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Interaction between spring temperature-photoperiod and experimentally induced transient cold shock influencing proliferative activity in the brain of an adult terrestrial heterothermic vertebrate, *Rana bergeri* (Günther, 1986)

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Abstract

The seasonal thermal cycle and correlated variations in photoperiod exert antithetical influences on the proliferation of the reserve brain stem cells, which are mostly ependymal and subependymal, of adult earth-dwelling heterothermic vertebrates upon deprivation of an encephalic area. Also, an induced sudden, transient thermal stress preceding surgical cerebral maiming increases or depresses the proliferation of these stand-by cells, depending on the season. In particular, the concomitance of spring temperature and photoperiod with a cold stimulus increases proliferation. To re-evaluate these findings, normal adults of *Rana bergeri* were exposed to a cold shock in spring time. The outlined patterns, as revealed by immunocytochemical detection of a proliferation-linked antigen, showed that those conditions affect only the forebrain, where immunoreactivity was identifiable in quiescent cells mostly located in peculiar telencephalic ependymal sites, known as *zonae germinativae dorsales* and *ventrales*, while the regions lying behind had no substantial proliferative response. These results may be due to the absence of further proliferative stimuli (surgical stress, cerebral ablation), so that only the stand-by cells in the encephalic areas more rich in such cells are activated to proliferate. The findings are in line with the subordinate position of Anurans as compared with Urodeles, which are the most gifted with spontaneous and experimentally induced reparative and regenerative capacity among vertebrates.

Key words

Adult frog, Brain, Cold shock, PCNA spring expression

Introduction

Detailed investigations in the last forty years have evaluated the plasticity of the adult brain of sea, fresh water or earth-dwelling Anamnia, as well as of poikilothermal and homeothermic Amniota; these studies have been performed rarely in normal conditions and more frequently in experimental conditions (see review by Margotta and Morelli, 1996).

The observed plasticity depends on peculiar, post-embryonic, undifferentiated cells, which appear small and basophilic and are more numerous in young than in

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old individuals and in lower than in higher vertebrates; these cells can proliferate and give rise to neurons and glial cells (Kirsche, 1967, 1983).

These putative stem, or precursor cells have been termed by Kirsche (1967) "matrix cells" if scattered and "Matrixzonen" (now "matrix areas") if grouped. They are mostly observed in the ependymal and sub-ependymal layers of the forebrain, sometimes in the depth of other tissues belonging to the hindbrain and are endowed with latent neurogenic capacity, which becomes apparent through the generation of descendants capables of a differentiating into neuronal or glial cells (Kirsche, 1967), thus contributing to repair or regeneration processes.

Several studies on this issue have been conducted on terrestrial heterothermic vertebrates (Urodeles, Anurans, lacertilian Reptiles), some performing experiments in which surgical removal of encephalic areas was preceded by a sudden, transient drop in body temperature.

A thermal stimulus was introduced by Del Grande and Minelli (1971), Minelli and Del Grande (1974a, b) in adult *Triturus cristatus carnifex* (now better named *T. carnifex,* as ascertained by Bonifazi, 2000) to ease experiments by limiting hemorrhage and consequent high mortality of animals upon deprivation of a portion of the optic tectum, and it was found to give rise to attractive, encouraging regeneration patterns in the remaining brain.

These unexpected results were taken as starting point for further studies, mostly based on histoautoradiography and performed again in *T. cristatus carnifex* (Del Grande et al., 1982a; Minelli et al., 1987; Del Grande et al., 1990; Minelli et al., 1990; Franceschini et al., 1992) and also in *Lacerta viridis* (Minelli et al., 1978; Minelli and Del Grande, 1980; Del Grande et al., 1981) and *Rana esculenta* (Minelli et al., 1982a; Del Grande et al., 1984) subjected to ablation of telencephalic, diencephalic or mesencephalic areas; restorative results beyond hope were obtained.

The re-appearance or the amplification of reparative/regenerative processes, allowing for nervous plasticity, were due to neural-like cells, still present in the adult brain and responsibles for proliferation upon those experimental conditions or otherwise silent.

Complementary contributions on these issues were supplied for *L. viridis* (Del Grande and Minelli, 1980) and *T. cristatus carnifex* (Del Grande et al., 1982b; Minelli et al., 1987).

Minelli et al. (1982a) and Franceschini et al. (1992) turned the attention to frogs and newts, respectively, which received only a cold shock and were compared with individuals of the same species subjected to cerebral ablations.

To evaluate whether this thermal stimulus, without any encephalic ablation, could itself stimulate brain cell proliferation, we have carried immunocytochemical investigations in adult individuals of *T. carnifex* (Chimenti and Margotta, 2013) and *Podarcis sicula* (Margotta, 2014b); the latter species is now synonymous (according to Capula, 2000) of *L. viridis* (Tortonese and Lanza, 1968).

Antithetical season influences on proliferative patterns were described by Minelli et al. (1982a) in cerebral injured and uninjured, cold shocked adults of *R. esculenta*. The observations on individuals subjected only to thermal stress were carried out on a little number of samples we felt that the issue deserved further investigation. Reconsidering the literature, where most reports regard the newt, we realized that only the degree of cold shock was unequivocally determined, while there was defec-

tive, ambiguous or conflicting information about the other experimental conditions. This information totally lacks for lizards, while for frogs the season of the year when the observations were carried out was known (Minelli et al., 1982a).

We have applied here to *R. bergeri* (according to Capula, 2000), at times designed *R. esculenta* L (Tortonese and Lanza, 1968), the same experimental procedures previously applied to *T. carnifex* (Chimenti and Margotta, 2013) and *P. sicula* (Margotta, 2014), except for the seasonal timing for which we followed Minelli et al. (1982a). We have analysed, by immunohistochemistry, at first animals taken from their habitat in spring and then animals collected in autumn and have used Proliferating Cell Nuclear Antigen (PCNA; Miyachi et al., 1978) as indicator of proliferative events, since this antigen is expressed during DNA synthesis and is easy to detect in the specific experimental conditions (for further details, see Margotta et al., 2007).

Material and methods

Specimens of adult *Rana bergeri* (Günther, 1986; Capula, 2000) of both sexes were caught from their habitat near Sora (Frosinone, Latium, Italy) at the end of April and raised in captivity in an open environment (with temperature varying between 10 °C and 16 °C) until the first days of May when they were separated in two groups. The first group continued to living in open environment, while the second group was first kept at 4 °C for 24 hours (temperature reached abruptly) and then brought back to an open environment. After a week, the specimens of both 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 alcohol, 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. Two parallel sets of slides were obtained for each brain: one was stained with haematoxylin-eosin, while the other was used for immunohistochemistry as follows: the sections 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 streptavidin/ peroxidase 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 bromodeoxyuridine, was used as positive control.

Results

In the cold-shocked *Rana bergeri*, more immunoreactive cells were identifiable in the forebrain than in controls. These cells were mainly clustered in the *zonae germinativae*, less frequently they were scattered elsewhere in the ependymal epithelium and, occasionally, in the sub ependymal grey matter.

In the olfactory bulbs, each of which supplied with a wide cavity, in specimens subjected to cold shock scanty labelled cells were seen in the ependymal epithelium and at times in the sub-ependymal layer (Fig. 1a), while in control frogs stained cells were rarely present in the ependyma or in the periventricular grey matter (Fig. 1b).

In the telencephalon, where each hemisphere was provided with a falciform cavity, a few PCNA labelled cells were found in the ventricular wall of cold-stressed indi-

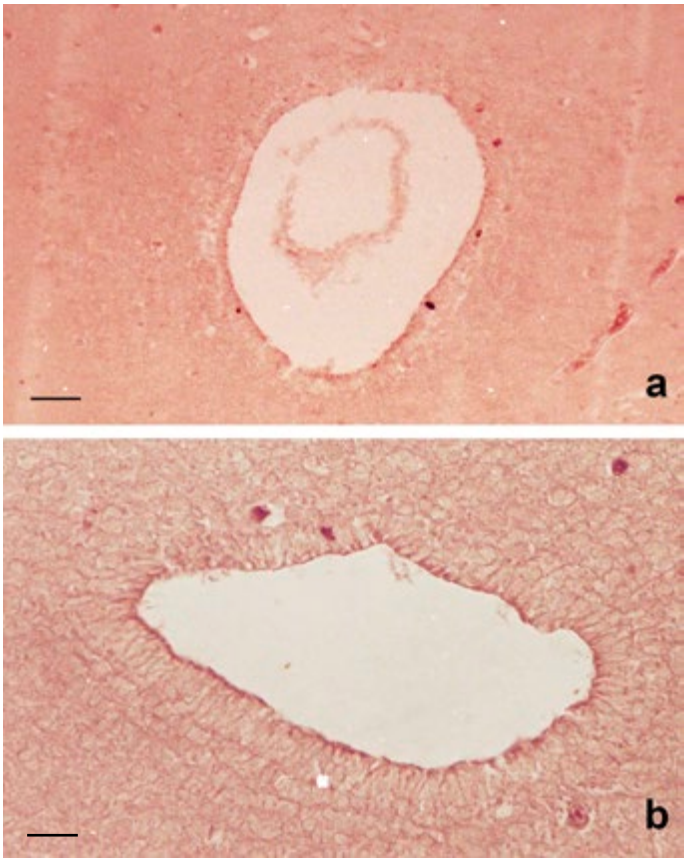

Figure 1 – Transverse sections of olfactory bulbs of adult *Rana bergeri*. a) Specimen subjected to cold shock. Some scattered PCNA-positive cells are visible in the ependyma. b) Control specimen. Scanty PCNA-positive cells are present in the sub-ependymal grey matter. In both instances the ependyma also shows a weak, diffuse, apical immunoreactivity. PCNA immunoytochemistry without nuclear counterstaining. Bar = 100 μm for (a) and 50 μm for (b).

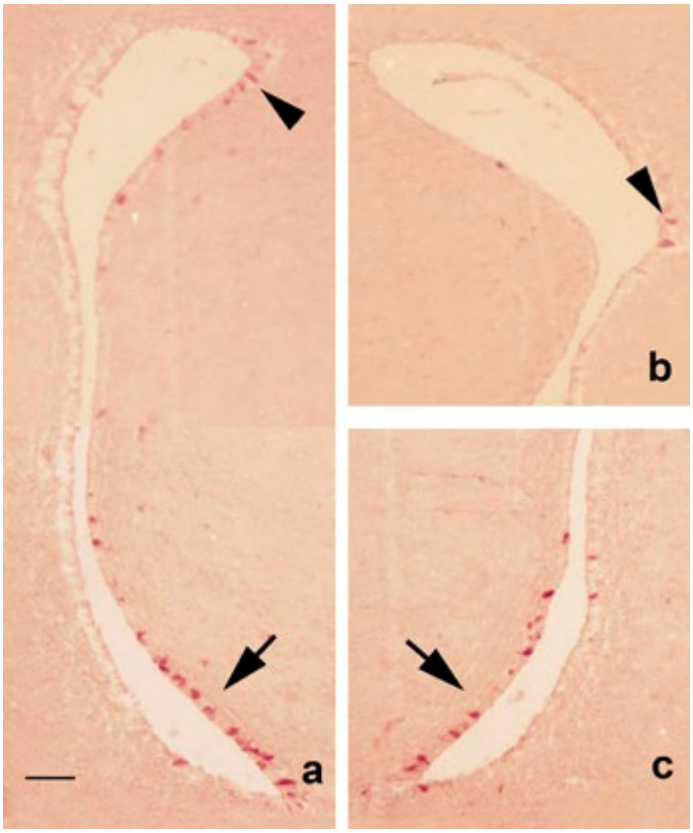

Figure 2 – Transverse sections of telencephalic hemispheres of adult *Rana bergeri*. a) Specimen subjected to cold shock. Ependymal PCNA labelled cells appear scattered or in clusters forming the *zonae germinativae dorsales* (arrowhead) and *ventrales* (arrow). b and c) Control specimen. Few scattered ependymal PCNA-positive cells are visible in the *zonae germinativae dorsales* (arrowhead) and *ventrales* (arrow). PCNA immunocytochemistry without nuclear counterstaining. Bar = 100 µm.

viduals, for the most part in the medial ventricular wall near the upper and lower corners (Fig. 2a). The position of these cells corresponds to the *zonae germinativae dorsales* and *zonae germinativae ventrales*, respectively. Immunoreactivity was rarely found in the surrounding grey matter. In control frogs rare stained cells were seen in dorsal part of the ventricle lining, corresponding to the *zonae germinativae dorsales* (Fig. 2b), while few immunoreactive cells were identifiable in the *zonae germinativae ventrales* (Fig. 2c). Sometimes a widespread weak immunoreaction was observed in the epithelium lining this cavity or in the grey matter.

In the diencephalon, which contains a sagittal, narrow III ventricle, few labelled cells were present in the ependyma, occasionally also in the sub-ependymal layer and near the pre-optic (Fig. 3a) and infundibular recesses of specimens subjected to cold shock, similar to control frogs (Fig. 3b).

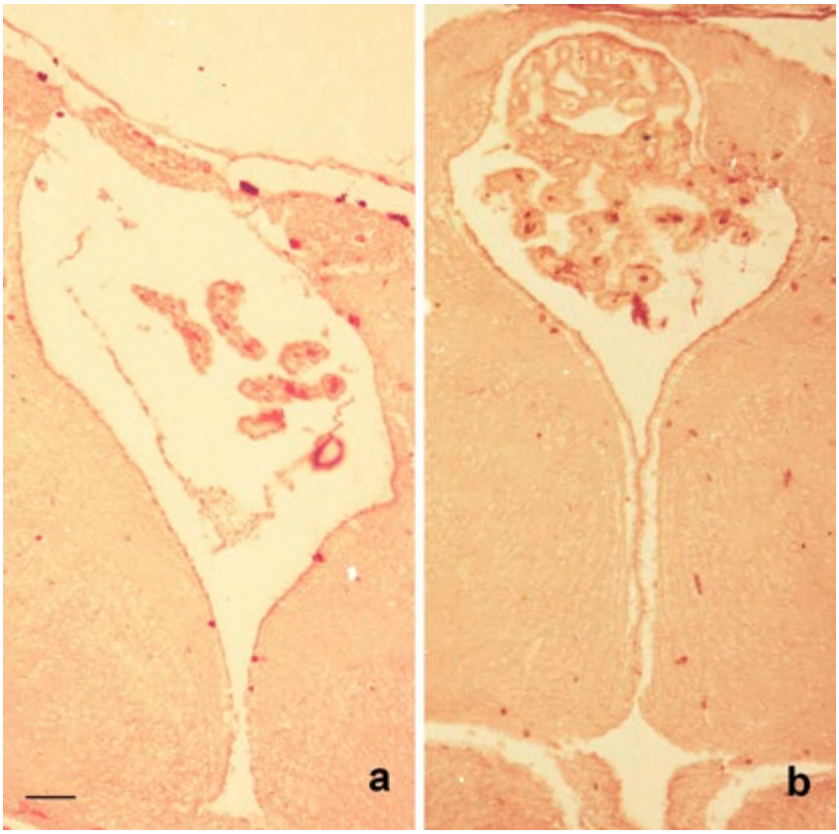

Figure 3 – Transverse sections of diencephalon of adult *Rana bergeri*. a) Specimen subjected to cold shock. Few PCNA-positive cells are present in the ependyma. b) Control specimen. Few labelled cells appear in the sub-ependymal grey matter. In both instances the ependyma also shows a weak, diffuse, apical immunoreactivity. PCNA immunoytochemistry without nuclear counterstaining. Bar = 100 µm.

In the mesencephalon of cold-shocked individuals, PCNA expressions were visible occasionally in the deepest layers of the optic lobes, while no immunoreactivity was found in normal frogs.

PCNA expression was not seen in the *cerebellum* and *medulla oblongata* of treated and of control animals as well.

Discussion

In various adult heterothermic vertebrates the seasonal thermal cycle and the correlated variations in photoperiod can exert at different times a positive or an unfavourable influence on the proliferation of stem cells.

As better specified in previous papers (Margotta, 2012; Chimenti and Margotta, 2013; Margotta, 2014a, b), the above mentioned influence can be observed in the brain

of Anamniota, not only terricolous (Rothstein et al., 1975; Minelli et al., 1982a; Bernocchi et al., 1990; Chetverukhin and Polenov, 1993; Polenov and Chetverukhin, 1993; Chieffi Baccari et al., 1994; Dawley et al., 2000), but also living in fresh or sea water (Velasco et al., 2001; Vidal Pizarro et al., 2004), as well as of Amniota (Ramirez et al., 1997; Margotta et al., 2005).

Similar effects have been described for cell populations of other organs (Rothstein et al., 1975; Velasco et al., 2001) or tissues (Dawley et al., 2000).

Such relationships between annual temperature and daytime cycle and proliferative events is supported by recent immunohistochemical observations into adulthood. In fact, the autumnal conditions provokes a widespread inhibition of encephalic cell proliferation in *R. bergeri* (Margotta, 2012) while the summer environment stimulates these activity in the anterior brain portions of *P. sicula* (Margotta, 2014a).

Also an artificially applied temperature change can stimulate the proliferation of the neural-like cells still present in adult brain and potentiate the regeneration upon cerebral injury as observed in *T. cristatus carnifex* (Del Grande and Minelli, 1971; Minelli and Del Grande, 1974a, b; Del Grande et al., 1982a, 1990; Minelli et al., 1987, 1990; Franceschini et al., 1992), *L. viridis* (Minelli et al., 1978; Minelli and Del Grande, 1980; Del Grande et al., 1981) and *R. esculenta* (Minelli et al., 1982a; Del Grande et al., 1984).

Del Grande et al. (1982a) and Minelli et al. (1982b), upon reconsidering the studies of other authors, hypothesized that the obtained results were due to modifications in the hemato-encephalic barrier and therefore in the cerebral metabolism; more details on this subject can be found in Chimenti and Margotta (2013).

We have addressed the issue whether a cold stimulus can stimulate proliferation in not injured brain of adult terrestrial heterothermic vertebrates. Immunohistochemical analyses on *T. carnifex* (Chimenti and Margotta, 2013) and *P. sicula* (Margotta, 2014b) have indicated that the response seems circumscribed to the olfactory district and to the telencephalic matrix areas.

We have then focused on adult *R. bergeri*, following the seminal but scanty observations by Minelli et al. (1982a) on adult *R. esculenta* collected from the wild in three different periods of the year. These authors observed an influence of the temperature on the encephalic thymidine uptake expressive of proliferative events both in brain injured and normal specimens; the influence appeared both in relation to the seasonal cycle and to experimental thermal stimulation. In detail, in normal specimens thymidine uptake by brain putative stem cells appeared weak in spring, strong in autumn and waning again in proximity of winter. Upon artificial cold stress, on the contrary, thymidine uptake increased above control values in spring and decreased in autumn.

The present study on cold exposed *R. bergeri* taken from their habitat in spring has allowed to record PCNA immunoreactivity in the olfactory district and in general in the telencephalon; in the hemispheres the position of immunolabelled cells was in agreement with that identified by Kirsche (1967) at autoradiography, *i.e.* the *zonae germinativae dorsales* and *ventrales*. Similar but tiny signs were present in the diencephalon, while the remaining portions of the encephalon not show substantial labelling. Single stained cells or weak widespread labelling were rarely observed in control individuals. Therefore it may be proposed that a sudden, transient thermal stimulus interacts with the baseline proliferative activity regulated by the spring temperature and photoperiod leading to a higher tendency to proliferate.

The naturally waning proliferation previously seen in individuals captured in proximity of the winter (Margotta. 2012) and the present findings in normal and thermal-shocked specimens caught in May confirm what had been referred by Minelli et al. (1982a) on the basis of a small number samples: the spontaneous proliferation rate in spring is weak but is emphasized by the administration of a cold shock.

However it appears highly improbable that the amount of immunohistochemically detectable proliferation observed here can sustain the regeneration boosted by Minelli et al. (1982a) and Del Grande et al. (1984) in adult cold-stressed and brain-injured individuals of *R. esculenta*. The proliferative answer of *R. bergeri* exposed to a cold stimulus in the absence of mechanical injury appears rather low and restricted to the only encephalic areas provided with a potential stock of silent cells, like in other terricolous heterothermic vertebrates (Chimenti and Margotta, 2013; Margotta, 2014b). In this respect in adult condition Anura, for regenerative capacity occupy a less favourable position than Teleosts and Urodeles, the most gifted vertebrates for tissue reparation and regeneration.

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