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Relationships between seasonal (spring, summer, autumnal) thermal variations and cell proliferation in heterothermic vertebrates, as revealed by PCNA expression in the brain of adult *Triturus carnifex*

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Abstract

Inspired both by the literature reports and our previous findings on the question if a seasonal cycle alone, consisting of temperature and photoperiod variations, might impact on or activate natural proliferative fluctuations or unmask a latent spontaneous proliferative power in adult brain of poikilothermal Anamnia (fresh water, earth-dwelling) and Amniota (terrestrial), consequently allowing for encephalic reparative and even regenerative potentialities, an investigation has been carried on in normal adult brain of *Triturus carnifex* caught in nature in spring, summer, autumn. Cells immunostained for PCNA, *i.e.* cycling cells, were found scattered ("matrix cells") in the olfactory territories, where they appeared scarce in spring, more frequent in summer, noticeable in autumn; also, immunostained cells were found clustered in "matrix areas", also named *zonae germinativae dorsales* and *ventrales*, in the telencephalic hemispheres: few clusters in spring, an intermediate condition in summer, frequent cell groups in autumn. These results reveal an increasing trend in proliferation from spring, through summer, to autumn. This scenario was appreciable in the forebrain, mainly in the olfactory and telencephalic districts, which is the typical site of stem cells. Signs of potential proliferative activity are well appreciable in the urodele Amphibians, which are the best provided among vertebrates with reparative and regenerative power and possess the richest endowment of dormant cells susceptible to be recruited to proliferation.

Key words

Seasonal influence, neural matrix cells/areas, *Triturus*.

Introduction

In vertebrates, among the great amount of investigations testifying the plasticity, now well known, of the brain in adult poikilothermal Anamnia (fresh water, like Teleosts, earth-dwelling, like Amphibians) and heterothermic Amniota (terrestrial, like lacertilian Reptiles), the literature refers a handful of observations about whether seasonal cycle, including temperature and photoperiod variations, alone or coupled with various experimental approaches, might activate natural proliferative fluctuations or unmask a latent spontaneous proliferative power at the level of adult brain, conse-

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quently making evident some encephalic reparative and even regenerative potential. This potential is most pronounced in Urodeles Amphibians, intermediate in Teleosts, lower in Anuran Amphibia, most limited in lacertilian Reptiles.

Kirscke (1967) always takes the credit for a wide, systematic study on such issue in adult non-mammalian vertebrates ranging from Teleosts to Birds, passing through urodelan and anuran Amphibia and lacertilian Reptiles. This author must be acknowledged for the identification of the sites and features of the cells responsible of proliferative events which appear linked to the survival of stem cells in adults. These cells are capable of self-reproduction and can start cycling again giving rise to descendants which may evolve into neuronal or glial cells.

These normally quiescent cells, small and basophilic, are remnants of the embryonic neural layer responsible of central nervous system histogenesis (Kahle,1951; Fujita, 1963; Kirsche, 1967) and their number decreases during life from the embryonic to adult age through - if present - the larval stages.

The number of these neural-like cells varies among vertebrates. It is higher in lower than in higher vertebrates and depends on the different encephalic districts: cycling cells are easy to find in the anterior portion of the brain (olfactory bulbs/peduncles, telencephalon), but not in the diencephalon, in contrast they are absent from the *cerebellum* (except in Teleosts); as regards the *truncus cerebri*, they cannot be easily found in the midbrain (except in Teleosts) nor in the *medulla oblongata*.

These cycling cells appear scattered ("matrix cells") in the olfactory districts, otherwise clustered, often layered, in circumscribed areas ("matrix areas", or *Matrixzonen* of Kirsche, 1967) in the telencephalic hemispheres, at the dorsal and ventral edges of the lateral surface of each semi-lunar shaped ventricle which are named *zonae germinativae dorsales* and *ventrales*, respectively, which are extended antero-posteriourly. The cells in the former areas are exhausted before those in the latter areas, which is also generally wider and richer in cells (Kirsche, 1967).

In detail, matrix cells appear more frequently in relationship with the peri-ventricular grey matter or within the brain tissues, while matrix areas are typically located among the ependymal cells and in the sub-ependymal layer lining each encephalic cavity.

The knowledge on these putative precursor or perhaps stem cells has expanded through a great amount of observations derived from experimental procedures such as brain-injury, ablation of encephalic portions sometimes with subsequent heterotopic heterotransplantation (even of the whole brain), *in vitro* culture. In those studies several techniques: first classical histology, then autoradiography and immunohistochemistry targeting proliferation-related enzymes, seldom electron microscopy.

Past investigations in brain-injured or normal adult *Rana esculenta* (Minelli et al., 1982) and in brain-damaged adult *Podarcis hispanica* (Ramirez et al., 1997) have raised the question if seasonal cycle alone, that is made of temperature and photoperiod variations, might impact encephalic proliferative fluctuations. Since we have recently investigated this same question by immunohistochemistry in adult normal *Rana bergeri* (now synonymous of *R. esculenta*: Capula, 2000) (Margotta and Chimenti, 2017, 2018), we have now extended the observations to normal adult *Triturus carnifex*, addressing the presence and localization of proliferating cell nuclear antigen (PCNA: Miyachi et al., 1978), a reliable marker of cycling cells (see Margotta and Chimenti, 2016).

Materials and methods

Normal adult *Triturus carnifex* - ascertained following Bonifazi (2000) - of both sexes involved in the actual research originated from three captures, performed on purpose, in the wild near Rome: the first in spring (environmental temperature varying between 12°C to 18°C), the second in summer (environmental temperature varying between 14°C and 24°C), the third in autumn (temperature varying between 8°C to 18°C). The newts were sacrificed under anaesthesia with tricaine methanesulfonate (Ms 222 from Sandoz, Holzkirchen, Germany: 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 plastic paraffin under *vacuum*. Transverse, 8 μm thick serial sections were cut in antero-posterior direction with a rotary microtome.

For immunohistochemistry the sections, upon removal of paraffin and hydratation, were soaked 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-Aldrich, 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-Aldrich) 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 dehydrated and mounted with Entellan (Merck, Darmstadt, Germany). The specificity of the immunostaining was tested by replacing the primary antibody with non-immune goat serum.

Results

In the olfactory bulbs immunoreactive PCNA-positive cells were present scattered among the ependymal cells lining the symmetrical falciform-shaped ventricles, at times in the sub-ependymal layer. That appeared rarely in "spring" specimens (Fig. 1a), in a more pronounced manner in "summer" individuals (Fig. 1b), in a noticeable amount in "autumn" samples (Fig. 1c).

In the telencephalic hemispheres (each including a wide, irregular, falciform-shaped vertical cavity) among the ependymal epithelial cells and in the peri-ventricular grey matter layer at the level of the latero-dorsal edges and latero-ventral-medial bottom some clustered labelled cells were positioned at the surface of the ventricles, sometimes associate to other scattered stained cells. There were few clusters in "spring" specimens (Fig. 2a), an intermediate amount in "summer" individuals (Fig. 2b) and many in "autumn" samples (Fig. 2c). These cells occurred in the sites where the *zonae germinativae dorsales* (Figs. 2a, 2b, 2c) and *ventrales* (Figs. 2a, 2b, 2c)

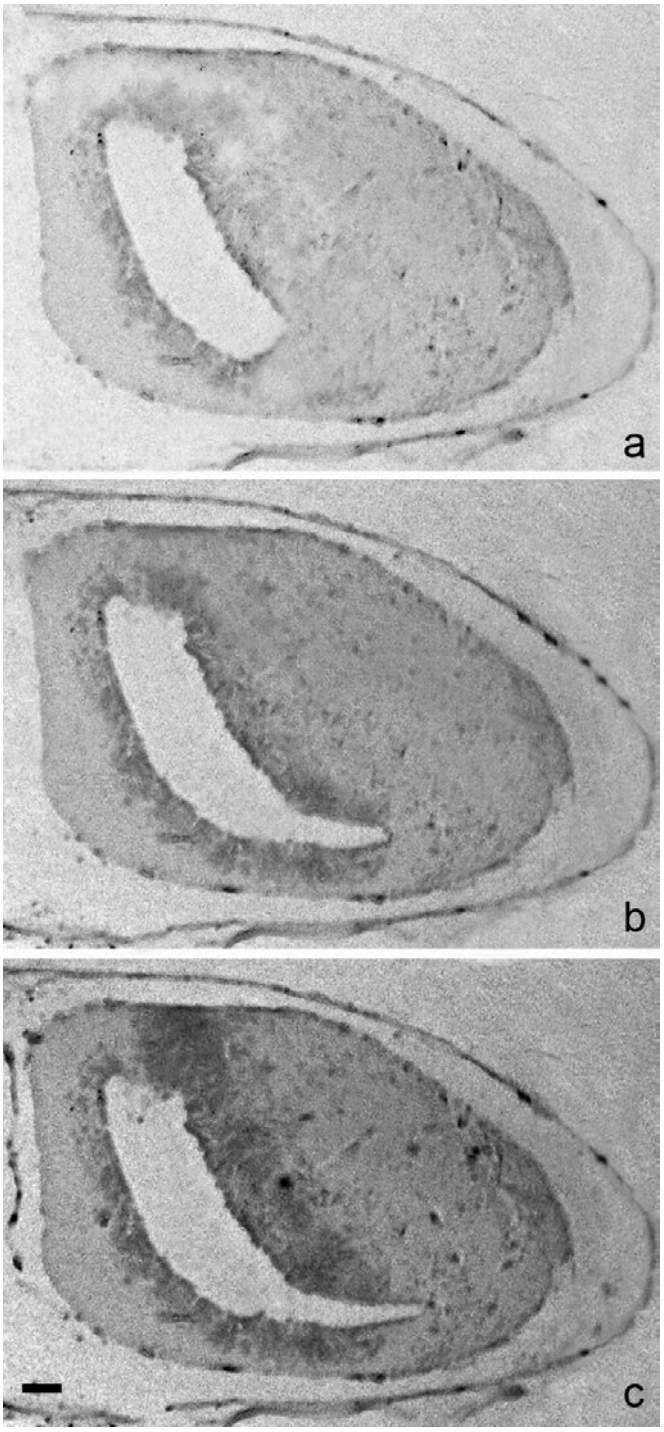

Figure 1. Transverse sections of olfactory bulbs of normal adult *Triturus carnifex*. Labelling appear mainly scattered in the ependyma, rarely in the sub-ependyma, around the ventricles. PCNA-positivity was scanty in "spring" specimens (a), intermediate in "summer" specimens (b) and abundant in "autumn" specimens (c). PCNA immunocytochemistry without nuclear conterstaining. Calibration bar = 50 µm.

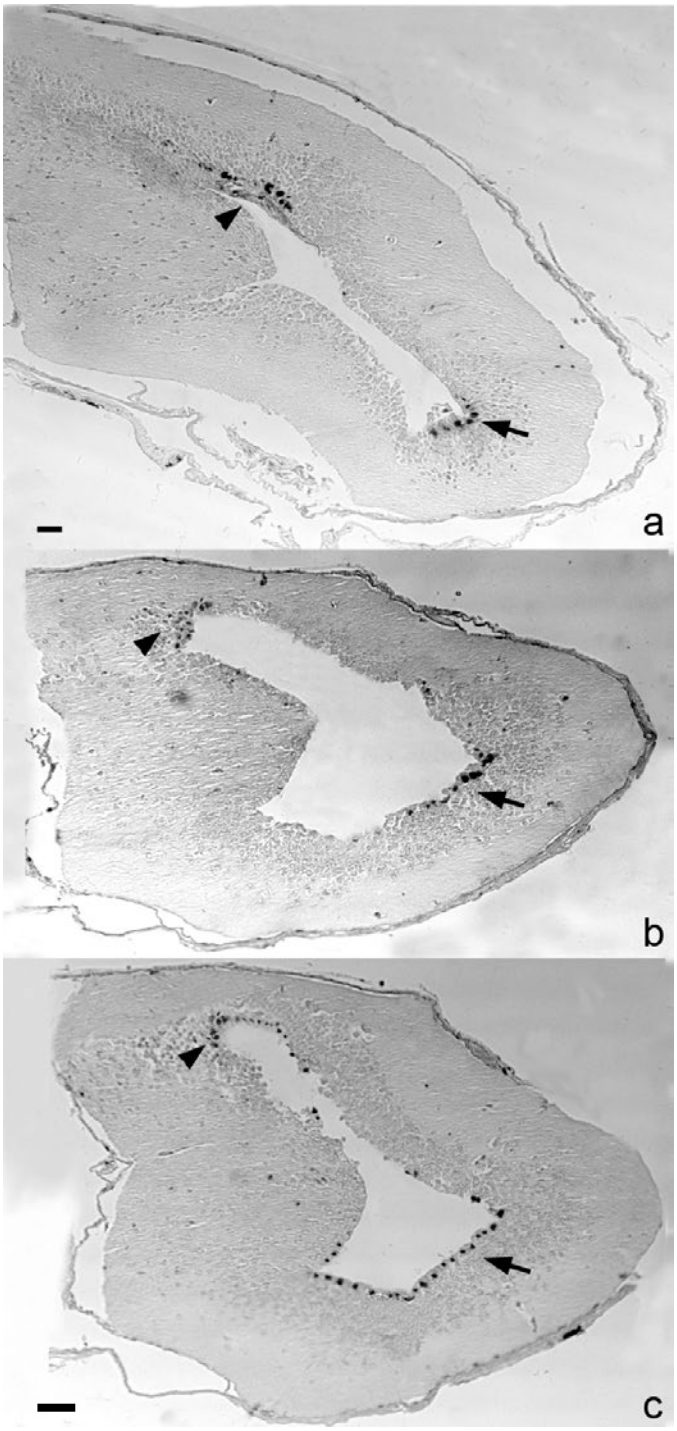

Figure 2. Transverse sections of telencephalic hemispheres of normal adult *Triturus carnifex*. PCNA-positive grouped and scattered cells appear in the ependymal and periependymal layers at the latero-dorsal edges (*zonae germinativae dorsales*, arrowheads) and at the bottom (*zonae germinativae ventrales*, arrows) of the lateral ventricles: labelled cells were scanty in "spring" specimens (Fig. 2a), some more in "summer" specimens (Fig. 2b) and numerous in "autumn" specimens (Fig. 2c). PCNA immunocytochemistry without nuclear conterstaining. Calibration bar = 50 µm.

are known to be located. Both *zonae germinativae* were extended antero-posteriorly, but the *zonae ventrales* were provided with more PCNA-labelled cells than the *zonae dorsales*.

In the diencephalon of "spring", "summer" and "autumn" individuals, weakly stained cells appeared in the ependyma and in the peri-ventricular grey matter around the narrow IIIth ventricle; pronounced immuno-positivity could be identified dorsally and ventrally where the symmetrical habenular ganglia and the impair preoptic and infundibular recesses, respectively, are located, without appreciable differences among "spring", "summer" and "autumn" samples.

In the midbrain the immuno-reaction was pale and hard to see in all samples, while in the remaining brain districts lying behind no labelling was traceable in any specimen.

Discussion

Numberless researches on plasticity of normal adult brain of vertebrates have clearly stated that the entity of proliferative phenomena in such organisms vary depending on the place in the systematic scale and structural complexity, so these events appear be limited in some poikilothermal, anamniotic and amniotic, groups. They depend on the survival of stem cells among the adult brain tissues, which appear scattered ("matrix cells") or clustered, often layered, in circumscribed areas ("matrix areas"): the first are found mainly in the olfactory, diencephalic, cerebellar and medullary districts, the latter in other regions (telencephalic, mesencephalic) limited to circumscribed areas: the *zonae germinativae*. Such quiescent cells make their appearance most frequently within the peri-ventricular grey matter (the matrix cells) and, among the ependymal cells (the matrix areas).

Investigations have addressed the question if seasonal cycle by itself, with variations only of temperature and photoperiod, might activate encephalic proliferative fluctuations or unmask latent spontaneous proliferative potentialities, otherwise hidden, consequently favouring reparative and even regenerative activity. This activity is most pronounced in urodele Amphibians, the vertebrates richest in putative stem cells, and decreases through Teleosts and anuran Amphibia down to lacertilian Reptiles.

From the evaluation of spontaneous fluctuations in the number of PCNA labelled cells in adult *T. carnifex* it has emerged that in the olfactory districts scattered labelled cells appeared occasionally in "spring" conditions, some more in "summer" conditions and numerous in "autumn" conditions, at the level of the ependyma and peri-ventricular grey matter. In the telencephalic hemispheres, the *zonae germinativae dorsales* and *ventrales* were identifiable as clusters of stained cells, which were few in "spring" conditions, in intermediate amount in "summer" conditions and most abundant in "autumn" conditions, mainly among the ependymal cells and rarely in the peri-ventricular grey matter.

Summarizing, these results reveal an increasing proliferation trend from spring through summer to autumn in the forebrain, precisely in the olfactory and telencephalic portions which are the typical sites of matrix cells/areas, while the putative proliferating cells diminished harshly or totally disappeared in the more caudal districts in all conditions.

Studies have also been published regarding both brain-injures and few normal brain of adult *R. esculenta* (Minelli et al., 1982) and brain-damaged adult *P. hispanica* (Ramirez et al., 1997). Minelli et al. (1982) observed by autoradiography an increase in proliferating cells in autumn with respect to spring and then a strong reduction in winter. Coherently, Ramirez et al. (1997), also by immunocytochemistry, noticed a proliferative peak in summer and stated that: "cold (winter) temperature prevented migration of the newly generated immature neurons", an observation possibly correlated with an involvement of radial glial cells in that migration (for details see Margotta and Morelli, 1997).

The seasonal gap in the studies of Minelli et al.(1982) and Ramirez et al.(1997) has been filled for the normal adult brain of *R. bergeri* by Margotta and Chimenti (2017, 2018), of *P. sicula* by Margotta and Chimenti (in press) and of *T. carnifex* by the actual investigation. We have considered unnecessary to repeat observations on "winter" specimens given the findings for this season of Minelli al. (1982) and Ramirez et al. (1997), which should reasonably apply also to newts.

In adulthood besides in different taxonomic species, the immunocytochemical patterns described here for *T. carnifex* seem to be in substantial agreement with those in different other species (frogs, lizards) including the findings of Minelli et al. (1982), related to spring and autumn, and of Ramirez et al. (1997), related to summer.

In experimental conditions similar to present ones, an increase in proliferation from spring through summer to autumn has been described for the adult brain of normal earth-dwelling Anamnia and terrestrial Amniota, including *R. bergeri* in spring, autumn (Margotta and Chimenti, 2017) and summer (Margotta and Chimenti, 2018). This report informs on *T. carnifex* and attention is worth being devoted also to *P. sicula* (Margotta and Chimenti, in press).

The previous and present findings on encephalic proliferative capacity in normal adult poikilothermal Anamnia (fresh water, like Teleosts, and earth-dwelling, like Amphibians) and in heterothermic terrestrial Amniota (like lacertilian Reptiles) may explain the different extent of reparative or regenerative events obtained by previous authors, if one admits that a role in this respect may also be played - among others - by the type of experimental stress applied (traumatic, surgical, thermal).

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References

Bonifazi A. (2000) *Triturus carnifex*. In: Bologna M.A., Capula M., Carpaneto G.M. (Eds.) Anfibi e Rettili del Lazio. Fratelli Palombi Editori, Roma. Pp. 42-43.

Capula M. (2000) *Rana bergeri* (Günther, 1986). *Rana kl. hispanica* (Bonaparte, 1839). In: Bologna M.A., Capula M., Carpaneto G.M. (Eds.) Anfibi e Rettili del Lazio. Fratelli Palombi Editori, Roma. Pp. 56-57.

Fujita S. (1963) The matrix cell and cytogenesis in the developing central nervous system. J. Comp. Neur. 120: 37-42.

Kahle W. (1951) Studien über die Matrixphasen und die örtliken Reifungsunterschiede im embryonalen menschlichen Gehirn. I. Mitteilung: Die Matrixphasen im allgemeinen: Dtsch. Zschr. Nervenheilk. 166: 273-302.

Kirsche W. (1967) Über postembryonale Matrixzonen im Gehirn verschiedener Vertebraten und deren Beziehung zur Hirnbauplanlehre. Z. mikrosk.-anat. Forsch. 77: 313-406.

Kirsche W. (1983) The significance of matrix zones for brain regeneration and brain transplantation with special consideration of lower vertebrates. In: Wallace R.B., Das G.D. (Eds.), Neural Tissue Transplatation Research. Spring-Verlag, New York Berlin Heidelberg Tokyo. Pp. 65-104.

Margotta V., Chimenti C. (2016) Plasticity of the central nervous system in adult vertebrates: immunohistochemical report on the effects of seasonal variations alone or coupled with induced cold shock on brain proliferation in fresh water or earth-dwelling Anamnia and heterothermic Amniota. Ital. J. Anat. Embryol. 121: 265-283.

Margotta V., Chimenti C. (2017) 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). Ital. J. Anat. Embryol. 122: 89-97.

Margotta V., Chimenti C. (2018) 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). Ital. J. Anat. Embryol. 123: 100-107.

Margotta V., Chimenti C. (2019) Relationships between seasonal (spring, summer, autumnal) thermal variations and cell proliferation in heterothermic vertebrates, as revealed by PCNA expression in the brain of adult *Podarcis sicula* (in press)

Margotta V., Morelli A. (1996) Encephalic matrix areas and post-natal neurogenesis under natural and experimental conditions. Anim. Biol. 5: 117-131.

Margotta V., Morelli A. (1997) Contribution of radial glial cells to neurogenesis and plasticity of central nervous system in adult Vertebrates. Anim. Biol. 6: 101-108.

Margotta V., Morelli A., Alfei L. (1999) PCNA positivity in the telencephalic matrix areas in the adult of a newt. J. Brain Res. 39: 525-530.

Margotta V., Morelli A., Caronti B. (2005) Expression of PCNA positivity in the brain of normal adult heterothermic Vertebrates: further observations. Ital. J. Anat. Embryol. 110: 59-74.

Minelli G., Del Grande P., Franceschini V. (1982) Uptake of 6-H3 thymidine in the normal and regenerating CNS of *Rana esculenta*. Z. mikrosk.-anat. Forsch. 96: 201-213.

Miyachi K., Fritzler M.J., Tan E.M. (1978) Autoantibody to a nuclear antigen in proliferating cells: J. Immunol. 121: 2228-2234.

Ramirez C., Nacher J., Molowny A., Sancez-Sancez F., Irurzun A., Lopez-Garcia C. (1997) Photoperiod-temperature and neuroblast proliferation-migration in the adult lizard cortex. NeuroReport 8: 2337-2342.