

Circulatory system

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*

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Abstract

Among the literature reports on the possible effects of the seasonal cycle, made of temperature and photoperiod variations, on spontaneous proliferation in the brain of adult fresh water, earth-dwelling Anamnia and heterothermic Amniota, one autoradiographic study was conducted on experimentally brain injured and normal *Rana esculenta*, collected in the wild in spring and in autumn, and another immunohistochemical study was conducted on brain injured *Podarcis hispanica* caught in nature in summer. To expand that knowledge an immunohistochemical investigation has been performed on brain of normal adult *Podarcis sicula* captured in the wild in spring, summer and autumn with the aim to analyse exclusively the seasonal (temperature and photoperiodic) cycle impact on latent spontaneous proliferation. Cycling cells have been labeled for PCNA. The results show that the cycling cells are rare in spring, in intermediate numbers in summer and frequent in autumn. Therefore environmental conditions affect the proliferative capacity of the cells in stand-by, that are typically mainly positioned in some telencephalic areas: the *zonae germinativae latero-dorsales, medio-dorsales* and *ventrales*. An investigation on the winter aspect was purposely omitted, since such lack of cycling cells in winter was already known from previous literature reports. With the present findings the time course of proliferation of putative brain stem cells - as demonstrated by immunolabeling for PCNA - is assessed in lizard for the whole year.

Keywords

Season influence, neural matrix cells/areas, *Podarcis*.

Introduction

The plasticity of adult brain has been ascertained for some anamniotic and amniotic vertebrates, mainly in fresh water fish, like Teleosts, in earth-dwelling Amphibia, like Urodeles and Anurans, and in terrestrial Reptiles, like lacertilians. That plasticity depends on the presence of surviving stem cells, which are responsible for the persistence of proliferative potential and therefore, probably, of reparative and even regenerative power.

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The proliferative potential is linked to the presence of small, basophilic, neural-like cells, that are remnants of the germinative neural layer of early embryonic development (Kahle, 1951; Fujita, 1963; Kirsche, 1967). Their number decreases from earlier to more advanced embryonic stages, then to the subsequent larval stages, if present, and eventually to adult life.

The number of these sleeping cells can vary among the different vertebrates; generally speaking they appear much greater in lower vertebrates than in higher ones. An increasing plasticity is shown from lacertilian Reptiles to anuran Amphibians to Teleosts to urodelan Amphibians; the last animals are the vertebrates most gifted with putative stem cells.

These putative precursor or stem cell are normally silent, but they are capable of self-reproduction and can start cycling again giving rise to descendants which may undergo late differentiation into neuronal or glial cells (Kirsche, 1967, 1983).

An exhaustive, comparative, autoradiographic study was carried out by Kirsche (1967) on the normal adult brain of vertebrates, ranging from Teleosts to Birds passing through urodelan and anuran Amphibians and lacertilian Reptiles and focusing on the presence of such cells in stand-by which can be found scattered ("matrix cells") or clustered ("matrix areas") in the layers lining the cerebral cavities. In details, the matrix areas ("Matrixzonen" according to Kirsche, 1967) are at the dorsal and ventral edges and at the bottom of each ventricular surface of the telencephalic hemispheres, making up, respectively, the *zonae germinativae dorsales* and *ventrales*, extended antero-posteriorly. The latter areas are generally wider and more populated in cells, whose persistence also appears more prolonged than those in the other areas (Kirsche, 1967).

In the meantime, Kirsche (1967) testified proliferative events in the *medulla oblongata* of *Triturus cristatus carnifex*, *T. vulgaris* and *Rana esculenta*, linked to the presence of matrix cells.

Subsequently, the presence of stem cells was reported in other encephalic sites and in other animal species mainly by autoradiographic, rarely by immunohistochemistry. In the layers delimiting the cerebral ventricles of Petromyzontidae (Margotta et al., 2007) and Selacians (Margotta, 2007), in ependymal and sub-ependymal layers of the olfactory districts of Teleosts, urodelan and anuran Amphibians and lacertilians Reptiles (Alonso et al., 1989; Garcia-Verdugo et al., 1989; Byrd and Brunjes, 2001; Margotta et al., 2005). In lacertilian Reptiles each *zona germinativa dorsalis* is distinguishable into two portions, *lateralis* and *medialis* (Minelli and Del Grande, 1980). A midbrain additional symmetrical matrix area (*zona germinativa caudalis*) was described in Teleosts, as well as matrix cells in the deep cerebellar tissues of Teleosts. Grouped matrix cells were found scattered in the forebrain of male songbirds ("hot spots") and "matrix tissue" was described in some Mammals (for further literature review see Margotta and Morelli, 1996).

Most investigations included submitting the samples to surgical ablations of encephalic plugs or areas, some were based on heterotopic hetero- (rarely homo-) transplants, *in vitro* culture of cerebral tissues and other experimental conditions and only a few were made on normal specimens. Methods were at first traditional histology, then autoradiography, seldom electron microscopy or immunohistochemistry, the last method being applied to target proliferation-related enzyme activity.

The awareness on the plasticity of adult brain in fresh water and earth-dwelling Anamnia and poikilothermal Amniota was sometimes obtained evaluating, among

other issues, if a seasonal cycle, made of temperature and photoperiod variations, alone or coupled with various experimental procedures might activate proliferative fluctuations or unmask an encephalic latent spontaneous proliferative potential, thus showing reparative and even regenerative potentiality due to an otherwise hidden mitotic activity of quiescent cells still present in the adult brain.

In particular, Minelli et al. (1982) in an autographic study on acutely injured and uninjured brain of adult *R. esculenta*, ascertained that the trend of labeled nucleoside uptake both in brain-injured and normal specimens was extremely low in advanced spring, was higher in autumn and waned again in proximity of winter. This trend was inverted in spring and autumn by first submitting the samples to cold.

Ramirez et al. (1997), by autoradiography and immunostaining, made similar investigations on adult brain-injured *Podarcis hispanica* caught in nature in summer and stated that cerebral proliferation was increased in summer and migration of newly generated immature neurons was inhibited in winter.

These studies were incomplete in that one lacked information on summer events (Minelli et al., 1982) and the other lacked information on spring and autumn ones (Ramirez et al., 1997). Therefore we have now carried out a study on normal adult brain of *P. sicula* (once *Lacerta viridis* Rafinesque: Tortonese and Lanza, 1968), captured in nature in spring, summer and autumn. The study was also aimed at ascertaining if the findings of Minelli et al. (1982) for spring and autumn might be extended to adult *P. sicula*, i.e. across different species.

The present study was performed with an immunocytochemical method, by revealing the Proliferating Cell Nuclear Antigen (PCNA: Miyachi et al., 1978). This marker had previously proved to reliable and suitable as a proliferation test (for further details see Margotta and Chimenti, 2016).

Materials and methods

Normal adult *Podarcis sicula* - as ascertained according to Capula (2000) - have been involved in the actual research. On the whole, six samples (three males and three females) were considered for each season. All specimens were collected in the wild near Roma, Italy. The individuals here employed as controls belonged to past catches and related investigations: some were caught in late spring (environmental temperature between 10° and 16°C) (Margotta et al., 1999, 2005), others at the end of July (environmental temperature between 12° and 24°C) (Margotta, 2014). Furthermore, a portion of the specimens belonging to lizards of the second capture were maintained in a stable. put in an open environment, to prevent difficult availability (diapause) during advanced autumn, and then were sacrificed in that season (temperature between 8° and 18°C). The lizards 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 antero-posterior direction with a rotary microtome.

For immunohistochemistry the sections were deparaffined and hydrated, rinsed in isotonic, 0.01 mol/litre phosphate buffered saline, pH 7.4 (PBS), incubated in 3% H₂O₂ 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% NiSO₄ and 0.017% H₂O₂ in 0.05 mol/litre Tris-HCl, pH 7.6. Slides were then dehydrated and mounted with Entellan (Merck, Germany).

Results

The actual account originate from an analysis carried out in normal adult specimens of *P. sicula*, in part from past catches in spring and at the end of July, previously published (Margotta et al., 1999, 2005, Margotta, 2014), and in part from present samples maintained in a stable kept in an open environment till advanced autumn before sacrifice. The following results were drawn.

In the olfactory peduncles PCNA positive cells appeared scanty in spring (Fig. 1a), numerous in summer (Fig. 1b) and even more abundant in autumn (Fig. 1c). These cells were scattered among the ependymal epithelium lining the ventricles, rarely were seen also in the sub-ependymal layer.

In each telencephalic hemisphere PCNA positive cells were found in areas located dorsally (each subdivided in two portions, lateral and medial) and ventrally, corresponding to *zonae germinativae latero-dorsales, medio-dorsales* (Figs. 2a, b, c) and *ventrales* (Figs. 3a, b, c) as known after Kirsche (1967), Minelli and Del Grande (1980). Few labeled cells were observed in spring (Figs. 2a, 3a), an intermediate number in summer (Figs. 2b, 3b) and many in autumn (Figs. 2c, 3c). The ventral matrix areas, in contrast to the dorsal ones, were more extended in antero-posterior direction and were better provided with putative stem cells, as anticipated by Kirsche (1967).

In the diencephalon of samples sacrificed in spring, summer and autumn weak labeling appeared in the ependyma and in the periventricular grey lining the 3rd ventricle without distinction among seasons; pronounced staining was observed dorsally and ventrally, where the symmetrical habenular ganglia and the unpaired pre-othic and infundibular recesses are respectively located.

In the midbrain the immune positivity was faint and even absent from some specimens in any season.

In the encephalic districts lying behind no labeling was identifiable in any specimen.

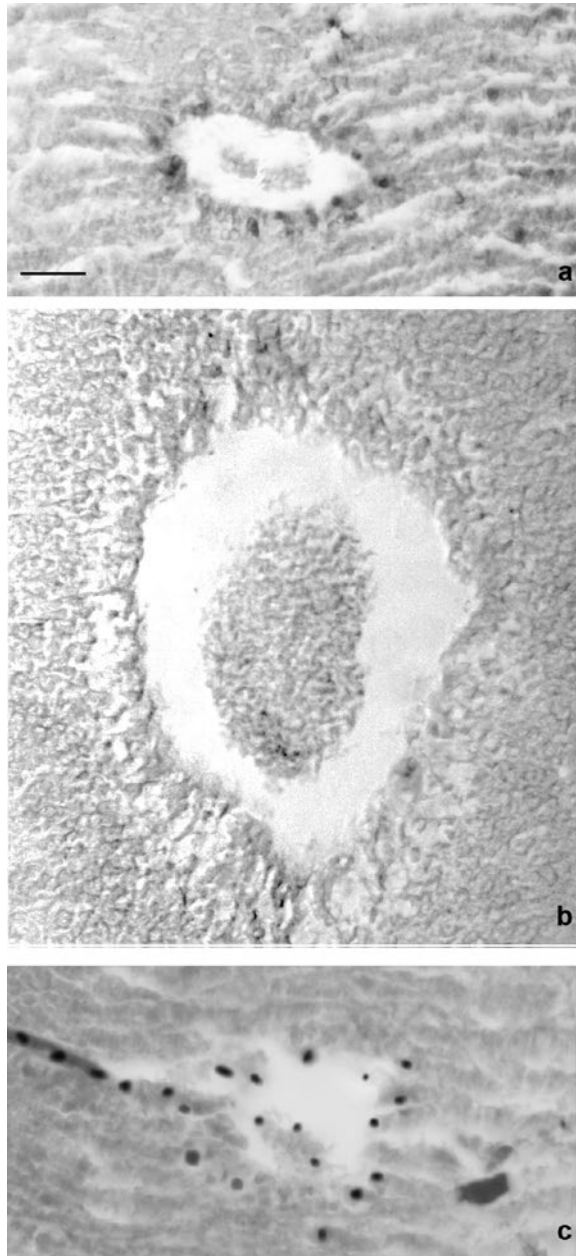


Fig. 1. Olfactory peduncles of normal adult *Podarcis sicula*. Labelling was mainly distributed in the layer lining the ventricles and rarely in the sub-ependyma. Scanty scattered PCNA-positive cells were found in specimens caught in late spring (Fig 1a), a more pronounced staining was present in specimens caught at the end of July (Fig. 1b) and numerous labeled cells were found in specimens caught in autumn. (Fig. 1c). [Figs. 1a, 1b: reprinted from Margotta, 2014a with permission]. Transverse sections. PCNA immunocytochemistry without nuclear counterstaining. Calibration bar=20 μ m.

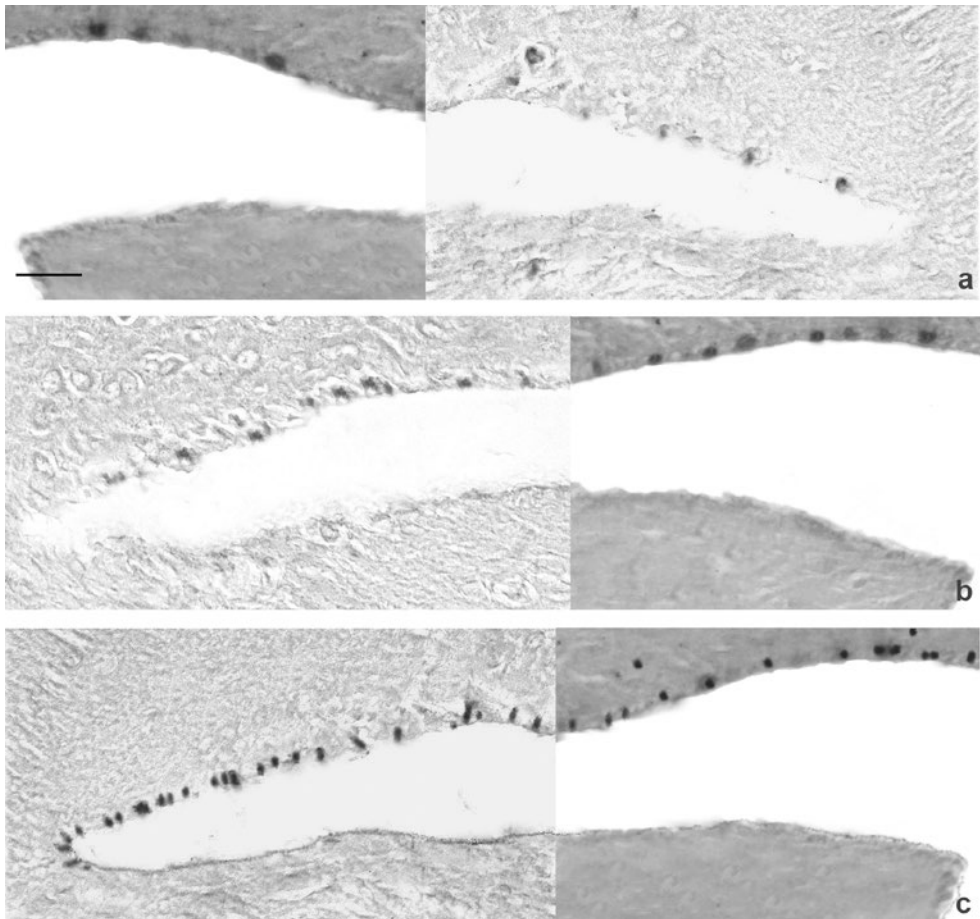


Fig. 2. Telencephalic hemisphere of normal adult *Podarcis sicula*. The immunolabeling was recognized in scattered ependymal cells and rare cells in the sub-ependymal layer in latero-dorsal and medio-dorsal position (*zonae germinativae latero-dorsales* and *zonae germinativae medio-dorsales* respectively). The labeled cells appeared increased from late spring (Fig. 2a) to the end of July (Fig. 2b) to autumn (Fig. 2c). [Figs. 2a, 2b: reprinted from Margotta, 2014a with permission]. Transverse sections. PCNA immunocytochemistry without nuclear counterstaining. Calibration bar = 20 μm .

Discussion

Many investigations support the opinion that cyclic fluctuations of seasonal environmental factors (temperature and photoperiod) could activate proliferation or impact on the natural or induced variations of proliferative potential in several tissues or organs of various systematic groups of heterothermal vertebrates, mainly Amphibians. That was ascertained for the cornea and eye lens (Rothstein et al., 1975), chemosensory epithelium (Dawley et al., 2000), retinal cells (Velasco et al., 2001), and the brain (Minelli et al., 1982; Bernocchi et al., 1990; Chetverukhin and Polenov 1993;

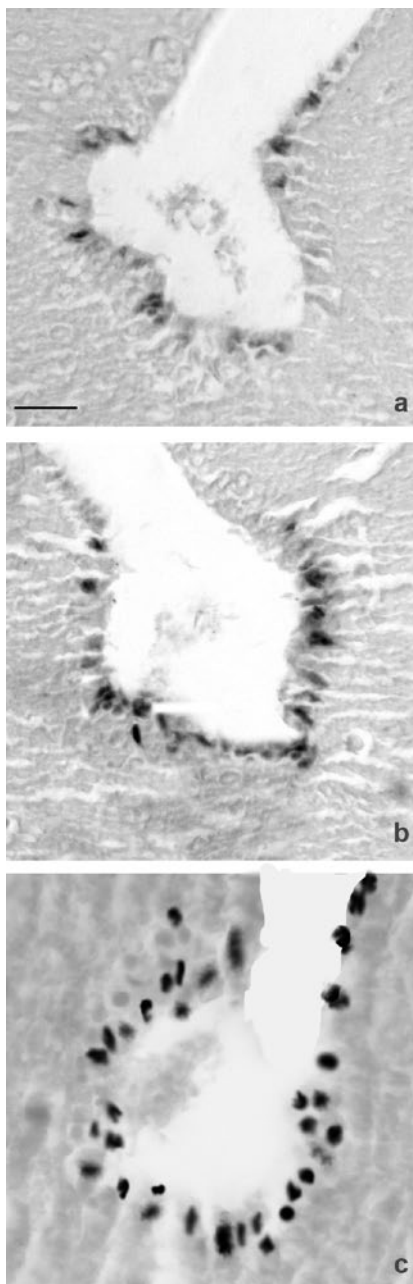


Fig. 3. Telencephalic hemisphere of normal adult *Podarcis sicula*. The immunolabeling was recognized in scattered ependymal cells and in the sub-ependyma layer in the vertical medio-ventral position (*zonae generativae ventrales*). The labeled cells were increased from late spring (Fig. 3a) to the end of July (Fig. 3b) to autumn (Fig. 3c). [Figs. 3a, 3b: reprinted from Margotta, 2014a with permission]. Transverse sections. PCNA immunocytochemistry without nuclear counterstaining. Calibration bar = 20 μ m

Polenov and Chetverukhin, 1993; Chieffi Baccari et al., 1994; Ramirez et al., 1997; Dawley et al., 2000; Vidal Pizarro et al., 2004; Margotta, 2012, 2014a; Margotta and Chimenti, 2016, 2017, 2018, 2019), These studies have also shown that one same season can stimulate different proliferative answers in different species.

In particular, for the brain the already mentioned papers of Minelli et al. (1982) and Ramirez et al. (1997) on *R. esculenta* and *P. hispanica* respectively, must be exposed in detail.

Minelli et al. (1982) studied by autoradiography adult specimens of *R. esculenta* subjected to surgical injury and cold shock or to cold shock alone. The uptake of 6-H³ thymidine in the brain showed a strict correlation between natural or artificial cooling and cerebral proliferative and regenerative potential. The mitotic activity and the regenerative capacity were low in May/June, became very high in September/October, declined in advanced November and reached a minimum at the beginning of winter. A temporary cooling (24 h at 4°C) led to an increase in proliferation in May/June and a decrease in September/October. The differences in mitotic rhythms along the year could explain the conflicting results reached by previous authors on the regenerative power of central nervous system in adult anuran Amphibians. Following previous studies (Rosomoff and Gilbert, 1955; Stone et al., 1956, Loughheed et al., 1960; Kiernan, 1979; Kiernan and Contestabile, 1980; Minelli and Del Grande, 1980), Minelli et al. (1982) proposed that a correlation exists between an influence of cold on blood brain barrier and regenerative capacity of brain tissue.

Ramirez et al. (1997) studied by autoradiography and immunohistochemistry adult specimens of *P. hispanica* subjected to experimental surgical injury and found a proliferative peak in summer. These authors also stated that cold (winter) temperature prevented migration of the newly generated immature neurons. This latter finding might hint to an influence of cold on radial glial cells, that are responsible for the translation of undifferentiated cells from the site of origin to the definitive place in the central nervous system in adult vertebrates (Margotta and Morelli, 1997).

A direct comparison between the report of Minelli et al. (1982) and that of Ramirez (1997) would be inappropriate because of relevant differences among species (in the sensitivity to seasonal conditions, experimental cooling and surgical injury) depending on the place in the evolutionary scale, habitat, timing of capture, laboratory environment and details of experimental procedures.

The physiological potential for proliferation of the uninjured, unstressed adult brain has been the subject of research in *P. sicula* (Margotta et al. 1999, 2005; Margotta, 2014a), *R. esculenta* (Margotta et al. 2000, 2005), *R. bergeri* (Margotta, 2012; Margotta and Chimenti, 2016, 2017, 2018) and *T. carnifex* (Margotta and Chimenti, 2019).

On the basis of the present and previously published data it may be stated that in normal adult brain of *P. sicula* the labeling for PCNA, indicating cycling cells, is scanty in spring, evident in summer, more evident in autumn; mainly restricted to the forebrain. "Matrix cells" are identifiable in olfactory ventricular surfaces, while "matrix areas" are identifiable in the telencephalic hemispheres as *zonae germinativae latero-dorsales*, *medio-dorsales* and *ventrales*. In the diencephalon labeling is found in the ventricular ependyma and periventricular grey matter, besides scattered labeled cells there are in the epithalamus, habenular ganglia, hypothalamic (infundibular and pre-optic) recesses. No labeling was seen in encephalic districts lying more behind.

Also the present observations in lizards seem to support and reaffirm some remarks, originated from previous investigations carried out in frogs (Margotta and Chimenti, 2017, 2018) and newts (Margotta and Chimenti, 2019), that the entity of the spontaneous proliferation seems too low to explain the reparative or even regenerative processes, obtained sometimes in that species by previous authors, reason why these processes could also rather depend on stimulation by the various (surgical, traumatic, thermal) experimental stimuli.

Autumn condition seems to remind a similar positivity exerted by an transient cold shock on brain proliferation in adult lizard (Margotta, 2014b).

The autumnal actual findings complete those previously reported for the same *P. sicula* caught in spring (Margotta et al., 1999, 2005) and summer (Margotta, 2014), showing an increasing trend in spontaneous proliferation of putative brain stem cells from springtime to autumn. We didn't make observations on winter specimens, assuming that those of Minelli et al. (1982) in *R. esculenta* and above all of Ramirez et al. (1997) in *P. hispanica* could hold also for *P. sicula*.

The overall moderate entity of the proliferative answer to environmental inputs, observed here, is in line with the diffuse awareness of a relative low regenerative potential of adult lacertilian Reptiles among poikilothermal vertebrates.

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