and complementary one. On a handful of specimens it was demonstrated that in spring thymidine uptake is extremely low and is increased by cold shock, whereas in autumn it is higher and is decreased by cold shock. The encephalic regenerative processes were also influenced by keeping the animals at 4 °C for one day.

At the light of these seminal findings on a handful of brain-uninjured, but thermally (seasonally/artificially) stressed specimens, we wish to address the same issue with a different, qualitative technique, in normal adult *R. bergeri* (once synonymous of *R. esculenta*: Tortonese and Lanza, 1968), caught in nature in spring and in autumn.

An immunocytochemically detectable marker, the proliferating cell nuclear antigen (PCNA: Miyachi et al., 1978), will be assayed as probe for proliferating cells. This method has been shown to be highly reliabile (Margotta and Chimenti, 2016).

The results were compared with previous related ones obtained by the same authors (Chimenti and Margotta, 2015; Margotta, 2015).

Materials and methods

Specimens of normal adult *Rana bergeri* (Günther, 1986) - as ascertained by Capula (2000) - of both sexes were caught from their habitat near Sora (Frosinone, Latium, Italy) both at the end of April (environmental temperature between 10 and 16 °C; five animals) and at the end of October (environmental temperature between 8 and 18 °C; five animals). The frogs of the two groups were 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 and then transferred to 80% ethyl alcohol, where the brain was removed under a stereomicroscope. The tissue was dehydrated through graded ethyl alcohols, cleared in histolemon and embedded in paraffin under vacuum. Transverse, 8 μm thick serial sections were cut in antero-posterior direction with a rotary microtome.

Immunohistochemistry was performed as follows. The sections of "spring" and "autumnal" samples were heated in an oven at 60 °C for 20 min until the paraffin melted, deparaffinised and rehydrated through graded ethyl alcohol. A Vectastain Universal Quick Kit (Vector Labs, Burlingame, CA, USA) and 0.01 mol/L phosphate buffer, pH 7.5, with 0.02% Triton X100 were used, at room temperature. The procedure was as follows. 10 min in 3% (v/v) H_2O_2 followed by rinse, 10 min in blocking serum, 15 min + 15 min in avidin/biotin blockingKit (Vector Labs) followed by rinse, 90 min (in a moisted chamber) in monoclonal antibody against PCNA (Sigma, Milan, Italy; cod. P8825), diluted 1:500 with 1,5% blocking serum, followed by rinse, 10 min in biotinylated universal secondary antibody (Vector Lab) followed by rinse, 10 min in streptavidin/ peroxidase complex followed by rinse, 10-15 min incubation in Nova Red or DAB substrate Kits (Vector), with or without nickel enhancement. The sections were then washed and mounted in Kaiser's glycerol gelatine (Sigma). Control sections of representative tissues were prepared substituting the primary antibody with normal mouse serum. A section of regenerating rat liver, in which a high cell proliferative activity had been documented by incorporation of bromodeoxyuridine, was used as positive control.

Results

In the olfactory bulbs, PCNA labelling appeared diffuse in the layer lining the ventricles both in "spring" and "autumn" specimens (Figs. 1a,b), while positive cells were seen scattered among the ependymal epithelium and rarely in the grey matter of "autumn" individuals (Fig. 1b).

In the telencephalon of "spring" specimens immunoreactive scattered cells were visible among the ependymal cells positioned dorsally and ventrally with respect to

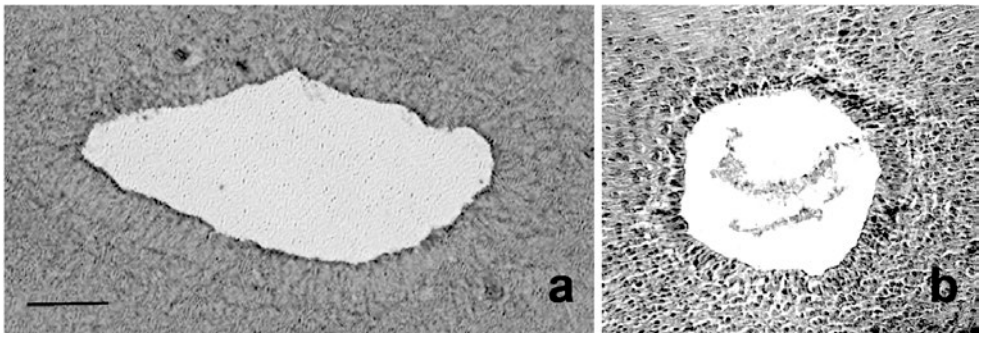

Figure 1 – Olfactory bulbs of normal adult *Rana bergeri*. Immunolabelling appeared diffuse in the layer lining the ventricles both in spring and autumnal specimens (Figs. 1a, b), while scanty positive cells were seen scattered among the ependymal epithelium and in sub-ependyma of autumnal specimens (Fig.1b). Transverse sections. PCNA immunocytochemistry without nuclear conterstaining. Calibration bars = 50 µm in a, 200 µm in b. Fig. 1a: reprinted from Chimenti and Margotta, 2015. with permission; Fig. 1b: reprinted from Margotta, 2015. with permission.

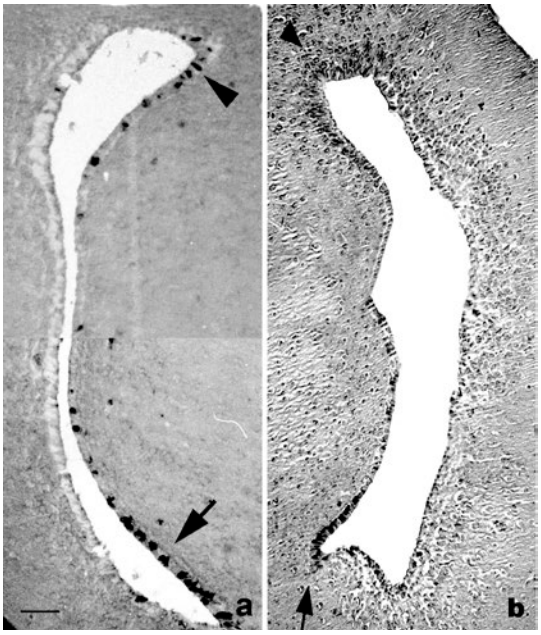

Figure 2 – Telencephalic hemispheres of normal adult *Rana bergeri*. In spring specimens immunoreaction was circumscribed to scanty cells scattered among the ependymal epithelial cells, dorsally (zonae germinativae dorsales) and ventrally (zonae germinativae ventrales) with respect to falciform cavities (Fig. 2a), while in autumnal specimens there were both diffuse immuno-labelling in the layer lining the ventricles and clusters of numerous PCNA-positive cells among the ependymal epithelial cells dorsally (zonae germinativae dorsales) and ventrally (zonae germinativae ventrales) with respect to falciform cavities (Fig. 2b). Transverse sections. PCNA immunocytochemistry without nuclear conterstaining. Calibration bars = 100 µm in a, b. Fig. 2a: reprinted from Chimenti and Margotta, 2015, with permission; Fig. 2b: reprinted enlarged and cropped from Margotta, 2015, with permission.

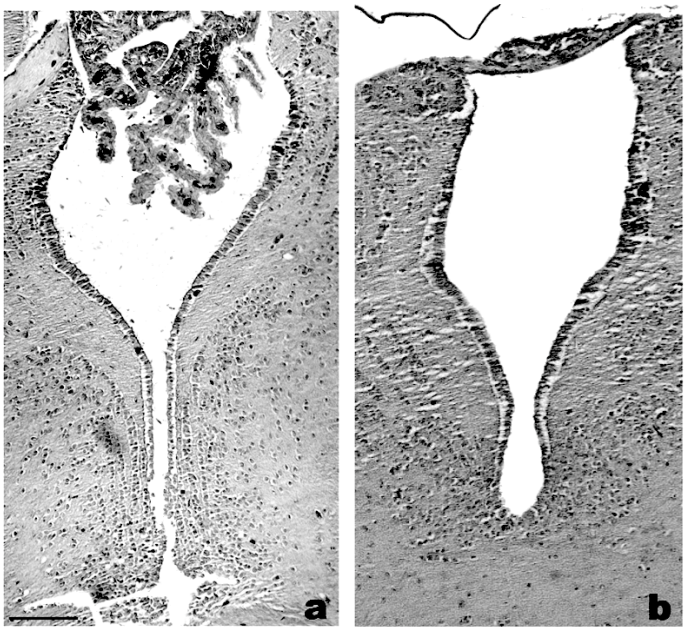

Figure 3 – Diencephalon of normal adult *Rana bergeri*. In spring samples PCNA-positivity appeared diffuse in the superficial and sub-ependymal layers which enveloped the ventricle, and weak dorsally at the level of the habenular ganglia (Fig. 3a). In these same sites more pronounced PCNA-positivity was noticed in the autumnal samples (Fig.3b). Tranverse sections. PCNA immunocytochemistry without nuclear conterstaining. Calibration bars = 100 µm in a, 200 µm in b. Fig. 3a: reprinted from Chimenti and Margotta, 2015 with permission; Fig. 3b: reprinted from Margotta, 2015, with permission, magnified.

hemispheric falciform cavities, i.e. they were located in the same sites of *zonae germinativae dorsales* and *ventrales* (Fig. 2a). In the telencephalon of "autumn" individuals, besides a diffuse immuno-labelling in the ependyma and sub-ependyma, large clusters of labelled cells were identifiable dorsally and ventrally to the ventricular cavities (*zonae germinativae dorsales* and *ventrales*) (Fig. 2b).

In the diencephalon of "spring" frogs, PCNA-positivity appeared diffuse in the ependyma and in the sub-ependymal layer which enveloped the ventricle (Fig. 3a), weak labelling could be seen dorsally at the level of the habenular ganglia. More evident reactivity was noticed in the same sites in "autumn" specimens (Fig.3b).

In the midbrain the immuno-positivity was scarce in "spring" samples, more pronounced in "autumn" ones, while in the hindbrain no labelling was found either in "spring" or "autumnal" specimens.

Discussion

In the present observations on normal brain of adult *R. bergeri* captured in a same area in spring and in autumn, PCNA expression was diffuse in ependymal and sub-

ependymal grey matter of the forebrain. More precisely, labelling was found on scattered cells on the ventricular surfaces of the olfactory bulbs, on clustered cells in the ventricular walls of the telencephalic hemispheres (*zonae germinativae*) and on circumscribed cells in the diencephalic epithamic habenular ganglia and ipothalamic recesses. In the midbrain the immuno-positivity was less clearly evident and in the hindbrain no labelling was found.

Our present results suggest that spring conditions may exert a soft proliferative stimulation, while autumnal ones may exert a strong stimulus on normal adult brain, with consequent vanishing or alternatively substantial proliferative response in spring and in autumn respectively.

This study adds to those which ascertained in some fresh water (like Teleosts), in earth-dwelling Anamnia (like urodelan and anuran Amphibia) and in some poikilothermal Amniota (like lacertilian Reptiles) a more or less marked impact of the seasonal (thermal and photoperiod) cycle on spontaneous or induced fluctuations of proliferation of neural stem cells survived to adulthood.

Some of those researches addressed the question if seasonal cyclic variations could activate proliferation of quiescent cells in the brain (Minelli et al., 1982; Bernocchi et al., 1990; Chetverukhin and Polenov 1993; Polenov and Chetverukhin, 1993; Chieffi Baccari et al., 1994; Ramirez et al., 1997; Dawley et al., 2000; Vidal Pizarro et al., 2004) and several tissues (ocular, nervous: Rothstein et al., 1975; chemosensory epithelium: Dawley et al., 2000; retinal cells: Velasco et al., 2001) of adult marine (*Petromyzon marinus*: Vidal Pizarro et al., 2004), fresh water (*Tinca tinca*: Velasco et al., 2001), terricolous (*R. esculenta*: Rothstein et al., 1975; Minelli et al., 1982; Bernocchi et al., 1990; Chieffi Baccari et al., 1994; *R. temporaria*: Chetverukhin and Polenov 1993; Polenov and Chetverukhin, 1993; *Plethodon cinereus*: Dawley et al., 2000) and terrestrial (*Podarcis hispanica*: Ramirez et al., 1997) vertebrates. The response to the seasonal cycle was not univocal among the various systematic groups.

In detail, Minelli et al. (1982) in an enlightening study on adult brain of *R. esculenta* collected in their wild habitat in different times of the year (spring, late and advanced autumn, winter) focused on the effect of low (natural or experimentally applied) or high (natural alone) temperature on the uptake of 6-H^3 thymidine by normal or injured brain. Those authors noticed that in May/June such uptake was extremely low and mitoses were very scanty, whereas label uptake became very high in September/October, indicating a strong increase in the mitotic activity. Later on proliferation declined, becoming of intermediate intensity in advanced November and further decreasing in proximity of winter. Minelli et al. (1982) also recorded that the observed high or low values could be changed to their opposite if an experimental, transient thermal stress was administered (cooling at 4 °C for 24 hours): in such conditions the proliferation appeared increased in May/June and decreased in September/October. Such findings could light upon the conflicting results previously reached also by other authors on the encephalic events in anurans. Therefore Minelli et al. (1982) demonstrated in *R. esculenta* an environmental input on fluctuations of experimentally induced and spontaneous proliferation of encephalic cells.

Furthermore, Minelli and Del Grande (1980) and Minelli et al. (1982), following previous authors (Rosomoff and Gilbert, 1955; Stone et al., 1956, Lougheed et al., 1960; Kiernan, 1979; Kiernan and Contestabile, 1980), proposed the existence of a relationship among cold temperature, reduction of blood brain barrier and regenera-

tive capacity and for elucidate owen results attributed that due probable low doven metabolism operating fürther reduction in the autumnal proliferative rates.

Perhaps the explanation of the mentioned winter event could be due, besides to different among systematic groups of vertebrates, to what Ramirez et al. (1997) discovered by autography and immunostaining in adult brain-injured *P. hispanica*, *i.e.* that "…cold (winter) temperature prevented migration of the newly generated neurons".

Among fresh water or earth-dwelling Anamnia and poikilothermal Amniota, Anura occupy a less privileged position than Teleosts and even less than Urodeles; the latter are the most gifted vertebrates for matrix cells, which are especially well identifiable in the telencephalic *zonae germinativae*, that are the areas best endowed with putative stem cells in physiological conditions (Kirsche, 1967).

Relationships linked to a natural cell proliferation have been investigated immunocytochemically in the brain of adult *R. bergeri* and *P. sicula* captured in nature in late autumn (Margotta, 2012) and in summer (Margotta, 2014), respectively. In the former study (Margotta, 2012) a widespread reduction in proliferation was observed as compared with what had been observed in *R. esculenta* caught in late autumn but stabled for many days in the laboratory before being analyzed (Margotta et al., 2000, 2005).

The immunocytological results obtained previously in normal adult brain of *R.esculenta* (Margotta et al., 2000, 2005) and *R. bergeri* (Margotta, 2012), and the present results as well, are in agreement with the autoradiographic seminal findings of Minelli et al. (1982) on the normal brain of a small number of adult *R. esculenta*, which gives support to our previous and present investigations.

The immunohistochemical investigations of Chimenti and Margotta (2015) and Margotta (2015) constitute a triad the results of which have been in agreement with those obtained by autoradiography by Minelli et al. (1982) in the same species.

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