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# **Proliferative events experimentally induced by a transient cold shock in the brain of adult terrestrial heterothermic vertebrates: preliminary analysis of PCNA expression in *Podarcis sicula***

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## Abstract

In past studies on the encephalic regenerative phenomena some authors adopted a pre-surgical stratagem (drastic, sudden, transient thermal stimulus) to adult brain-injured newts to limit death rate upon surgery and with this method, unexpected tissue reparation was obtained. This procedure became a routine technicality also in frog and lizard to stimulate an increase in the neurogenesis, attributable to putative stem cells which appear either in clusters ("matrix areas" or "matrix zones"), mostly at or near the telencephalic ventricular surfaces, or scattered ("matrix cells") within other cerebral districts. On the basis of this literature background, planning an immunocytochemical re-evaluation of the survival of latent proliferative properties in these adult cold shocked organisms, as already studied in *Triturus carnifex*, the actual investigation was carried out on the brain of *Podarcis sicula* not subjected to cerebral injury. The immunohistochemical expression of the proliferating cell nuclear antigen (PCNA) seemed moderate only in those encephalic portions better provided with cells in stand-by: the olfactory peduncles and the telencephalic *zonae germinativae ventrales*. This scenario appeared rather disappointed and inadequate with respect to the unexpected, widespread restoration of the removed portions observed by previous authors. These findings could be due both to a small effectiveness of a relatively mild thermal stress, depending on the difference between the applied temperature and seasonal one, to the lack of other experimental conditions (surgical trauma, encephalic injury) adopted in the past, and to the interspecific differences in sensitivity, since lacertilian Reptiles are less endowed with proliferative/regenerative power than Amphibians, mainly Urodela, and Teleosts.

## Key words

Adult lizard, brain, cold shock, PCNA expression.

## Introduction

Since several decades it has been demonstrated the survival in the ependyma and periventricular grey matter of the forebrain, sometimes also deeper in few hindbrain portions, of peculiar basophil, small stem cells, vestiges of the germinative layers during morphogenesis.

These quiescent cells, more numerous in young than in old individuals and in lower than in higher vertebrates, appear grouped in circumscribed telencephalic

areas (“matrix zones”, now “matrix areas”) and scattered in the remaining districts (“matrix cells”; Kirsche 1967, 1983). They can undergo a neuronal or glial differentiation and allow for natural reparative phenomena or experimental regenerative events (for review see Margotta and Morelli, 1996).

Del Grande and Minelli (1971), Minelli and Del Grande (1974a, b) first applied a sudden, transient hypothermic stress to adult Urodele Amphibian *Triturus cristatus carnifex* before the surgical ablation of a cerebral area, aimed at limiting cardiac activity thus reducing post-operative massive haemorrhage and the consequent high experimental mortality. With this method, unexpected tissue reparation was obtained.

Cold stress before surgery should be recalled among the experimental procedures useful to emphasize in adulthood the neurogenic potentialities and then evaluate the brain plasticity.

The above mentioned studies involved terrestrial heterothermic vertebrates surgically deprived of encephalic areas, especially Amphibia, mainly Urodela and in lesser amount Anura; only a small number of investigations regarded lacertilian Reptiles.

For the latter Amniota must be reminded only histoautoradiographic studies carried out by Minelli et al. (1978), Minelli and Del Grande (1980), Del Grande et al. (1981) in *Lacerta viridis* and by Minelli et al. (1982b) in the meantime in the same *L. viridis*, *T. cristatus carnifex* and *Rana esculenta*.

On the basis of this bibliographic background, while attempting an immunocytochemical re-appraisal of the influence of cold stress on the latent proliferative capacity of these organisms in adulthood, in the absence of mechanical cerebral damage and following investigations in *T. carnifex* (Chimenti and Margotta, 2013), now synonymous (Bonifazi, 2000) of *T. cristatus carnifex* (Giacoma and Balletto, 1988) the present investigation has addressed this issue in *Podarcis sicula* (Capula, 2000), at times designed *L. viridis* Rafinesque (Tortonese and Lanza, 1968).

To allow comparison with previous studies upon cold stress associated to surgical trauma, detailed information on previous studies should be available. Which happens for the newt (Del Grande and Minelli, 1971; Minelli and Del Grande, 1974a, b; Del Grande et al., 1982, 1990; Minelli et al., 1987, 1990; Franceschini et al., 1992), the lizard (Minelli et al., 1978, 1982b; Minelli and Del Grande, 1980; Del Grande et al., 1981) and the frog (Minelli et al., 1982a; Del Grande et al., 1984).

These news, while univocal about the degree of the cold stimulus, appear conflicting, defective or ambiguous about the remaining parameters (how cold temperature was reached, if rapidly or gradually, and how was it protracted in time, at which temperature the specimens were housed after cold shock, the length of their stalling after the thermal treatment, how long was the interval between the end of hypothermia and sacrifice). Among this information which can be found in literature, the most part regards *T. cristatus carnifex* and specimens deprived of encephalic areas.

In the present investigation on adult *P. sicula* submitted to sudden, transient cold shock, I have chosen to adopt the procedure put into effect previously in the newt and already followed in a recent study on cold-shocked *T. carnifex* (Chimenti and Margotta, 2012).

For the present observations I have used the immunolabelling of proliferating cell nuclear antigen (PCNA; Miyachi et al., 1978), which had proved valuable in our former investigations. PCNA is a member of the cyclin family and an auxiliary protein of DNA polymerase  $\delta$  closely associated with sites of DNA replication. It reaches

appreciable levels when DNA is synthesized in the cell cycle, thus revealing cells in S phase (Bravo and Macdonald-Bravo, 1985, 1987; Bravo et al., 1987; Jaskulski et al., 1988; Liu et al., 1989; Fairman, 1990; Diffley, 1992). Therefore, PCNA is an indirect marker which reveals cell proliferation through DNA synthesis.

## Material and methods

Sexually mature specimens of *Podarcis sicula* of both sexes were taken from their habitat near Rome at the end of April. In this species such period corresponds to the breeding season (Capula, 2000), the external temperature may vary between 12 °C and 19 °C.

The samples were separated into two groups. The specimens of the first group continued to live in this environment, while those of the second group were kept at 4 °C (temperature reached abruptly) for 24 hours, after which they were returned outside. After a week, the lizards of both groups were sacrificed under anaesthesia with tricainemethanesulfonate (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. 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.

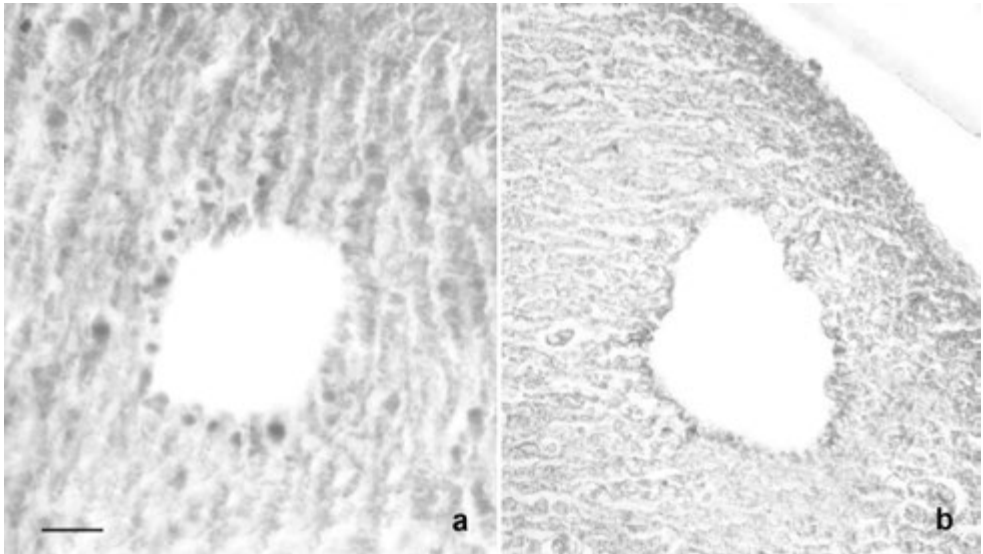
Upon removal of paraffin and hydration, the sections were rinsed in isotonic, 0.01 mol/litre phosphate buffered saline, pH 7.4 (PBS), incubated in 3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min to block endogenous peroxidase, washed in PBS, incubated in 20% normal horse serum to block unspecific 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 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<sub>4</sub> 1% and 0.017 H<sub>2</sub>O<sub>2</sub> 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 peduncles, in the proximity of the telencephalic hemispheres, in the cold-shocked specimens showed labelled cells in the ependymal epithelium and in the periependymal grey matter (Fig. 1a), while in the normal individuals the immunoreactive cells appeared less numerous in such tissues (Fig. 1b).



**Figure 1** – In the olfactory peduncles of adult *Podarcis sicula*, PCNA expression is seen in the ependymal epithelium, periventricular grey matter and internal granular layer: a) specimen subjected to cold shock; b) normal specimen. Transverse sections, PCNA immunocytochemistry without nuclear counterstaining. Calibration bars = 20  $\mu$ m.

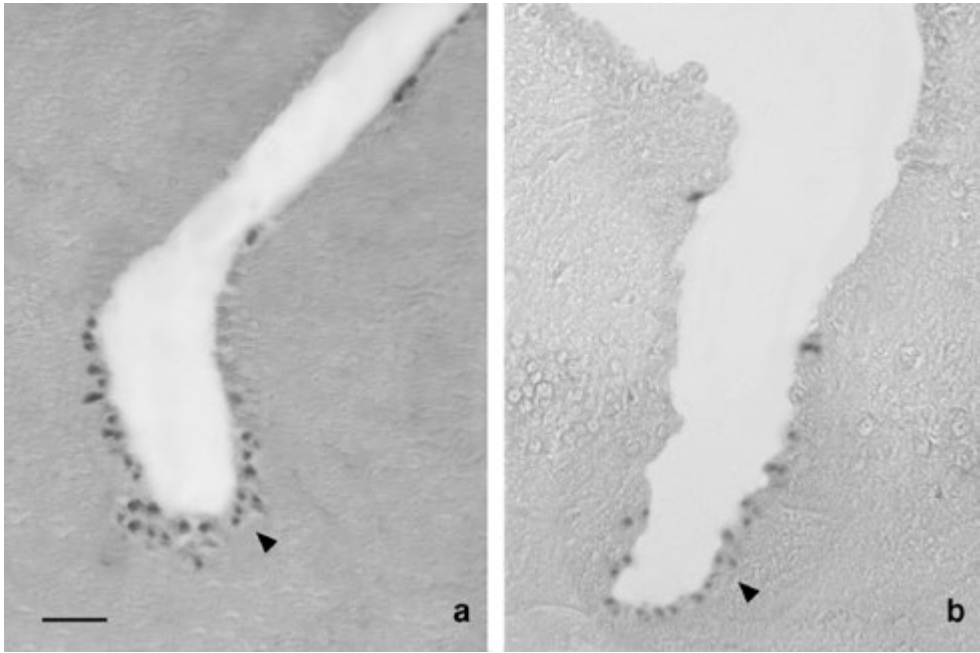
In the telencephalon of the specimens submitted to a thermal stimulus, PCNA positive isolated cells were observed in the ependymal and in the sub-ependymal layers, anteriorly in the outer corners and posteriorly in the inner edge of the hemispheric ventricular roof where formed, respectively, the *laterales* and *mediales* components of the *zonae germinativae dorsales*. Besides, in the ventral ependymal epithelium and periventricular layer of each hemisphere, at the same levels of the *pars lateralis* of the *zona germinativa dorsalis*, several groups of immunoreactive cells formed a *zona germinativa ventralis* (Fig. 2a). Outside these areas, scanty immunostaining was present in the ependyma. In the normal individuals, among the three components of the *zonae germinativae* only the *ventrales* ones (Fig. 2b) showed a small number of labelled cells.

In the diencephalon of both specimens subjected to a cold shock and normal individuals no difference in immunolabelling was observed in the habenular ganglia, nor in the ependymal cells and in the periventricular grey matter of the epithalamic, thalamic and hypothalamic (preoptic and infundibular recesses) regions.

Also in the districts lying behind (midbrain, *cerebellum*, *medulla oblongata*), the immunoreactive patterns appeared quite moderate both in thermal-shocked and in normal lizards.

## Discussion

Past studies on adult terrestrial heterothermic vertebrates focused on both an artificially applied cold stimulus and the cyclic, seasonal thermal rhythm, correlated with



**Figure 2** – In the telencephalon of adult *Podarcis sicula*, immunolabelled cells are visible in the *zonae germinativae ventrales* (arrowheads), in the ependyma and sub-ependymal grey matter (arrows): a) specimen subjected to cold shock; b) normal specimen. Transverse sections, PCNA immunocytochemistry without nuclear counterstaining. Calibration bars = 20  $\mu$ m.

variations in photoperiod; these studies gave evidence that these events can induce in the brain experimental or natural fluctuations in cell proliferation and even reparative or even regenerative phenomena, due to increased neuronal or glial differentiation of the stem cells which populate the matrix areas or are scattered as individual matrix cells.

The lacertilian Reptiles *L. viridis* (Minelli et al., 1978, 1982b; Minelli and Del Grande, 1980; Del Grande et al., 1981) and *P. hispanica* (Ramirez et al., 1997), the Urodele Amphibian *T. cristatus carnifex* (Del Grande and Minelli, 1971; Minelli and Del Grande, 1974a, b; Del Grande et al., 1982; Minelli et al., 1982b; Franceschini et al., 1992), the Anuran Amphibians *R. esculenta* (Minelli et al., 1982a, b) and *R. temporaria* (Chetverukhin and Polenov, 1993) were involved in the experiments about the influence of an induced thermal shock.

The differential influence of temperature and/or photoperiod was investigated in various organs (like the central nervous system and the eye) and tissues (like the chemosensory epithelium) (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; Ramirez et al., 1997; Dawley et al., 2000; Velasco et al., 2001; Vidal Pizarro et al., 2004; Margotta and Caronti, 2005; Margotta et al., 2005a; Margotta, 2012a, b) belonging to Anamnia (living in sea and fresh water or terricolous) and to heterothermic and homeothermic Amniota.

In these organisms a hypothesis on the mechanisms mediating the influence of the low natural temperature or experimentally applied has been advanced in terms of a distortion of the blood-brain barrier (Rosomoff and Gilbert, 1955; Stone et al., 1956; Loughheed et al., 1960; Kienan, 1979; Kienan and Contestabile, 1980), which has been reconsidered by Del Grande et al. (1982) (for details see: Chimenti and Margotta, 2012).

The presence and distribution of such cells, which are in stand-by, in the normal brain adult of lacertilian Reptiles, have been studied by classical or autoradiographic histological methods and Kirsche (1967) in *L. agilis agilis* was the first to describe clusters of undifferentiated cells in the dorsal and ventral ventricular walls of the telencephalic hemispheres, which he named *zonae germinativae dorsales* and *ventrales*; this finding has been confirmed for the same lizard by Schulz (1969).

Later two components, *lateralis* and *medialis*, were identified in each *zona germinativa dorsalis* of *L. viridis*, (Minelli and Del Grande, 1980).

Furthermore, other neural-like cells were found in *L. agilis agilis*, scattered in various diencephalic areas (Kirsche, 1967), similar to what had been described for *Testudo graeca* (Fleischhauer, 1957).

Moreover a few, scattered cells of this nature were identified in the innermost layers of the mesencephalic optic lobes of *L. viridis* (Del Grande et al., 1981).

Postnatal neurogenesis was discovered in *L. galloti* also in the olfactory peduncles (Garcia-Verdugo et al., 1989).

Regarding the identification of the location of matrix areas or matrix cells, the actual findings were in agreement with what had been shown by Kirsche (1967), Del Grande and Minelli (1980), Del Grande et al. (1981). Garcia-Verdugo et al. (1989) and also with the data obtained by Del Grande and Minelli (1980), Del Grande et al. (1981) in lizards deprived of an encephalic area.

Contrary to expectation, in spite of the thermal shock the present immunocytochemical results, qualitative evaluated, do not show widespread proliferative activity in the forebrain, where such activity was only moderate in those territories best provided with cells in stand-by: the olfactory portions and the telencephalic *zonae germinativae ventrales*,

It is highly improbable that the entity of the proliferative events observed here may explain the restoring patterns obtained by Minelli et al. (1978, 1982b), Minelli and Del Grande (1980), Del Grande et al. (1981) in adult, brain-injured, cold-stressed *L. viridis* and by Ramirez et al. (1997) in adult *P. hispanica* taken from the wild under warm temperature–long photoperiod conditions.

For an evaluation of the actual observations there are, as reference, the previous immunocytochemical our own remarks about the adult brain of normal *P. sicula* caught from the wild in late spring (Margotta et al., 1999, 2005b) and in summer (Margotta, 2012b), and in cold-shocked *T. carnifex*, captured in their habitat at the beginning of spring (Chimenti and Margotta, 2013). Indeed, we saw proliferative signs of a certain amount only in the forebrain: in the olfactory peduncles and in the *zonae germinativae, dorsales (laterales and mediales)* and *ventrales* (Margotta et al., 1999, 2005b) of the telencephalon, the only portions of the brain supplied with a certain number of cells in stand-by.

Similar conclusions have been reached on the basis of an observation (inspired from a research in adult *P. hispanica*, caught in summer in another geographic habitat and subjected to cerebral damage by Ramirez et al. (1997) where, likewise, the sum-

mer season and the correlated photoperiod appeared to encourage proliferation only in the forebrain (Margotta, 2012b).

At present, proliferative immunoreactions have been seen in the olfactory district and in the telencephalon, but circumscribed to the *zonae germinativae ventrales*.

Comparing the preceding findings obtained in late spring (Margotta et al., 1999, 2005b) and in summer (Margotta, 2012b) with the actual ones obtained about at half spring upon cold shock, it appears that the thermal stimulus becomes less effective when the difference between the seasonal temperature - which is correlated with the photoperiod - and the thermal stress applied is less pronounced.

A more pronounced cell proliferation appeared restricted to the olfactory portions and to the telencephalic *zonae germinativae, dorsales* and *ventrales* also in the adult brain of cold-stressed *T. carnifex* (Chimenti and Margotta, 2012).

An interpretation of the previous (Margotta et al., 1999, 2005b), recent (Margotta, 2012b; Chimenti and Margotta, 2012) and present findings - quite at variance with those upon thermal plus surgical stress and cerebral damage - might be that the influence of the thermal stimulus (seasonal or artificial) alone, not coupled with another experimental stress (surgery and encephalic injury), exerts a low stimulation that is effective only in those vertebrates and in those areas of the brain more equipped with these sleepy cells.

A further explanation may be offered by the fact that *P. sicula*, being a lacertilian Reptile, is not in a high rank for the proliferative and, consequently, the regenerative capacity, contrary to Amphibians, especially Urodele rather than Anuran, and, to a lower extent, the Teleosts.

The entity of these events in the bony fishes does not correspond with their taxonomic place, since such extent is most probably correlated with peculiar and related features involving also the central nervous system, in particular the fact that the determination of the cell number is not concluded when the individual has reached its specific somatic size and the definitive number is attained later, as shown by a physiological mitotic activity (Richter, 1966; Rahmann, 1968; Schlecht, 1969; Richter and Kranz, 1971, 1981; Birse et al., 1980; Raymond and Easter, 1983; Zupanc and Horschke, 1995; Zupanc, 1999; Zikopoulos et al., 2000; Ekström et al., 2001).

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