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High values of DNA content in the hypotalamic neurons of *Lophius piscatorius* and *Diodon holacanthus* (Osteichthyes)

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Abstract — Computerised image analysis on Feulgen-stained preparations indicated that in both *Lophius piscatorius* and *Diodon holacanthus* the Feulgen-DNA content of the hypothalamic magnocellular neurons in the preoptic and tuberal complexes largely exceeded 2C. Subsequent quantitative microfluorometric evaluation revealed that the DNA content of these cells was as higher as 68C in *L. piscatorius* and 84C in *D. holacanthus.* The presence of high levels of DNA content in neuron nuclei is well known in the case of the giant neurons of Molluscs but, although it has recently been found in the supramedullary neuron cluster of the same Teleosts used in this study, it must be considered exceptional for the nervous system of Vertebrates.

Key words: DNA content, Feulgen-DNA, Cytofluorometric evaluation, Hypothalamic neurons, Teleosts.

INTRODUCTION

The central nervous system (c.n.s.) of Teleosts belonging to the orders Batrachoidiformes, Tetraodontiformes and Lophiiformes presents a cluster of large neurons located dorsally at the boundary between the spinal cord and the medulla oblongata (FRITSCH 1884, 1886; TAGLIANI 1894, 1895, 1897; DAHLGREN 1897; BURR 1928; BENNETT *et al.* 1959a, b, c; BENNETT 1960; ISHIBASHI 1962; NAKAJIMA *et al.* 1965; BENNETT *et al.* 1967; LEHRER *et al.* 1968; LUKER 1975; BARRY *et al.* 1986; MOLA 1990; BENEDETTI and MOLA 1991). The function and the axon pathway of these nerve elements are still an open question (FUNAKOSHI *et al.* 1998).

Recently, microfluorometric analysis on the cluster neurons of *Lophius piscatorius* (Lophiiformes) revealed that the DNA content of these cells is very much higher than 2C (BENEDETTI and MOLA 1991; BENEDETTI *et al.* 1993), reaching values of over 5000C (SASSI *et al.* 1995).

Observations carried out on the cluster neurons of *Diodon holacanthus* (Tetraodontiformes) confirmed this peculiarity, which, although well known in the case of the giant neurons of Molluscs, is exceptional in that of the nervous system of Vertebrates (MoLA *et al.* 1992).

Accordingly, we set out to verify the possible presence of this phenomenon in other areas of the central nervous system of both *L. piscatorius* and *D. holacanthus,* particular attention being paid to the hypothalamic region of the diencephalic vescicle, since it contains relatively large neurons, whose function and axon pathway are known (KUHLENBECK, 1977).

To perform this study, the Feulgen-DNA content of hypothalamic neurons in specimens of *L. piscatorius* and *D. holacanthus* was evaluated on Feulgen-stained preparations by means of a computerised image analysis system, and the real DNA content was measured cytofluorometrically after staining with ethidium bromide (EB).

MATERIALS AND METHODS

Material — Seven specimens of *Lophius piscatorius* L. (Lophiiformes, Osteichthyes), ranging in length from 15 to 22 cm, were caught in the Tyrrhenian Sea; seven specimens of *Diodon holacanthus* L. (Tetraodontiformes, Osteichthyes), ranging in length from '

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10 to 17 cm, were obtained from the Philippines and kept for some days in an aquarium.

All animals were killed immediately prior to analysis and the central nervous systems surgically isolated.

Feulgen staining — The isolated brains from four specimens of *L. piscatorius* and four specimens of D. *holacanthus* were fixed in 10% neutral formalin for 24 hours at room temperature, and then rinsed in tap water for 24 hours. The fixed brains were embedded in celloidin-paraffin and cut into 7 µm transversal sections. The histological slides were then subjected to Feulgen reaction with 5N HC1 hydrolysis for 60 min at room temperature and immersion in Schiff reagent for 45 min (BERNOCCHI *et al.* 1976).

After staining, the preparations were examined by means of a computerized image analysis system at the Centro Interdipartimentale Grandi Strumenti, University of Modena.

EB staining — Microfluorometric analysis of the DNA content of diencephalic neurons was carried on three specimens of L. *piscatorius* and three specimens of *D. holacanthus.* The diencephalic vescicles including the preoptic and tuberal complexes were surgically isolated and cut into small pieces; cytological imprints were made by gently touching the surface of the slides with these fragments.

The slides were air dried, fixed in methanol: acetic acid (3: 1, v: v) for 5 min at 4°C and buffered with PBS, pH 7.0, for 2 min at room temperature, and then treated with 0.8 mg/ml ribonuclease I-A in distilled water, pH 6.8, for 1 h at 37°C. Staining was performed with an 0.04% solution of ethidium bromide (EB) in PBS, pH 7.4, for 25 min in the dark. The preparations were then rinsed in PBS, pH 7.4, for 5 min in the dark and mounted in PBS, pH 7.4.

Fluorescence microscopy analysis — Fluorescence emissions were evaluated by means of a Zeiss Photomicroskop III equipped with Photometer 03 microfluorometer and HBO 100 mercury vapor light source. A specific combination of filters was employed: exciter BP 546/12, dicroic FT 580 and barrier LP 590.

Cytofluorometric evaluations were only carried out on nuclei that remained intact after dissection of the c.n.s. and cytological imprints; damaged nuclei were discarded. Nuclear size was measured with a micrometric eyepiece.

The C values were obtained by estimating the ratio between the emission values of the hypothalamic neurons and the average 2C value measured on glial cells from the same specimen. The coefficient of variation ranged between 5% and 10%.

Statistical analysis was performed using the SPSS statistical package.

RESULTS

Light microscopy analysis of histological sections subjected to Feulgen reaction showed that the nucleus of the hypothalamic magnocellular neurons was intensely stained in both L. *piscatorius* (Fig. la) and *D. holacanthus* (Fig. Ib).

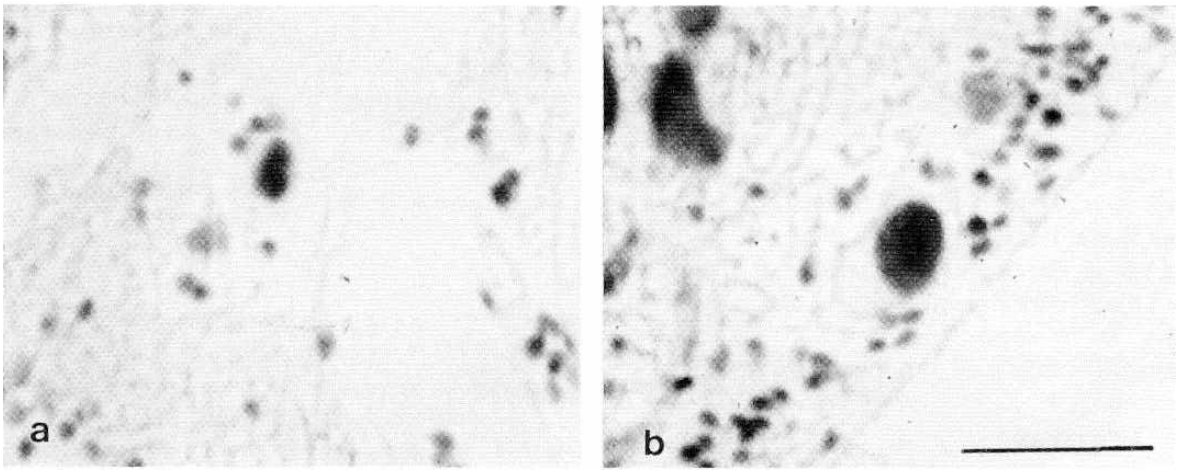

Fig. 1 — Hystological sections of hypothalamic neurosecretory neurons of L. *piscatorius* (a) and D. *holacanthus* (b) after Fuelgen reaction. Bar corresponds to 50 µm.

The above findings were confirmed by computerized image analysis, measuring the total nuclear area, the integrated optic density (i.o.d.) and the i.o.d. per area unit. This analysis was carried out both on the hypothalamic neurosecretory neurons (preoptic and tuberal complexes), and on the surrounding glial and ependimal cells. The results showed that the i.o.d. per area unit was comparable in all the cells in question, indicating that the increase in the nuclear area of the neurosecretory neurons was matched by an increase in their Feulgen-DNA content. On the other hand, the large rombencephalic reticular neurons and the mesencephalic trigeminal neurons, serving as controls, presented an extremely pale nucleus because of the dispersion of the chromatin.

After EB staining, microfluorometric analysis demonstrated that the hypothalamic neurons possess nuclei whose DNA content increased with the increase of their size, reaching values of about 68C in *L. piscatorius* (Fig. 2a) and about 84C in *D. holacanthus* (Fig. 2b). Furthermore, there was an evident correlation between DNA content and nuclear size (Fig. 3).

The percent distributions of C values in the nuclei of hypothalamic neurons from *L. piscatorius* (fig. 4a) and *D. holacanthus* (fig. 4b) showed that, in general, the peaks did not correspond to integral multiples of the 2C diploid amount.

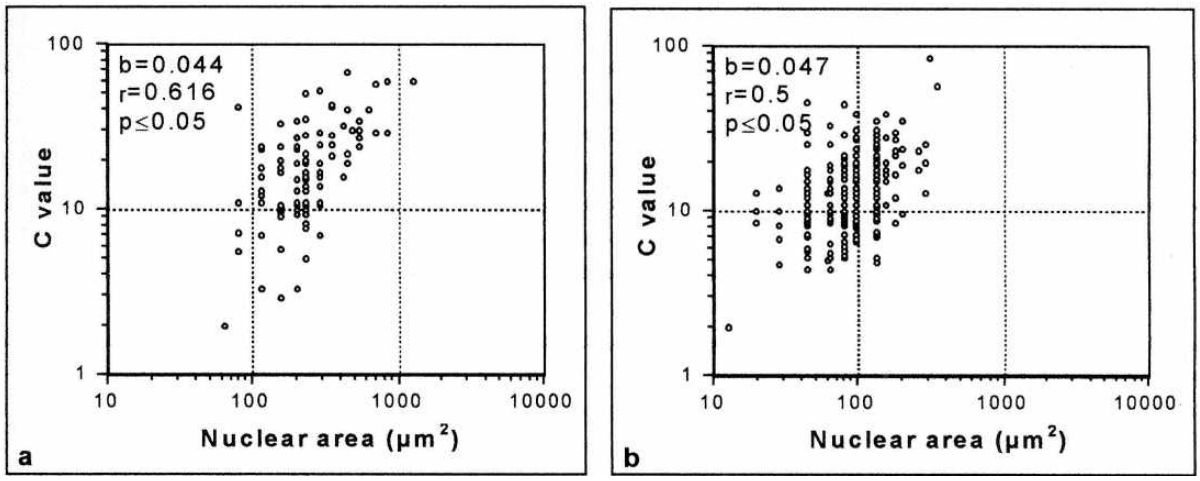

Fig. 2 — Relationship between nuclear area and DNA content (expressed as C value) in hypothalamic neurons o f *L. piscatorius* (a) and *D. holacanthus* (b).

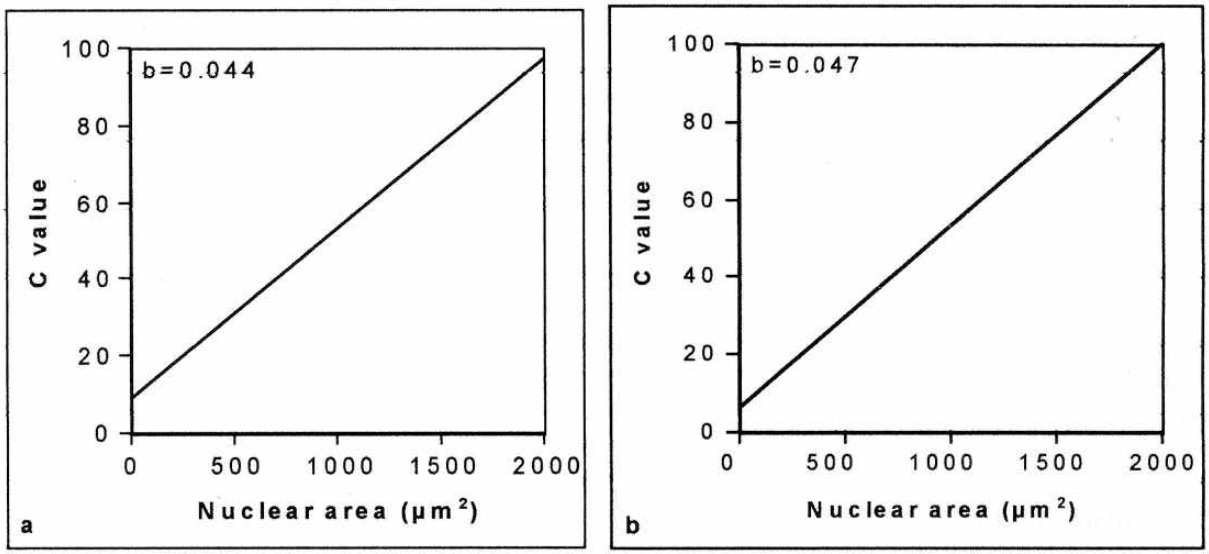

Fig. 3 — Regression coefficient of C value on nuclear area in hypothalamic neurons of L. *piscatorius* (a) and *D. holacanthus* (b).

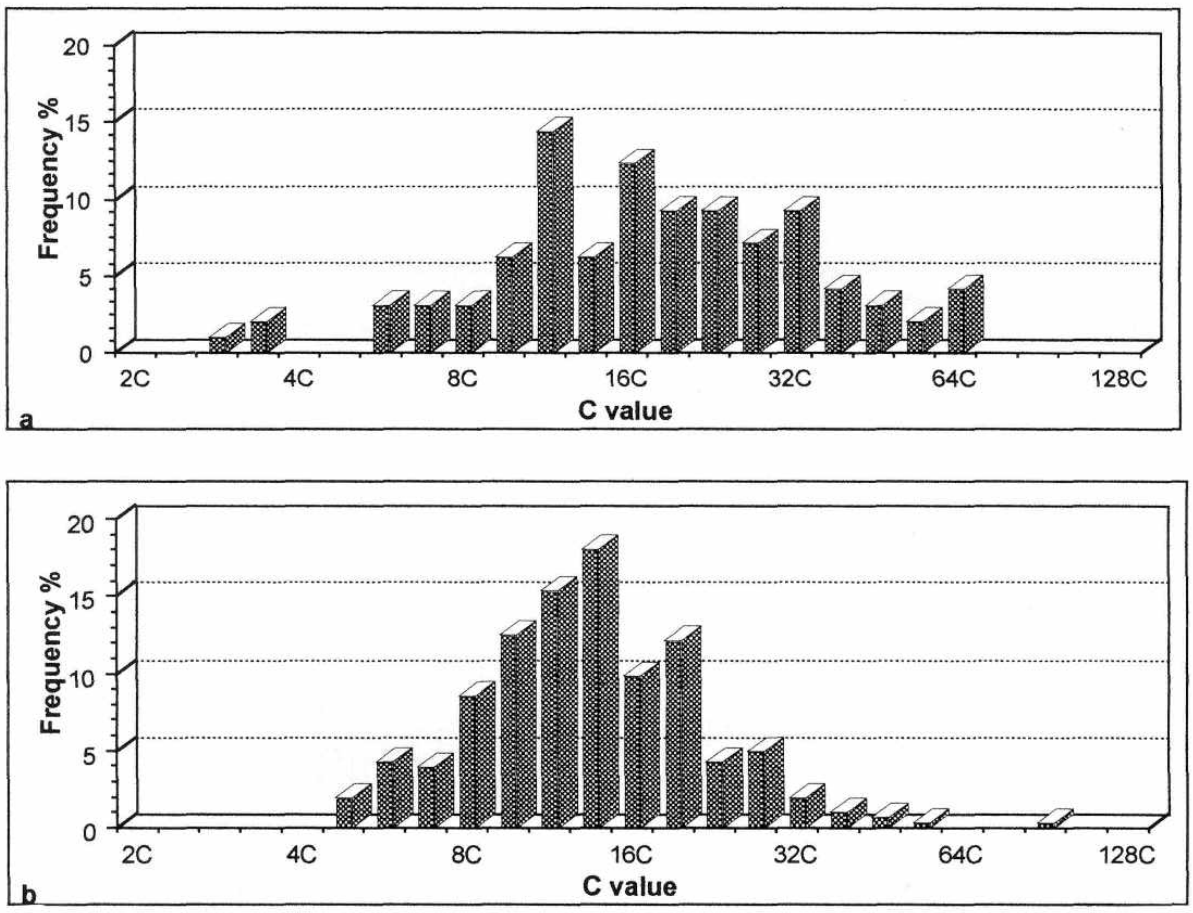

Fig. 4 — Percent distribution histograms of C value measured in hypothalamic neurons of *L. piscatonus* (a) and *D. holacanthus* (b).

DISCUSSION

It is known that the amount of nuclear DNA content in the neurons of Vertebrates is generally 2C, and that their chromatin appears more finely granular and dispersed as the nuclear area increases (MARINI 1956). On the other hand, Feulgen-DNA values reaching a maximum of 4C have been demonstrated in Purkinje cells in different classes of Vertebrates (BOHM *et al.* 1981; BRODSKY *et al.* 1979, 1984; SWARTZ and BHATNAGAR 1981). This finding has been correlated with different developmental and functional stages (BERNOCCHI and SCHERINI 1981; BERNOCCHI 1983, 1985; BERNOCCHI and BARNI 1985; Bernocchi *et al.*, 1986; Giacometti *et al.*, 1986), but variations in the Feulgen-DNA content of Purkinje cells have also been ascribed to experimental manipulations (SCHERINI *et al.* 1988; BERNOCCHI *et al.* 1989). Only recently, nuclei with a DNA content largely exceeding 2C have been found in the large cluster neurons, located at the boundary between the spinal cord and the medulla oblongata, in both L. *piscatonus* (BENEDETTI and MOLA 1991; BENEDETTI *et al.* 1993; SASSI *et al.* 1995) and D. *holacanthus* (MOLA *et al.* 1992).

The findings illustrated in this paper indicate that, in the above mentioned fish species, the presence of marked increases in neuron DNA content is not an exclusive characteristic of the supramedullary neuron cluster but it can also be found in neurons isolated by cytological imprints of diencephalic vesicle. Moreover, computerized image analysis of hystological sections of diencephalic vesicle showed that an increase in Feulgen-DNA content was present in preoptic and tuberal magnocellular neurons but was absent in other diencephalic cells. In this connection, it is reasonable to assume that the diencephalic neurons in which cytofluoro-metric analysis showed a nuclear DNA content

largely exceeding 2C belong to the preoptic and tuberal complexes. In both species, the neuron DNA content increased progressively with the increase of the nuclear area, although it could vary considerably, as shown by the correlation coefficients; indeed, neurons of a given nuclear size could present different DNA contents. This data could be influenced by the heterogeneity of the neuron population examined. Comparison of the regression coefficients (b=0.044 in *L. piscatorius* and b-0.047 in *D. holacanthus)* indicated that, in the hypothalamic magnocellular neurons, the relationship between the increase in nuclear area and that in DNA content followed a similar pattern in both species.

High levels of DNA content may be obtained by repeated duplications of the whole genome or by selected amplification of some genome fractions. The lack of peaks corresponding to the doubling classes of total DNA content suggest that the increase in DNA content observed in both species could be due to differential amplification of the genome. In this context, it must be emphasized that evidence of differential genome endoreplication has been found in the cluster neurons of L. *piscatorius (SASSI et al.* 1995).

Studies concerning the giant neurons of Molluscs suggest a direct relationship between neuron DNA content, nuclear size and area of innervation (GILLETTE 1991). This hypothesis may hold good also for the hypothalamic magnocellular elements of *L. piscatorius* and *D. holacanthus* since the neurosecretory neurons utilized for our research are relatively large and influence quite wide areas, albeit indirectly by means of the vascular system.

Taken as a whole, the data presented in this paper can serve as a starting-point for further studies aimed at identifying the mechanisms by which, and the reasons for which, neurons of Vertebrates increase their DNA content. In fact, whereas the functional meaning and axon pathway of the cluster neurons are as yet unclear, the nerve connections, neurosecretory functions and products of the preoptic and tuberal magnocellular elements are well known.

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