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

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Abstract

It has been shown previously that in adult *Rana esculenta*, caught in nature, cold-shocked and brain-injured, encephalic cell proliferation is increased when capture and experiment occurred in spring and depressed when they occurred in autumn. Upon exclusive thermal stress cell proliferation appeared weak in spring and intense in autumn. The present study has investigated cold-shocked, but otherwise uninjured *Rana bergeri* to assess the impact of autumnal environment on encephalic cell proliferation. Lowering temperature - natural or experimental - seemed to exert a mild stimulation on the proliferative activity only in the forebrain. These results complete those previously obtained in spring and appear in substantial agreement with past reports about antithetical interactions between natural (season climate–photoperiod) and experimental (cold stimulus) environmental factors. However, the present results do not seem sufficient to explain the regenerative events described by past authors. A possible explanation of this discrepancy might be that if the spread between the autumnal environmental conditions and the entity of the cold shock is small the latter would be less effective. Alternatively, cold shock may need to be accompanied by further stimuli, such as surgical trauma (partial resection of brain tissue), to achieve extended stimulation and in the absence of those stimuli it would promote cell proliferation only in the forebrain, which is the region best provided with stand-by putative stem cells.

Key words

Adult frog, Brain, Cold shock, PCNA

Introduction

A great amount of studies has been carried out on the regenerative events in the brain of adult terrestrial heterothermic vertebrates, frequently in experimental, rarely in normal conditions. A handful of researches has investigated if the seasonal thermal variations, also correlated with photoperiod, and/or an experimentally applied low temperature, sudden and transient, could exert an impact on putative stem or precursor cells thus unmasking their proliferative potential, otherwise hidden, giving rise to

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possible reparative or even regenerative phenomena, either upon surgery or spontaneous (see review by Margotta and Morelli, 1996).

Such investigations were conducted by different techniques, most by histo-autoradiography, seldom by electron microscopy or immunohistochemistry.

Stem/precursor cells, which live in a stand-by condition, are small and basophilic, clustered and sometimes stratified, and identifiable in circumscribed areas of the ependymal layer ("Matrixzonen", actually "matrix areas") of the telencephalon or scattered in the depth of other cerebral sites ("matrix cells").

"Matrix areas" and "matrix cells" may be considered vestiges of those embryonic territories where neural cells proliferated during morphogenesis of nervous central system; the stock of these neuroblasts decreases from early to advanced embryonic stages, then to post-embryonic and further down with increasing age of the organism (Kahle, 1951; Fujita, 1963; Kirsche, 1967).

The proliferative potential and the reparative and even regenerative power can vary among the various animal groups; generally speaking they are much greater in lower than in higher vertebrates.

Originally, the artificial temporary cold treatment was a pre-surgical device adopted by Del Grande and Minelli (1971) Minelli and Del Grande (1974a, b) in adult *Triturus cristatus carnifex* in order to limit cardiac activity, thus reducing post-operatory haemorrhage and therefore the high mortality of animals subjected to ablation of an encephalic area. In concomitance with the introduction of such procedure unexpected regenerative results were reached.

Owing to these tempting results, the suspicion was put forwards that a cold shock might be linked to a cell proliferative response due to some morpho-functional distortions of the haemato-encephalic barrier, as proposed by Del Grande et al. (1982b), which re-read and reconsidered a hypothesis expressed in the past (Rosomoff and Gilbert, 1955; Stone et al., 1956; Lougheed et al., 1960; Kienan, 1979; Kienan and Contestabile, 1980) on the mechanisms activated by a thermal stimulus (for wider details: Chimenti and Margotta, 2013; Margotta, 2014b).

This hypothesis, based on the effect of such contrivance, gave rise to further investigations, which have regarded, in adult condition, mainly Amphibians, in lower amount lacertilian Reptiles after ablation of various encephalic areas. In particular, *Lacerta viridis* (Minelli et al., 1978; Del Grande et al., 1981), *Rana esculenta* (Minelli et al., 1982a; Del Grande et al., 1984), *T. cristatus carnifex* (Del Grande et al., 1982a, b; Minelli et al., 1987; 1990; Del Grande et al., 1990; Franceschini et al., 1992) and these three species together (Minelli et al., 1982b) were studied. More recently immunohistochemical investigations on adult normal individuals have re-examined the influence of seasonal temperature - linked to the corresponding photoperiod - on the sleeping cells still present in the brain of *R. bergeri* (Margotta, 2012) and *Podarcis sicula* (Margotta, 2014a), and that of seasonal climate-photoperiod associated to sudden, temporary, drastic cooling in the otherwise uninjured brain of *T. carnifex* (Chimenti and Margotta, 2013), *P. sicula* (Margotta, 2014b), *R.. bergeri* (Chimenti and Margotta, 2015), formerly *T. cristatus carnifex* (Tortonese and Lanza, 1968; Giacoma and Balletto, 1988), and *L. viridis* (Tortonese and Lanza, 1968).

Minelli et al. (1982a) in an autoradiographic study on thermally stressed adult *R. esculenta*, either subjected to surgical brain injury or not, found that in both surgically injured and uninjured animals thymidine uptake by brain putative stem cells

appeared weak in spring, strong in autumn and waning again in proximity of winter. Such natural environmental factors interacted with an applied cold shock so that upon that shock thymidine uptake increased in spring and decreased in autumn, contrary to spontaneous seasonal variations. Since this study included only scanty observations on not surgically injured animals, it seemed worthy to be expanded, which was done on normal and to hypothermic stress-exposed, adult *R. bergeri*, formerly *R. esculenta* (Tortonese and Lanza, 1968), captured in spring (Chimenti and Margotta, 2014) and in autumn. The experimental procedures have been the same previously adopted for studies on *T. carnifex* (Chimenti and Margotta, 2013), *P. sicula* (Margotta, 2014b) and *R. bergeri* (Chimenti and Margotta, 2015).

In the present evaluation the immunohistochemical detection of proliferating cell nuclear antigen (PCNA; Miyachi et al., 1978) has been used as a proxy of cell proliferation. This antigen is expressed during DNA synthesis and had formerly proved to be reliable for that purpose (for further information see: Margotta, 2012; Chimenti and Margotta, 2013; Margotta, 2014a, b; Chimenti and Margotta, 2014).

Material and methods

Mature specimens of *Rana bergeri* (Günther, 1986) - as ascertained by Capula (2000) - of both sexes were captured in their habitat near Sora (Frosinone, Latium, Italy) at the end of October, with environmental temperature varying between 8 to 18 °C, and divided into two groups. The first group continued to live in open environment, while the second group was first kept at 4 °C for 24 hours (temperature reached abruptly) and then were brought back to an open environment. After a week, which had appeared as the time with maximal proliferation in similar experiments on adult newt brain (Franceschini et al., 1992), the animals 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 and the other was used for immunohistochemistry.

Upon removal of paraffin and hydratation, 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 com-

plex (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).

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

Results

The olfactory bulbs showed PCNA-positivity in cells scattered among those delimiting the roundish bulb cavity and in the peri-ependymal and internal granular layers (Fig. 1a). Labelled cells were more frequent in animals not subjected to cold shock (Fig.1b).

Each telencephalic hemisphere, provided with a falciform-shape cavity, may be divided in an anterior, an intermediate, a posterior and a caudal portion. Proceeding antero-posteriorly, scanty labelling was at first observed in clustered cells placed in the outer corners of the ventricular roof, then at the ventricular bottom and in the adjacent walls. Some reactivity was visible at the caudal poles, where a relatively small number of immunostained cells was seen in the ependymal layer both dorsally and ventrally. Some PCNA expression could be found in the sub-ependymal layer. Generally, such positive cells were more frequent at the ventral than dorsal levels (Fig. 2a). The immunolabelling was more evident in the animals not subjected to cold shock (Fig. 2b).

The stem cell rich areas corresponded to the sites of the *zonae germinativae dorsales* and *ventrales*, initially identified by Kirsche (1967).

The diencephalon, containing the III narrow ventricle extended in dorso-ventral direction, showed mild immunoreactivity in the habenular ganglia and in the hypothalamus, especially in the ependyma of the infundibular and preoptic recesses (Fig. 3a). More labelled cells were seen in normal specimens (Fig. 3b).

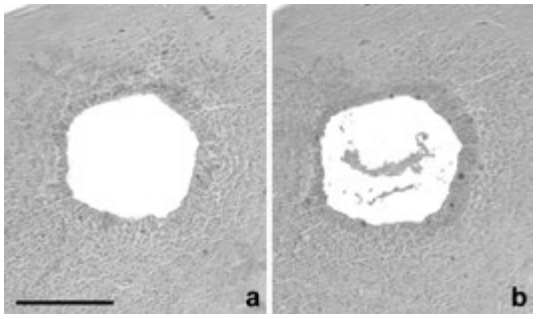

Fig. 1 – Transverse sections of olfactory bulbs of adult *Rana bergeri*. a) Specimen subjected to cold shock. Scattered PCNA-positivity is visible among the cells delimiting the round, wide cavity and those in the peri-ependymal and internal granular layers. b) Control specimen. More immunolabelled cells appear in the same areas. PCNA immunocytochemistry without nuclear counterstain. Calibration bar = 200 µm.

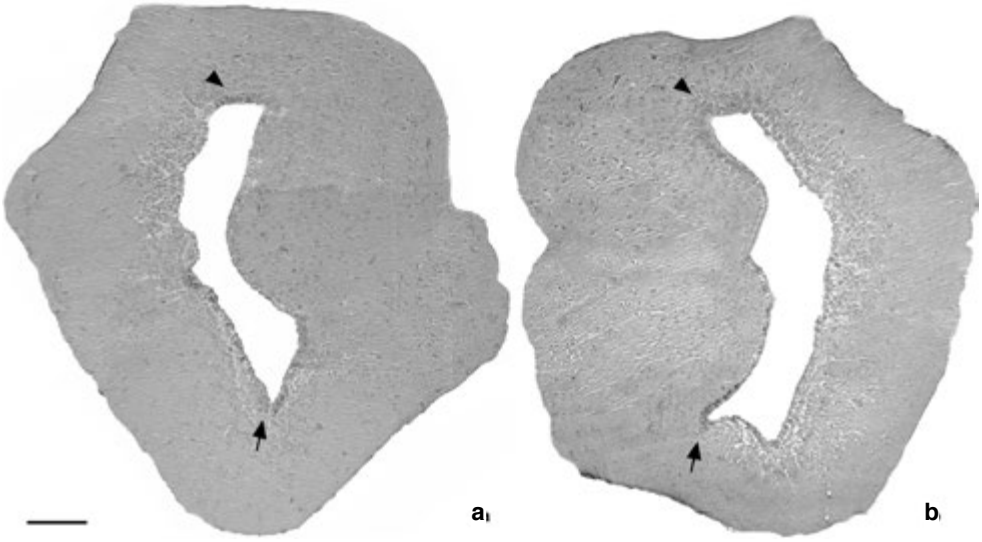

Fig. 2 – Transverse sections of telencephalic hemispheres of adult *Rana bergeri*. a) Specimen subjected to cold shock. Immunolabelled cells are identifiable where *zonae germinativae dorsales* (arrowheads) and *ventrales* (arrows) are known to be located. Some PCNA positivity is visible in the sub-ependymal layer. b) Control specimen. The immunocytochemical labelling in the same sites is more intense. PCNA immunocytochemistry without nuclear counterstain. Calibration bar = 200 μm.

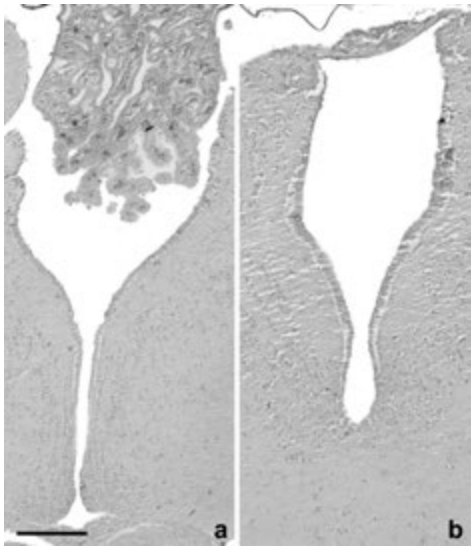

Fig. 3 – Transverse sections of diencephalon of adult *Rana bergeri*. a) Specimen subjected to cold shock. No substantial PCNA-positivity appears in the epithalamic habenular ganglia, in the epithelium of the III ventricle, nor in the hypothalamic infundibular and pre-optic recesses. b) Control specimen. Positive PCNA labelling appears in the those same areas. PCNA immunocytochemistry without nuclear counterstain. Calibration bar = 200 μm.

The mesencephalon and *cerebellum* did not express signs of proliferation both in individuals subjected to hypothermic stress and in not subjected ones.

Scarce, scattered stained cells were found in the walls of the rhombencephalic ventricle only of the animals not exposed to cold shock.

Discussion

By comparison with previous investigations in adult frogs (Minelli et al., 1982a; Chimenti and Margotta, 2015), the actual results suggest that a cold shock exerts a mild effect on the encephalic proliferative activity of animals treated upon catching in autumn, in which the immunoreaction appeared less pronounced and circumscribed to the forebrain. In the midbrain and hindbrain the signs of proliferation were scanty both in cold shocked and in not shocked specimens.

On the contrary, Chimenti and Margotta (2015) had found that the signs of proliferation in the forebrain of specimens caught in their habitat in spring and then exposed to cold (without any surgical injury) were increased over controls.

The scenario emerging from the present and the previous our investigations (Chimenti and Margotta, 2015) appears in agreement with what had been referred by Minelli et al. (1982a) on the basis of different analytical methods: they had observed that the interaction of the natural (spring or autumnal) environmental conditions and cold shock increased encephalic cell proliferation in spring and depressed it in autumn. Also the differences in the signs of proliferation between spring (Chimenti and Margotta, 2015), autumn and un-shocked specimens appear coherent with the experiments of Minelli et al. (1982a), despite the different methods used to evaluate proliferating cells.

It had already been shown that the spontaneous signs of proliferation waned moving from autumn to winter (Minelli et al., 1982a), except in the forebrain. Also in analogous researches on the influence of seasonal environment on normal *R. bergeri* (Margotta, 2012), *P. sicula* (Margotta, 2014a) and on cold exposed - without any other injury - *T. carnifex* (Chimenti and Margotta, 2013) and *P. sicula* (Margotta, 2014b), signs of proliferation had been observed only in the forebrain. It seems reasonable to conclude that, if proliferative activity is moderate, it is restricted to the forebrain (Margotta, 2012, Chimenti and Margotta, 2013; Margotta, 2014a, b; Chimenti and Margotta, 2015).

The present findings also seem to support the hypothesis that if the spread between seasonal climate-photoperiod and the entity of induced cooling is less marked, the shock to cells in stand-by is also less pronounced. The opposite may occur when such spread is wider, as indicated by the observations carried out in spring by Minelli et al. (1982a) and Chimenti and Margotta (2015).

It appears highly improbable that the proliferation shown by immunohistochemistry here as well as in previous investigations (Margotta, 2012; Chimenti and Margotta, 2013; Margotta, 2014a, b; Chimenti and Margotta, 2015) is enough to sustain restoring or regenerative processes; however it is conceivable that it may increase further in response to brain surgery.

At last, it is worth remarking that the telencephalic *zonae germinativae dorsales* and *ventrales*, which are the areas best endowed with post-embryonic putative stem cells (Kirsche, 1967), best respond to thermal fluctuations both in physiological and

experimental conditions. It should however be kept in mind that the reparative and regenerative processes may reach different intensity depending on various vertebrate systematic groups, since among heterothermic vertebrates they are progressively less efficient from Urodela to Anura to lacertian Reptiles (Kirsche, 1983).

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