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Relationships between summer 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

About the issue if the natural encephalic proliferative activities can be influenced by the seasonal (thermal and photoperiodic) cyclic fluctuations in adult earth-dwelling Anamnia and poikilothermal Amniota there is a gap regarding expressly summer season alone in literature reports on autoradiographic studies in frog. The actual study suggests that the brain proliferative pattern in *Rana bergeri* collected in the wild in summer is intermediate between those previously observed in spring and autumn. This would seem to indicate a proliferative increase from spring to autumn. This pattern mainly appears in the forebrain, typically in the olfactory portion where the stem cells are identifiable as scattered "matrix" cells in the ependyma and grey matter, and in the telencephalic district, where these cells are grouped as "matrix" areas in the well-known *zonae germinativae dorsales* and *ventrales*.

Key words

Seasonal influence, neural-like cells, *Rana*.

Introduction

Among the many investigations carried on adult brain of Anamnia and Amniota from the second half of the past century, a handful of studies has addressed the issue if a seasonal cycle, made of temperature and photoperiodic variations, might impact on natural proliferative fluctuations or unmask an encephalic latent spontaneous proliferative potential, consequently showing reparative and even regenerative potentialities thanks to otherwise hidden mitotic activity of quiescent cells, which are still present in the adult brain of normal fresh water, earth-dwelling Anamnia and heterothermic (Kirsche, 1983) and homothermic (Margotta and Morelli, 1996) Amniota.

These events are linked to small, basophilic neural-like stem cells, remnants of the periventricular germinative layer in the early embryo (Kahle, 1951; Fujita, 1963; Kirsche, 1967) whose number decreases from the earlier to the more advanced embryonic stages, then to larval ones and furthermore through youth until advanced life.

These putative precursors, or surviving stem cells, are normally silent but are capable of self-reproductions and can start cycling again given rise to descendants and even undergo late differentiation, so evolving into neuronal or glial cells (Kiesche, 1967; 1983). These cells in stand-by vary in number among vertebrates, gener-

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ally speaking they appear much more numerous in lower than in higher vertebrates.

Such “sleeping” cells are mainly located in the forebrain, sometimes in the midbrain, *cerebellum* (like in Teleosts) and *medulla oblongata*. In particular, they can appear scattered (so called matrix cells) in the ependymal and sub-ependymal layers of the olfactory district and clustered and extended antero-posteriourly in the telencephalic hemispheres (so called dorsal and ventral matrix areas, sometimes these areas may also be caudal like in Teleosts). As compared with isolated “matrix” cells, “matrix” areas (especially the ventral ones) are wide and rich in long lasting cells (Kirsche, 1967]. These areas, also named *zonae germinativae* (Kirsche (1967), are located among the ependymal cells on the walls of the lateral ventricles in the telencephalic hemispheres.

Such telencephalic groups of cells are located in the so-called “hot spots” of male songbirds or in the so-called “matrix tissue” of various Mammals (for further details: Margotta and Morelli, 1996).

The knowledge of the presence of such cells has been reached by various experimental procedures (surgical ablations or heterotopic, seldom homotopic, transplants of encephalic plugs or areas, *in vitro* cultures of brain tissues), with results evaluated by histological techniques, classical and advanced.

Among others, the literature reports autoradiographic (Minelli et al., 1982) and immunohistochemical (Ramirez et al., 1997) studies about encephalic proliferative performances in the adulthood of *Rana esculenta* and *Podarcis hispanica*, respectively, linked to seasonal cyclic (thermal and photoperiodic) fluctuations in earth-dwelling Anamia and poikilothermal Amniota.

Minelli et al. (1982), as part of a wide research on adult brain-injured of *R. esculenta*, also focused on a handful of normal specimens collected from the wild in various periods of the year (May-July, September-October, advanced November) and observed that the encephalic proliferation appeared low in May-July, higher in September-October, intermediate in advanced November and waned again in proximity of winter.

Keeping in mind these findings and what had emerged from our immunohistochemical studies in normal adult brain of *R. bergeri* caught in its habitat in spring (Chimenti and Margotta, 2015; Margotta and Chimenti, 2017) and autumn (Margotta, 2012; 2015; Margotta and Chimenti, 2017), we have addressed the gap due to the lack of specific investigation on the behaviour of putative stem cells at the height of summer by Minelli et al. (1982) and by our immunohistochemical analyses on the relationships between summer thermal/photoperiodic variations and cell proliferative behaviour in normal adult brain of *R. bergeri*.

Winter behaviour was not addressed since we expected to find a negligible proliferation in this season, as ascertained by autoradiography by Minelli et al. (1982).

As in our previous researches we have employed to detect potentially proliferating cells the Proliferating Cell Nuclear Antigen (PCNA: Miyachi, 1978); for further details on theoretical principles, ubiquity, reliability and value of this test see: Margotta and Chimenti (2016).

Materials and methods

Normal adult *Rana bergeri*, as ascertained by Capula (2000) (once *Rana esculenta*: Tortonese and Lanza, 1968), of both sexes were used after their caught in the wild

near Sora (Frosinone, Latium). The specimens were caught at the end of July (environmental temperature varying between 13° to 26 °C). Other individuals, employed as controls as in our recent investigations (Chimenti and Margotta, 2015; Margotta, 2015; Margotta and Chimenti, 2017), had been previously collected in the same wild at the end of April (environmental temperature varying between 10° to 16° C) (Chimenti and Margotta, 2015) and at the end of October (environmental temperature varying between 8° to 18 °C) (Margotta, 2015; Margotta and Chimenti, 2017).

The frogs 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 anterior-posterior direction with a rotary microtome.

The brain slides were processed as follows:

1) the sections of experimental ("summer") specimens, upon removal of paraffin and hydration, 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. 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% $NiSO_4$ and 0.017% H_2O_2 in 0.05 mol/litre Tris-HCl, pH 7.6. Slides were then dehydrated and mounted with Entellan (Merck, Germany).

2) the sections of control ("spring" and "autumnal") specimens 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 M 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, 5 min rinse, 10 min in blocking serum, 15 min + 15 min in avidin/biotin blocking Kit (Vector Labs), brief rinse, 90 min (in a moisted chamber) in monoclonal antibody against PCNA (Sigma, Milan, Italy; cod. P8825), diluted 1:500 in buffer with 1.5% blocking serum, 5 min rinse, 10 min in biotinylated universal secondary antibody, 5 min rinse, 10 min in streptoavidin/peroxidise complex, 5 min 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 gelatin (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 bromo-deoxyuridine, was used as positive control.

Results

The present description of normal brain of adult *R. bergeri* derives from the images belonging to individuals caught in nature in summer (experimental specimens) compared with those of control specimens previously obtained from normal individuals captured in the same site in spring (Chimenti and Margotta, 2015; Margotta and Chimenti, 2017) and in autumn (Margotta, 2015; Margotta and Chimenti, 2017).

In the olfactory bulbs of control "spring" individuals scarce labelled cells were found isolated in the ependymal layer and sometimes in the adjacent grey matter (Fig. 1a), while several stained cells were found scattered in the same structures of the "summer" specimens (Fig. 1b) and even more in the control "autumn" samples (Fig. 1c) .

In the telencephalon a PCNA-positivity was noticed in the walls in the crescent-shaped hemispheric cavities. Labelled cells appeared concentrated at the dorsal and ventral edges of the ventricles forming the *zonae germinativae dorsales* and *ventrales*. Such areas seemed least populated by labelled cells and thinnest in control "spring" individuals (Fig.2a), intermediately populated in "summer" specimens (Fig. 2b) and most populated in control "autumn" samples (Fig. 2c).

In the diencephalon few stained cells were seen in the wall of the III ventricle and in habenular ganglia, pre-optic and infundibular recesses in control "spring" individuals (Fig. 3a); the amount of labelled cells was intermediate in "summer" specimens (Fig. 3b) and even higher in the control "autumn" samples (Fig. 3c).

In the midbrain immuno-staining was very rare in control "spring" individuals, some labelling was present in "summer" specimens and more labelled cells were seen in control "autumnal" samples.

In the brain laying behind midbrain no labelling was noted in any sample.

Discussion

Some researches refer that in various adult vertebrates (fresh water and earth-dwelling Anamnia, poikilothermal Amniota) the seasonal cycle, made of temperature

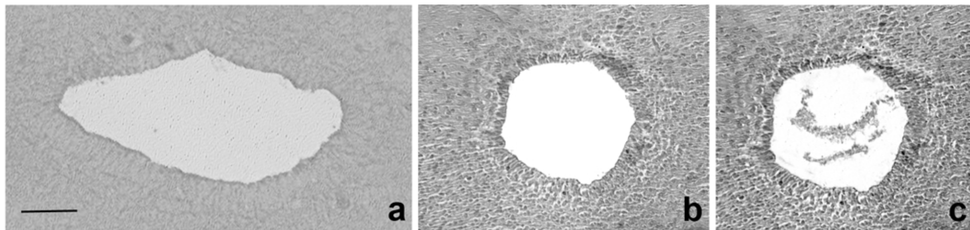

Figure 1. Transverse sections of olfactory bulbs in normal adult *Rana bergeri*. Immunoreaction was observed in scattered ependymal cells and in a few sub-ependymal cells: a) scarce labelling in a sample caught in spring; b) intermediate labelling in a sample caught in summer; c) more pronounced labelling in a sample caught in autumn. [Fig. 1a: reprinted from Chimenti and Margotta, 2015, and Margotta and Chimenti, in press, with permission; Fig. 1c: reprinted from Margotta, 2015, and Margotta and Chimenti, 2017, with permission]. PCNA immunocytochemistry without nuclear conterstain. Calibration bars = 50 µm.

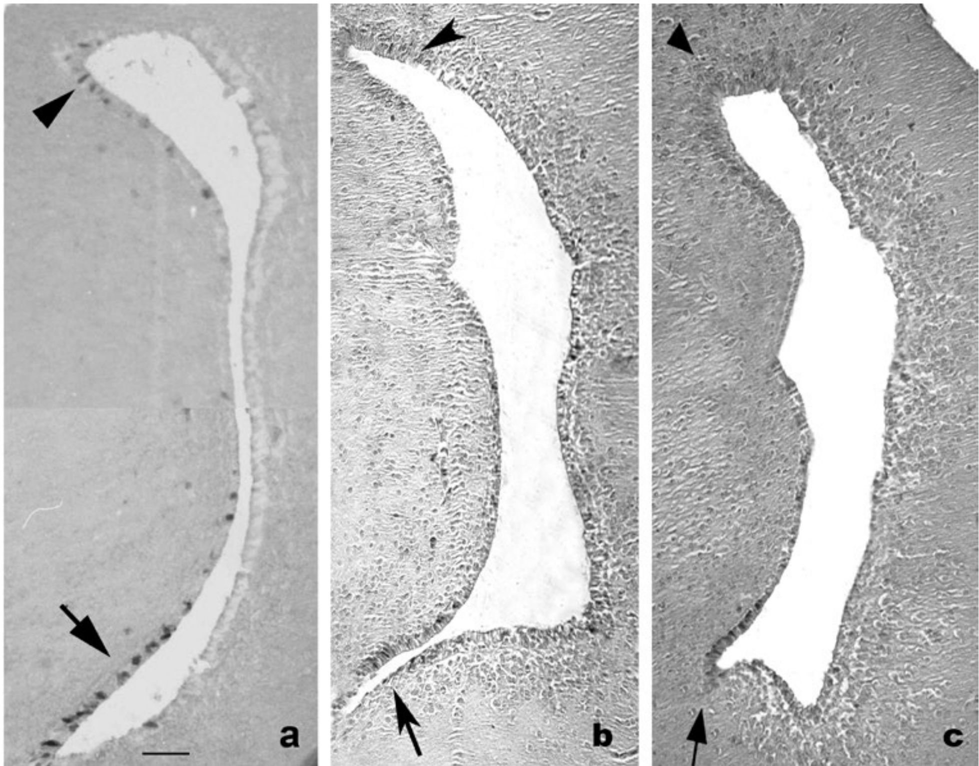

Figure 2. Transverse sections of telencephalic hemispheres in normal adult *Rana bergeri*. Immunoreaction is distinctly circumscribed to clustered cells among the ependyma, in the *zonae germinativae dorsales* and *zonae germinativae ventrales*: a) scanty labelling in a sample caught in spring at the level of left hemisphere; b) intermediate labelling in a sample caught in summer at the level of both hemispheres; c) more intense labelling in a simple caught in autumn at the level of right hemisphere [Fig. 2a: reprinted from Chimenti and Margotta, 2015, and Margotta and Chimenti, 2017, with permission; Fig. 2c: reprinted from Margotta, 2015, and Margotta and Chimenti, 2017, with permission]. PCNA immunocytochemistry without nuclear conter-stain. Calibration bars = 50 µm.

(natural or experimentally applied) and photoperiod variations, can exert a positive or alternatively negative stimulus on the proliferation of stem cells still surviving in adult tissues or organs as remains of the germinative layer in the early embryonic tissues and, in particular, in the central nervous system.

Minelli et al. (1982) carried on an extended autoradiographic study on this issue in brain-injured or not-brain-injured adult *R. esculenta,* collected from the wild in various periods of the year. They focused on May-July, September-October and advanced November. They observed that proliferation appeared low in spring and high in autumn, became intermediary in advanced November and waned later on in proximity of winter.

As a consequence of such remarks, it has been supposed that the different seasonal influence on proliferative rhythms could explain the controversial results obtained

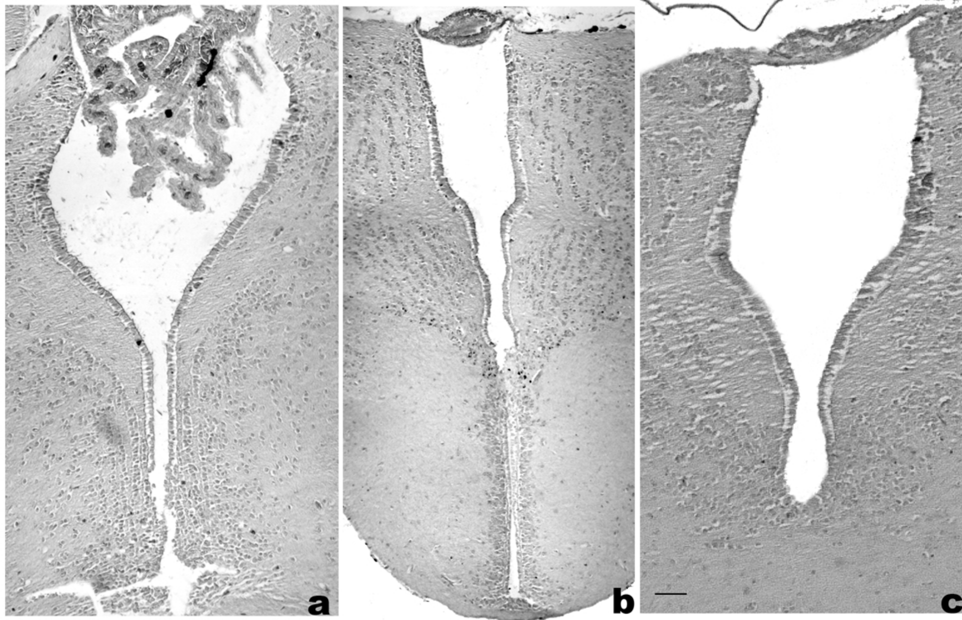

Figure 3. Transverse sections of diencephalon in normal adult *Rana bergeri*. Immunoreaction was found diffuse in the ependyma and periventricular grey matter enveloping the IIIrd ventricle, dorsally at the level of habenular ganglia, ventrally at the level of pre-optic and infundibular recesses: a) weak labelling in a sample caught in spring; b) more pronounced labelling in a sample caught in summer; c) higher labelling in a sample caught in autumnal [Fig. 3a: reprinted from Chimenti and Margotta, 2015, and Margotta and Chimenti, in press, with permission; Fig. 3c: reprinted from Margotta, 2015, and Margotta and Chimenti, 2017, with permission]. PCNA immunocytochemistry without nuclear conterstain. Calibration bars = 50 µm.

by some previous authors on the regenerative power of the nervous central system in adult anurans.

The seasonal results of Minelli et al. (1982) have been by re-challenged by PCNA immunohistochemistry: in normal adult brain of *R. esculenta* caught in late November, not immediately submitted to observation but instead housed in a thermally stable laboratory environment for several days before analysis (Margotta et al., 2000, 2005), and in normal adult brain of *R. bergeri* collected at the end of November (Margotta, 2012), April (Chimenti and Margotta, 2015; Margotta and Chimenti, 2017) and October (Margotta, 2015; Margotta and Chimenti, 2017) and examined without delay.

The results of our investigations were compared among each other revealing encephalic signs of different proliferative potentialities depending on season: a diffusely low immunolabelling was noticed in spring (Chimenti and Margotta, 2015; Margotta and Chimenti, 2017) and an increase in autumn (Margotta, 2012; 2015; Margotta and Chimenti, 2017), in agreement with the findings of Minelli et al. (1982).

In spite of the knowledge derived from the studies of Minelli et al. (1982) and confirmed by us with a different method, there was a gap of knowledge on the behaviour of encephalic proliferation at midsummer.

To fill this gap we have extended our studies and found that the encephalic label-

ling in summer was more pronounced than in spring (Chimenti and Margotta, 2015; Margotta and Chimenti, 2017) and less than in autumn (Margotta, 2015; Margotta and Chimenti, 2017).

These patterns appeared more evident in the encephalic districts more rich of matrix cells also in anurans, *i.e.* the olfactory and even more the telencephalic portions, in the latter mainly at the level of the *zonae germinativae dorsales* and *ventrales*.

These results seem to indicate that summer thermal and photoperiodic conditions exert an intermediate stimulus between spring and autumn conditions, with a consequently intermediate proliferative response deriving from only part of cerebral silent cells.

Therefore, taking into account the previous findings in spring, autumnal and winter (Minelli et al., 1982; Margotta, 2012, 2015; Chimenti and Margotta, 2015; Margotta and Chimenti, 2017) and the actual results in summer, the impact of the whole season cycle on adult brain proliferations is now known for the frog.

So, in an overall view covering a whole annual time one may claim that cell proliferation shows an increasing trend from spring through summer to autumn , then declines in the proximity of winter, as referred by Minelli et al. (1982).

Perhaps the winter decrease in encephalic proliferative capacity could be due, besides interspecific differences, to what was stated by Ramirez et al. (1997) for adult brain-injured *P. hispanica*: "…cold (winter) temperature prevented migration of the newly generated immature neurons", which in turn may be related in part to a possible role of radial glial cells in that migration (Margotta and Morelli, 1997).

Acknowledgements

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