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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

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Abstract

Latent morphogenetic potentials may be used by Vertebrates to reconstitute parts of the body gone lost, even of the central nervous system. This issue is reviewed here for the brain and, quickly, the spinal cord of Anamnia and Amniota up to Reptiles, paying special attention to germinative areas (*zonae germinativae*) in the forebrain of Amphibians as model systems. The results in this field may guide to understand plasticity of the central nervous system also in higher Vertebrates.

Key words

Neural stem cells, matrix cells/areas, brain, spinal cord, Teleosts, Amphibians, Reptiles, matrix areas.

Introduction

Among the properties of living organisms there is the possibility to use latent morphogenetic potentials to re-constitute body parts gone lost.

Many studies have shown the plasticity of the central nervous system of adult vertebrates in physiological and experimental conditions and upon trauma, depending on the survival of undifferentiated cells, possible remnants of the germinative layer of the embryo neural tube (Kahle, 1951; Fujita, 1963; Kirsche, 1967) which gives rise to neuroblasts and glioblasts.

This property, a typical regulative behaviour, decreases from the early embryonic phases to adult life and ageing (Kahle, 1951; Fujita, 1963; Kirsche, 1967) in parallel with a decrease in the number of the undifferentiated cells. It also decreases from lower to higher vertebrates, but may vary within the different systematic groups.

Knowledge on this issue has expanded through studies at first on spinal cord of newts mainly subjected to heterotopic heterotransplants (rarely homotransplants, sometimes autotransplants, in vitro coltures) of a medullary segment after amputation of the tail, partial or total resection of the trunk, and then - less frequently - on brain subjected to heterotopic heterotransplants upon ablation of encephalic plugs or portions, sometimes even of the whole encephalon.

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In these experiments Urodela have appeared best endowed with proliferative, reparative and regenerative ability, so they have been privileged for research.

Similar studies have also been performed on other poikilothermal vertebrates, especially fresh water or earth-dwelling Anamnia, and performance has appeared better in Teleosts than Anura, further lower in lacertian Reptiles.

The position of the bone fishes in this list is surprising since it does not corresponds with their place in evolution and taxonomy where, on the contrary, they come before the Amphibians. This might be correlated with the fact that in these fishes the cell number is not definitive when the organism has reached its specific somatic size, so a supply of undifferentiated cells survives for a longer time than in more advanced vertebrates also in the central nervous system (Richter, 1996; 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).

In homeothermic Amniota there is some regeneration but limited: less in Birds, circumscribed to moderate capacity of medullary central axons as in cocks (Margotta et al., 1989) and cerebral neurons as in male songbirds, and more in Mammals (see reviews: Kirsche, 1983; Margotta and Morelli, 1996). Several, some even contradictory explanations have been proposed to explain this trend, mainly based on a role of the micro-environment of central nervous system.

Many techniques have been used to achieve results in this field, from classic histology to autoradiography, electron microscopy, histochemistry and immunocytochemistry, the last methods being applied to target proliferation-related enzyme activity.

Brain

Awareness of the possible occurrence of reparative or even regenerative processes, depending on a certain number of stem cells surviving in the brain of adult vertebrates, was acquired at first in the second half of the past century after the intuition of Kirsche (1967) who identified brain matrix cells, using classical histological methods in a comparative, acute, exhaustive and enlightening study on normal adult vertebrates ranging from Anamnia (Teleosts belonging to genera *Carassius* - in particular *C. carassius* species - *Lebistes*, *Leuciscus*; some urodelan Amphibia: one *Ambystoma*, two newts among which *Triturus cristatus cristatus* species; some anuran Amphibia: two frogs among which *Rana esculenta* species, one toad) through poikilothermal Amniota (like Reptiles among which some tortoises, one lizard: *Lacerta agilis agilis* species, one crocodile) to Birds (several species).

These putative precursor or stem cells, small and basophilic, may occupy different sites and assume various features from species to species. They may be clustered in circumscribed areas and appear frequently layered, forming the so-called "Matrixzonen" (Kirsche, 1967), now designated "matrix areas". Some others of these peculiar cells may appear scattered within brain tissue and are then termed "matrix cells".

These cells are normally silent, but are capable of self-reproduction and can start cycling again and give rise to descendants or even undergo late differentiation and evolve into neurons or glia (Kirsche, 1967, 1983).

That would be the mechanism responsible for encephalic repair or even regeneration, however accompanied by progressive impoverishment of the stock of such sleeping cells.

Kirsche (1967) proposed that the distribution of the newly formed brain cells in normal and experimental conditions in post-natal life depends on targeted migration from proliferating areas to the definitive sites. Alvarez-Buylla et al. (1987, 1988, 1990b) and Alvarez-Buylla and Nottebohm (1988) supported that hypothesis by means of autoradiography coupled with immunocytochemistry for markers of radial glia. They found that the migration of neuroblasts is supported by the persistence of radial glia, which also showed considerable proliferative activity. Alvarez-Buylla et al. (1990b) hypothesized that radial glia exert a double role, at first favouring the production of neuroblasts from quiescent cells and then guiding the migration of neuroblasts to definitive sites. It is noteworthy that the body of the radial cells, observed by those authors, co-localizes with the *zonae germinativae*.

The possible concomitance between persistence of radial glia and basic or post-traumatic cell proliferation correlating with the plasticity of the central nervous system is also supported by reviews offering critical analysis of published autoradiographic or immunocytochemical reports (Margotta and Morelli, 1997; Alvarez-Buylla et al. 2002).

In the olfactory bulbs of adult teleostean genera *Carassius*, *Barbus*, *Cyprinus*, *Salmo* (Alonso et al., 1989), *Brachydanio* (Byrd and Brunjes, 2001) and in the olfactory peduncles of adult heterothermic Amniota, like *Podarcis hispanica* (Garcia-Verdugo et al., 1989), such sleeping cells are located at the ventricular borders and often also in the sub-ependymal and internal granular layers.

The telencephalic *Matrixzonen* were discovered by Kirsche (1967) in the above-mentioned adult vertebrates (they were also investigated in some Teleosts by Schlecht, 1969). Those studies showed the presence of *zonae germinativae dorsales* and *ventrales*, the location and overall configuration of which are the result of a process named "external eversion" (more pronounced among the Teleosts than in other Osteichthyes, however absent in some other bony fishes) which takes place during neuro-morphogenesis and gives rise to the appearance of only one, not hollow, hemisphere enveloped by an ependymal epithelium and binding what seems a dorso-ventral ventricle. Owing to such process, the *pallium* and the *epistriatum* lie dorso-medial-laterally (site of the *zonae germinativae dorsales*), while the *sub-pallium* (*primordium* of the *striatum*) and the *septum* with the *recessus medialis* lie ventral-medially (site of the *zonae germinativae ventrales*) (Beccari, 1943; Nieuwenhuys et al., 1998; for further details: Margotta et al., 2001)

In the remaining vertebrates another, opposite, process of "internal inversion" take place with the consequent appearance of the two hollow hemispheres.

In adult earth-dwelling Anamnia (like urodelan and anuran Amphibia) and in adult poikilothermal Amniota (like lacertilian Reptiles) these quiescent cells appear, as described by Kirsche (1967), clustered among the ependymal cells or scattered in the grey matter in the dorsal and ventral edges of each ventricular lateral surface, *i.e.* in the areas named *zonae germinativae dorsales* and *ventrales*; proceeding antero-posteriourly, the latter areas are generally wider and richer in cells than the *zonae germinativae dorsales* (Kirsche, 1967).

Kirsche (1967) at the end of his observations stated, among other things, that "… The matrix areas are used up with different time courses. In telencephalon the ventral

matrix area is distinctly delayed in respect to the dorsal one, while in truncus cerebri the descendants of basal plate are distinctly quicker [to be generated] than those of the alar plate."

Later on, Minelli and Del Grande (1980) found in adult *L. viridis* that each *zona germinativa dorsalis* is subdivided into two parts, *lateralis* and *medialis*.

What holds true in adult Amphibians and lacertilian Reptiles is even true in adult homothermic Amniota, such as many Birds (Kirsche, 1967), while in adult male songbirds quiescent precursor cells are distributed here and there in the ependymal layer, forming the so-called "hot spots" (Nottebohm, 1981). Such cells can be retained also in human newborns (Rydberg, 1932), where they are designated as "matrix tissue", and in various Mammals well beyond birth (Kershman, 1938), where they are named "zonae" (for details on Mammals: Margotta and Morelli, 1996).

In the diencephalon of adult fresh water and earth-dwelling Anamnia and heterothermic Amniota a restricted number of such quiescent cells are present mainly in *habenular* ganglia - ventral portions (*recessus praeopticus*, *infundibulum*) - and also in the ependyma and grey matter (Kirsche, 1967). This had been previously shown by Fleischhauer (1957) in *Testudo graeca*.

As regards the *truncus cerebri*, in the mesencephalon of adult Teleosts clusters of neural-like cells named *zonae germinativae dorsales*, *ventrales* and *caudales* were described first by Kirsche (1967); such cells appear scanty and spread in the tectal deep grey layers of adult other Anamnia, like Amphibia (Kirsche, 1967; Minelli and Quaglia, 1968; Leghissa et al., 1980; Ciani and Franceschini, 1982) and of adult heterothermic Amniota, like lacertilian Reptiles (Kirsche, 1967; Del Grande et al., 1981).

The *cerebellum*, as shown at first by Kirsche (1967) in aforesaid adult Teleosts and then by many authors in other adult teleostean genera (*Xiphophorus*, *Salmo*, *Sparus,* Gymnotiform), is characterized by many stem cells located deep in the tissue. This feature might be related to the role played by such district in activities as, for instance, social behaviour, linked to apoptotic events or to onset of reparative and even regenerative phenomena (for further details see Margotta et al., 2004).

In the *medulla oblongata* of various adult fresh water, earth-dwelling Anamnia and heterothermic Amniota putative stem cells may be observed in the epithelium lining the rhombo-encephalic ventricle (Kirsche, 1967).

Preceded by the knowledge of the literature data regarding the persistence during the adult life of a proliferative potential in the encephalon both in natural and experimental conditions (Margotta and Morelli, 1996), qualitative, correlative observations were carried out in normal adult animals in the last years, proceeding in opposite direction to evolution from *P. sicula* (Margotta et al., 1999a, 2005b) through *T. carnifex* (Margotta et al., 1999b; 2005b), *R. esculenta* (Margotta et al., 2000, 2005b), *C. carassius* (Margotta et al., 2001, 2002, 2004) and *Torpedo marmorata* (Margotta, 2007) to *Lampetra planeri* (Margotta et al., 2007). The proliferative power of such peculiar neural-like cells, indispensable to give raise to reparative or even regenerative events, has been re-evaluated through immunohistochemistry for proliferating cell nuclear antigen (PCNA: Miyachi et al., 1978).

Our investigations on *T. marmorata* and *L. planeri* (Franceschini et al., 1992) offered the first available evidence about Chondrichtyes (Margotta and Morelli, 1996) and greatly increased that available for Petromyzontidae (Margotta and Morelli, 1996), since the only foregoing researches in *L. planeri* lacked detailed information on the

adult condition and were focused mainly on their larval and metamorphic stages (Pfister, 1971/1972a, b).

Through this re-appraisal, matrix cells have been described for the first time in the ependymal and sub-ependymal layers delimiting the ventricles of the olfactory bulbs of adult earth-dwelling Anamnia like *T. carnifex* (Margotta et al., 2005b) and *R. esculenta* (Margotta el al., 2005b) and surrounding the whole brain cavity of *T. marmorata* (Margotta, 2007) and *L. planeri* (Margotta et al., 2007).

Good correspondence was found between our immunohistochemical observations and the results of studies performed mainly by autoradiography and often on brain-injured individuals of the same species as we had studied: *T. carnifex* (Bonifazi, 2000) and *P. sicula* (Capula, 2000); these two species were previously named *T. cristatus carnifex* (Tortonese and Lanza, 1968; Giacoma and Balletto, 1988) and *L. viridis* (Tortonese and Lanza, 1968) respectively.

The results have suggested that stem cells are constantly present in the forebrain, in a much smaller quantity in the midbrain and sometimes in the hindbrain, as is the case of Teleosts.

A handful of studies on adult Anamnia living in sea and fresh water or earth-dwelling and in heterothermic Amniota investigated if the environmental variations linked to seasonal cycle, made of temperature and photoperiod variations, might impact, sometimes with the aid of an experimental exposure to cold stress, on cell proliferation and activate latent spontaneous proliferative fluctuations or unmask the potentialities responsible for these events due to an otherwise hidden mitotic capability still present in the brain.

In particular, environmental influence was observed in the brain of Amphibians *R. esculenta* (Rothstein et al., 1975; Minelli et al., 1982a; Bernocchi et al., 1990; Chieffi Baccari et al., 1994), *R. temporaria* (Chetverukhin and Polenov, 1993; Polenov and Chetverukhin, 1993), *Plethodon cinereus* (Dawley et al., 2000), of lacertilian Reptiles *P. hispanica* (Ramirez et al., 1997), *P. sicula* (Margotta, 2014a), and of *Tinca tinca* (Velasco et al., 2001) and *Petromyzon marinus* (Vidal Pizzarro et al., 2004). A similar influence had been also seen in others sites like spinal cord and ocular tissues (Rothstein et al., 1975), gonads (Nottebohm, 1981), chemosensory epithelium (Dawley et al., 2000) and retinal cells (Velasco et al., 2001).

The photoperiod influences the reproductive cycle-related differentiation processes in the song encephalic centres of male canaries (Nottebohm, 1981; see also: Margotta et al., 2005a; Margotta and Caronti, 2005).

About the issue if an artificial exposure to thermal shock might exert an influence on the encephalic proliferation it must be remember that an experimental cold shock was at first adopted by Del Grande and Minelli (1971) and Minelli and Del Grande (1974a, b) to face post-operatory massive haemorrhage in adult *T. cristatus carnifex* deprived of a plug of the optic tectum. In those experiments unexpected regenerative patterns were obtained. Hoping to stimulate regenerative processes and reach the same results, later on this contrivance was introduced before the ablation of various encephalic areas in adult *L. viridis* (Minelli et al., 1978; Del Grande et., 1981; Minelli et al., 1982b), *R. esculenta* (Minelli et al., 1982a, b; Del Grande et al., 1984), *T. cristatus carnifex* (Del Grande et al., 1982a, b; Minelli et al., 1982b, 1987, 1990; Del Grande et al., 1990; Franceschini et al., 1992).

The suspicion arose that this effect might be linked to cell proliferation and various hypotheses were proposed in this respect. In particolar, Minelli and Del Grande

(1980), Minelli et al. (1982a) proposed that decrease could be due to metabolic disease which could provoke a diminution of the rithmic proliferative activities yet present in the nervous tissue during the autumn. Furthermore, also in account of observations of other authors (Rosomoff and Gilbert, 1955; Stone et al., 1956; Lougheed et al., 1960; Kienan, 1979; Kienan and Contestabile, 1980), proposed that a cold stimulus could lead to loosening the blood-brain barrier, which is practically non-existent in early embryonic stages and is imperfect during morphogenesis, but becomes fully formed upon full development. This might lead to re-establish a pseudo-embryonic condition and the reactivation of mitotic activity in matrix cells still present in the brain. Reversely, the formation of the haemato-encephalic barrier might be responsible for the decline of spontaneous proliferation.

Since literature sources referred, as said, of interactions between the natural environmental conditions - alone or linked to a sudden, temporary, experimental cooling - and proliferative performances, we have carried out two other series of qualitative immunohistochemical studies on adult brain in the same species of fresh water, earth-dwelling Anamnia and heterothermic Amniota previously investigated in similar studies by past authors: first to assess the influence of the different season conditions alone and then to assess the influence of those conditions in association with lowered body temperature on cold-shocked, but not brain damaged animals.

In such investigations we have used as indicator of proliferative events PCNA (Miyachi et al., 1978). This marker is expressed by proliferating cells and is easy to detect upon immunostaining. PCNA is a member of the cyclin family, an auxiliary protein of DNA polymerase δ closely associated with the sites of DNA replication. It reaches an appreciable level when DNA is synthesized in the S phase of the cell cycle (Bravo and Macdonald-Bravo, 1985, 1987; Bravo et al., 1987; Jaskulski et al., 1988; Liu et al., 1989; Fairmann, 1990; Diffley, 1992). This method had given proof of independence of tissue type (Almendral et al., 1987; Matsumoto et al., 1987; Olins et al., 1989; O'Reilly et al., 1989; Suzuka et al., 1989; Bauer and Burges, 1990; Leibovici et al., 1990; Nurse, 1990; Yamaguchi et al., 1990; Lew et al., 1992; Nacher et al., 1996; Ramirez et al., 1997) and had proved valuable also in our previous researches because it does not require housing animal in the laboratory between catch and sacrifice, at variance with methods based on the labelling of newly synthesized DNA; in turn, PCNA labelling does not allow to trace the destiny of daughter cells.

The impact of season climate and photoperiod on physiological cell proliferation in the encephalon was investigated in normal adult *R. bergeri* (Capula, 2000; now synonymous of *R. esculenta*: Tortonese and Lanza, 1968) and in normal adult *P. sicula* captured in the wild in late autumn (Margotta, 2012) and in summer (Margotta, 2014a).

As compared with their respective controls, *i.e. R. esculenta* housed in captivity in thermally regulated environment for several days before their sacrifice and experiments (Margotta et al., 2000, 2005b) and *P. sicula* caught in the wild in late autumn (Margotta et al., 1999, 2005b), the labelling in the olfactory districts and in the telencephalic *zonae germinativae dorsales* and *ventrales* was reduced in adult *R. bergeri* (Figs. 1a, b, c, d), while increased to a limited extent in adult *P. sicula* (Figs. 2a, b, c, d). In the diencephalon of both species PCNA immunoreactivity was observed in the epithalamus, hypothalamus and, diffuse, in other sites. No positivity was seen in the midbrain and hindbrain. These results were not immediately comparable but with our last findings on adult *R. esculenta* (Margotta et al., 2000, 2005b) and *P. sicula*

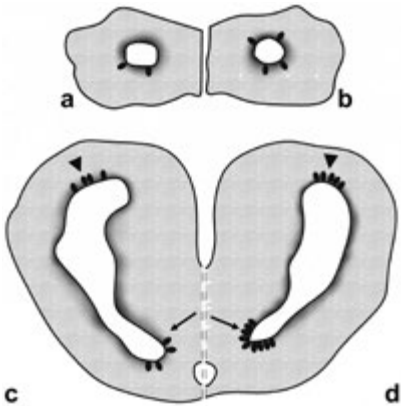

Figure 1. Drawings (not to scale) of a transverse view of olfactory bulbs (a, b) and telencephalic hemispheres (c, d) of normal adult *Rana bergeri* caught in the wild in late autumn. The dots represent PCNA immunolabelled matrix cells, isolated in the olfactory ependyma and periependymal grey matter (a, b) and clustered in the walls of telencephalic ventricular cavities to form the *zonae germinativae dorsales* (arrowheads) and *ventrales* (arrows) (c, d). a, c: Specimens sacrified immediately after capture. b, d: Specimens housed for some days in a thermally regulated environment between catch and sacrifice.

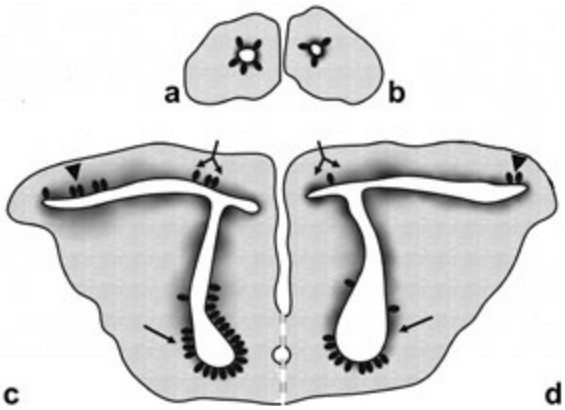

Figure 2. Drawings (not to scale) of a transverse view of the olfactory peduncles (a, b) and telencephalic hemispheres (c, d) of normal adult *Podarcis sicula*. The dots represent PCNA immuno-labelled matrix cells, isolated in the olfactory ependyma and periependymal grey matter (a, b) and clustered in the walls of telencephalic ventricular cavities to form the *zonae germinativae latero-dorsales* (arrowheads), *medio-dorsales* (arrowforkeds) and *ventrales* (arrows) (c, d). a, c: Specimens caught in summer. b. d: Specimens caught in late spring.

(Margotta et al., 1999, 2005b), not with previous ones on adult *R. esculenta* (Minelli et al., 1982a), *R. temporaria* (Chetveruklin and Polenov, 1993; Polenov and Chetveruklin, 1993) and *P. hispanica* (Ramirez et al., 1997) because of differences among the species studied, habitat, season of capture, acclimation to the laboratory, timing of sample collection in respect to breeding period (after that period in our studies on *P. sicula*, according to Capula, 2000), and experimental conditions (encephalic areas were removed in previous studies of other authors on *R. esculenta*, *R. temporaria* and *P. hispanica*, but not in our ones on *R. esculenta*, *R, bergeri* and *P. sicula*). All these factors may explain the disagreement among studies.

At the light of the outcomes of Minelli et al. (1982a) which would be here later on more in details exposed, as well as Ramirez et al. (1997), besides in another systematic group (adult brain-injured *P. hispanica*), by means autoradiographic and immunostaining techniques found that in summer the proliferation was increased, furthermore they referred that "…cold (winther) temperature prevented migration of the newly formed generated immature neurones", we have performed an investigation (Margotta and Chimenti, submitted for publification), programmed anothers on normal adult brain of *R. bergeri*, *P. sicula*, *T. carnifex* caughts in nature also in the remaining seasons, so the whole of these our past, recent and future investigations on such issue could to form an *unicum*.

The influence of the different season conditions in association with lowered body temperature was assayed on adult cold-shocked, but not brain damaged *T. carnifex* (Chimenti and Margotta, 2013) and *P. sicula* (Margotta, 2014b).

We gave priority to *T. carnifex* for these investigations since a much larger amount of experimental information is available for newts (Del Grande et al., 1982a; Minelli et al., 1987; Del Grande et al., 1990; Minelli et al., 1990; Franceschini et al., 1992), rather than frogs or lizards, subjected to cold-shock and ablation of telencephalic, diencephalic, or mesencephalic areas. Previous reports of other authors had not stated when *T. cristatus carnifex* or *L. viridis* were captured in nature, so we have privileged the period corresponding to the breeding season: the first week of spring for *T. carnifex* (Bonifazi, 2000), the fifth week of spring for *P. sicula* (Capula, 2000). After experimental cold exposure the interval between the transfer of the samples to the external temperature and their sacrifice in previous experiments was known for newt alone. For *T. carnifex* and *P. sicula* we chose that indicated by Franceschini et al. (1992) for *T. cristatus carnifex* as the most suitable for the expression at maximal brain cell proliferation, i.e. one week.

Upon lowering body temperature, the PCNA labelling of olfactory districts appeared more substantial in *T. carnifex* (Chimenti and Margotta, 2013; Figs. 3a, b) than in *P. sicula* (Margotta, 2014b; Figs. 4a, b). In the telencephalon labelled cells were found in the *zonae germinativae dorsales* and *ventrales* of newts (Chimenti and Margotta, 2013; Figs. 3c, d) and only in the *zonae germinativae ventrales* of lizards (Margotta, 2014b; Figs. 4c, d). In the diencephalon PCNA localized patterns appeared in the epithalamus and hypothalamus, and diffuse in other sites as had been observed in

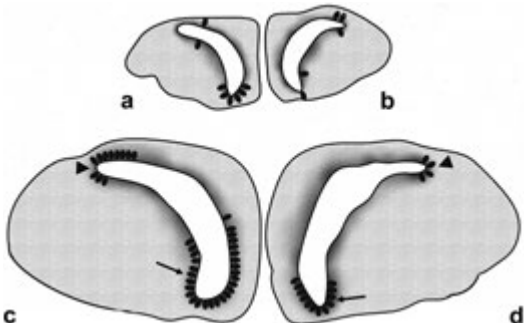

Figure 3. Drawings (not to scale) of a transverse view of the olfactory bulbs (a, b) and telencephalic hemispheres (c, d) of adult *Triturus carnifex* caught in the wild at the beginning of spring, not brain injured. The dots represent PCNA immuno-labelled matrix cells, isolated in the olfactory ependyma and periependymal grey matter (a, b) and clustered in the walls of telencephalic ventricular cavities to form the *zonae germinativae dorsales* (arrowheads) and *ventrales* (arrows) (c, d). a, c: Cold-shocked specimens. b, d: Specimens not subjected to cold shock.

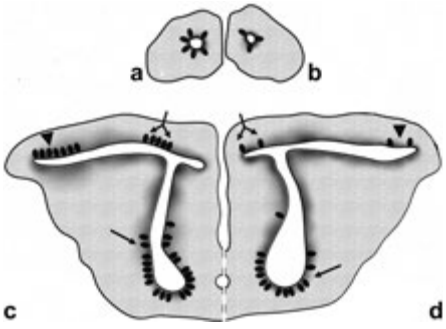

Figure 4. Drawings (not to scale) of a transverse view of the olfactory peduncles (a, b) and telencephalic hemispheres (c, d) of adult *Podarcis sicula* caught in the wild in spring, not brain injured. The dots represent PCNA immuno-labelled matrix cells, isolated in the olfactory ependyma and periependymal grey matter (a, b) and clustered in the walls of ventricular cavities to form the *zonae germinativae latero-dorsales* (arrowheads), *mediodorsales* (arrowforkeds) and *ventrales* (arrows) (c, d). a, c: Cold-shocked specimens. b, d: Specimens not subjected to cold shock.

our previous experimental and normal specimens, while no PCNA immunoreactivity were seen behind diencephalon.

The influence of the different seasonal conditions in association or not with lowered body temperature was in the past investigated by Minelli et al. (1982a) on normal and brain injured adult *R. esculenta* by means of autoradiography and quantitative analysis, carrying on two parallel, differently extended studies, the second of which on only a handful of samples. Altogether, a strict correlation was found between environmental or artificial environmental factors (climate and cold shock) and encephalic cell proliferative fluctuations. Spontaneous proliferation was extremely low and rapidly increased if coupled to a cold shock in samples caught in the wild in May-June, while it was very high and rapidly decreased if coupled to a cold shock in samples caught in the wild in September-October. The spontaneous expression of PCNA was intermediate and slightly decreased upon cold shock in samples caught in the wild in advanced November.

These authors also suggested that the different kariocynetic rithms of the various seasons could explain the contradictory results reached by some authors on the regenerative performances of the brain in adult Anuran Amphibia.

Therefore, the issue raised by the observations of Minelli et al. (1982a) was confirmed by our immunohistochemical analyses in normal brain of adult *R. bergeri*

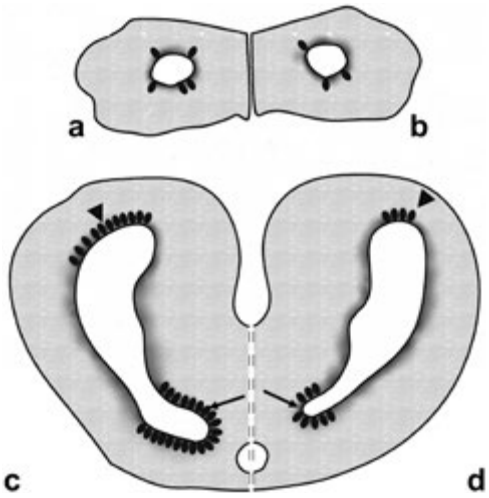

Figure 5. Drawings (not to scale) of a transverse view of the olfactory bulbs (a, b) and telencephalic hemispheres (c, d) of adult *Rana bergeri* caught in the wild in spring, not brain injured. The dots represent PCNA immuno-labelled matrix cells, isolated in the olfactory ependyma and periependymal grey matter (a, b) and clustered in the walls of ventricular cavities to form the *zonae germinativae dorsales* (arrowheads) and *ventrales* (arrows) (c, d). a, c: Cold-shocked, specimens. b, d: Specimens not subjected to cold shock (b, d).

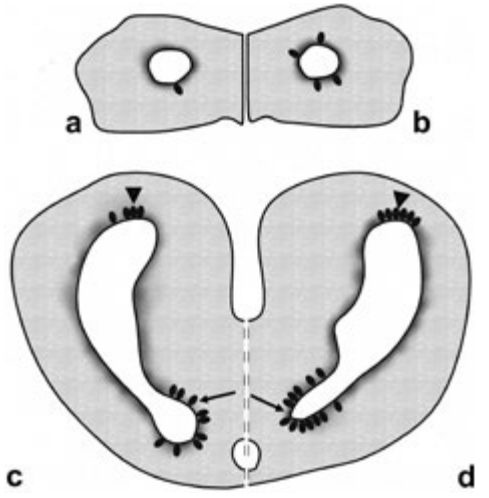

Figure 6. Drawings (not to scale) of a transverse view of the olfactory bulbs (a, b) and telencephalic hemispheres (c, d) of adult *Rana bergeri* caught in the wild in autumn, not brain injured. The dots represent PCNA immuno-labelled matrix cells, isolated in the olfactory ependyma and periependymal grey matter (a, b) and clustered in the walls of ventricular cavities to form the *zonae germinativae dorsales* (arrowheads) and *ventrales* (arrows) (c, d). a, c: Cold-shocked specimens. b, d: Specimens not subjected to cold shock (b, d).

caught in nature at the same times of Minelli et al. (1982a), *i.e.* spring and autumn, and then thermal-stressed. As previously done also in *T. carnifex* and *P. sicula*, the time lapse between the transfer of the cold-stressed frogs to the external temperature and sacrifice was one week, since it had been shown in newts that this interval allows for the expression of maximal brain cell proliferation (Franceschini et al., 1992).

Immunolabelling for PCNA was more substantial in spring (Chimenti and Margotta, 2015) than in autumn (Margotta, 2015) both at olfactory (Figs. 5a, b; Figs. 6a, b) and telencephalic levels where *zonae germinativae dorsales* and *ventrales* were clearly identified in the latter position (Figs. 5c, d; Figs. 6c, d). Also the diencephalic region showed in the two seasons the same labelling aspects as previously anticipated, both in experimentally treated animals and the respective controls. No immunoreaction was found behind the diencephalon, where the tissue is known to virtually lack such potential.

All these results supported and reinforced previous findings on the brain of adult specimens of Anamnia and poikilothermal Amniota, from lamprey (Margotta et al., 2007) to lizard (Margotta et al., 1999, 2005b), and strongly suggest that the input of the seasonal-photoperiodic cycle alone or combined with induced cooling on the brain of adult fresh water, earth-dwelling Anamnia and heterothermic Amniota can exert a similar, positive influence on the proliferative activity of the forebrain, mainly olfactory bulbs (newts and frogs) or peduncles (lizards) and the telencephalic *zonae germinativae dorsales* and *ventrales*, where matrix cells and hence *matrix zonae* are best represented in the analysed vertebrates.

Also in adult life, the proliferative power of newts surpassed that of frogs and even more that of lizards (according to the well-known, classical hierarchy of earth-dwelling Anamnia and heterothermic Amniota for regenerative potential), however never reached the extent supposed to be necessary for regeneration upon partial tissue excision.

These findings strongly suggest that the seasonal-photoperiodic cycle or an unnaturally lowered body temperature alone induce to proliferate a lower number of neural-like cells than the association of induced cooling and trauma, like surgery or cerebral injury.

Moreover, the results support the hypothesis that if the spread between thermal parameters (annual temperature-daytime cycle *versus* experimentally applied cooling) is small it has a low effect on the encephalic proliferative activity and conversely if that spread is wide the thermal stress becomes a powerful inducer to proliferation for many cells in stand-by. This hypothesis is also supported by our findings on thermal-stressed frogs analysed in spring (Chimenti and Margotta, 2015) and in autumn (Margotta, 2015) and may explain the discrepancy between such results and those on surgically injured animals. In any case, proliferation in adult fresh water, earth-dwelling Anamnia and heterothermic Amniota occurs where matrix cells - and hence matrix zonae - are best represented, *i.e.* in the forebrain, mainly in olfactoty bulbs (newts, frogs), peduncles (lizards), and the telencephalic *zonae germinativae dorsales* and *ventrales*.

All these results and related hypotheses reinforce previous findings on the encephalon of uninjured, not thermal-stressed adult specimens of Anamnia and poikilothermal Amniota from lamprey (Margotta et al., 2007) to lizard (Margotta et al., 1999, 2005b) and also support recent findings in adult *R. bergeri* (Margotta, 2012) and *P. sicula* (Margotta, 2014a) in similar conditions and in uninjured, thermal-stressed adult *T. carnifex* (Chimenti and Margotta, 2013), *P. sicula* (Margotta, 2014b)

and *R. bergeri* analysed in spring (Chimenti and Margotta, 2015) and in autumn (Margotta, 2015). These latest findings are in agreement also with the observations of Minelli et al. (1982a) on an adult cold-shocked, uninjured brain frogs examined - in small numbers - in spring and in autumn.

Spinal cord

The first news about regeneration of the spinal cord date back more than two centuries: the observations of Spallanzani (1768) on the adult salamanders deprived of the tail.

The morphogenetic and histogenetic potential responsible for the adult repair of the spinal cord appears still unclear A role has been proposed for epimorphosis, *i.e.* the appearance on the tail wound surface of a morphogenetic mesenchymal cell crowding ("blastema") which evolves into tissue anlage. However the nature of the ependymal cells of the tubule by which the newly formed spinal cord originates remains to be established: they might arise from de-differentiation of ependymal cells, of mesenchyme-derived tissues and of other tissues belonging to the tail stump (as sometimes suggested), from a stock of stand-by stem cells pre-existing to amputation including ependymal cells of the stump (especially in adult newts of *Triturus* genus: Colucci, 1884; Caporaso, 1889; Stefanelli and Capriata, 1943; Marini and Margotta, 1961; Marini, 1968; Filoni and Margotta, 1969; Margotta and Filoni, 1969, 1970; Donaldson and Wilson, 1975; Iten and Bryant, 1976), or from both these sources.

Margotta et al. (2002) and Margotta (2004, 2008) in adult *T. carnifex* carried out repeated tail amputation (until the 16th segment, equally staggered) and observed the rebuilding of an approximately normal morpho-functional organization in both the medullary and ganglionic components of the regenerating tail. Since on one hand such drastic experimental conditions might have provoked a depletion of the dowry of undifferentiated cells and on the another hand there is evidence of persistence of DNA synthesis and mitotic activity in the ependymal layer, the hypothesis of an integration between the activation of an undifferentiated cell reserve and the de-differentiation of other cells to achieve such regeneration seems especially attractive.

Another debated issue is the rebuilding of spinal ganglia. Most authors favour the hypothesis that it occurs at the expense of undifferentiated cells mingled with ependymal cells of the regenerating spinal cord, since neuroblasts have been observed to come out from the ependyma through the grey and white matter until emerging from the ventral medullary surface (especially in adult *Triturus* genus: Marini and Baffoni, 1967; Marini, 1968; Géraudie et al., 1988, 1989; Arsanto et al., 1992; Filoni et al., 1995; Margotta et al., 2002; Margotta, 2004, 2008). Others authors claim that the newly formed ganglia arise from the trans-differentiation of cells stemming from mesenchymal cells of meningeal origin or from de-differentiation of meningeal and Schwann cells (Anton et al., 1986; Anton and Döring, 1988; see also Géraudie et al., 1988, 1989). In the studies of these issues Urodela were privileged, the most investigated genus being *Triturus*.

Therefore in the examined organisms the cellular basis of plasticity would be at least in part similar between spinal cord and brain, lying in undifferentiated ependymal cells in both sites, and the nervous tissue should be numbered among "stable" rather than "perennial" tissues according to the traditional classification of Bizzozero (1894).

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