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Renewal of DNA in Purkinje Cell Nuclei of Mouse Cerebellum. A 9-Month Follow-up Autoradiographic Study

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Summary

^{3}H thymidine was injected into pregnant mice in order to label the DNA in the dividing Purkinje cell (PC) precursors of the embryonic cerebellum. The retention of ^{3}H-DNA was evaluated in PC nuclei of animals at the age of 25 days, 3, 6 or 9 months by light microscope autoradiography. The number of silver grains decreased in the whole nuclei by 13.6 % and 19.6 % in animals 6- and 9-month-old, respectively. In the nucleolar region, the loss of DNA radioactivity was more profound; the silver grain counts decreased by 22.6 % and 29.1 % in 6-and 9-month-old animals, respectively. No significant differences in the volume and dry mass concentration were found in the PC nuclei of 25 PD and 9 PM old animals. Therefore, the observed changes in grain density counts represent the actual measure of ^{3}H-DNA loss, and /or "spontaneous" renewal of the DNA molecule in PC nuclei, as well as its higher expression in the nucleolar region. Furthermore, it follows from the comparison of our data with those present in the literature, that DNA synthesized in nerve cell precursors before their withdrawal from the mitotic cycle is more stable than that synthesized in postmitotic neurones. This suggests that the repair of DNA in mature neurones might be of an error-prone type.

Key words

Nerve cell – ^{3}H thymidine autoradiography – DNA repair – unscheduled synthesis of DNA – nucleolus

Introduction

Maintenance of the integrity of cell populations, particularly those which cannot be renewed by cell division, such as neurones, requires effective repair of the transcribed part of the genome. Accumulation of DNA lesions may account for the aging of the brain and the origin of some neurodegenerative syndromes (Bradley and Krasin 1982). Indeed, defects in DNA repair have been observed in lymphocytes and fibroblasts of such patients (Kidson and Chen 1986, Tandan *et al.* 1987). The efficacy of DNA repair in the brain, particularly neurones, has, however, been a matter of controversy. Indications of the unscheduled synthesis of DNA as a measure of excision repair, have been reported in some, but not all, autoradiographic studies and/or experimental conditions (Watson 1965, Cameron and Adrian 1979, Dropp and Sodetz 1971, Korr *et al.* 1973, Korr and Schultze 1989, Mareš *et al.* 1973, 1974, Scherini *et al.* 1984, 1988, Williams *et al.* 1982). In the incorporation studies, this is mainly because of the low grain counts occurring over nuclei of postmitotic neurones which often cast reasonable doubts on the statistical and biological significance of data in some studies. Great divergences are, however, also apparent in the results of studies monitoring retention of relatively higher radioactivity incorporated into the DNA of dividing nerve cell precursors or mature neurones. As reviewed in Table 1, some authors found the radioactivity unchanged during long time intervals (Bennett *et al.* 1960, Haas *et al.* 1970, Mareš *et al.* 1974, Ishikawa 1986), while others reported its rapid decay (Haas and Werner 1973, Cameron and Adrian 1979,

Perrone-Capano *et al.* 1982). Indirect support for unscheduled DNA synthesis is provided by the biochemical data on the activity of DNA synthesizing enzymes in the mature brain (Kuenzle 1985, Ivanov 1988).

Thus, the proper estimation of DNA repair, both as to its existence *per se* or its real extent, is very difficult. Nevertheless, a comparison of the data obtained on different biological models, and/or conditions, led us to the assumption that the discrepancies may be partly caused by a higher stability of DNA synthesized before terminal mitosis and by a more effective repair of some parts of the genome (Table 1).

In order to re-examine the high degree of stability of premitotically synthesized DNA, we performed a follow-up study of ^{3}H-DNA in Purkinje cells (PC) of mice labelled with ^{3}H-thymidine prenatally. In contrast to our earlier study (Mareš *et al.* 1974), the animals were kept alive for a substantially longer time interval. Furthermore, in order to test the possibility of intranuclear differences in DNA renewal, the nucleolar region, as a part of the genome with a distinct structure and high template activity, was evaluated separately.

Table 1
Half-life time of DNA in nerve cell nuclei

Species	Half-life	Methods	Region/cells	Reference
Mouse	115-420 d	prem, arg	n.s.	Pelc 1968
Mouse	12 d	postm, arg	nc.n.XII	Cameron and Adrian 1979
Mouse	21d (502 h)	postm, arg	brain stem	Williams *et al.* 1982
Mouse	7-14 d	postm, bioch	whole brain	Kimberlin *et al.* 1974
Rat	6h, 100 h	postm, bioch	whole brain	Merrits and Cain 1969
Rat	20-60 h	postm, bioch	forebrain	Rattan 1989
Mouse	inf.	prem, arg	Purkinje cells	Mares *et al.* 1974
Rat	inf.	prem, arg	photoreceptors	Ishikawa 1986
Mouse	inf.	prem, bioch	whole brain	Bennett *et al.* 1960
Mouse	16 d, 257 d	postm, bioch	whole brain	Commerford *et al.* 1982
Human	inf.	prem, C12/13	cerebellum	Slatkin 1985
Rat*	8 d, inf.	prem, arg	Purkinje cells	Haas and Werner 1973
Rat*	3.3-31 d, inf	prem,arg	cortical neurones	Haas *et al.* 1970

Pairs or triples of figures refer to half-life values calculated from the initial and later parts of the radioactive DNA disappearance curves

Abbreviations and symbols:

prem: DNA labelled during premitotic S-phase of dividing precursors of nerve cells; postm: DNA labelled in postmitotic neurones; arg: autoradiographic study; bioch: biochemical study; n.s.: the region was not specified; inf.: no measurable turnover within a life span of the species was declared by author or could be calculated from the reported data; d, h: days, hours; * : approx. values calculated by us from the graphical data, nc.n.XII: hypoglossal nucleus.

Material and Methods

Animals, isotope injections and histological procedure.

Female NMRI mice (Zentralinstitut für Versuchstierzucht, Hannover) were mated overnight. Later, two animals with postcoital vaginal plugs received an intraperitoneal injection of 5 μCi of ^{3}H-thymidine per gram of body weight on the 13th to 14th day of pregnancy (specific activity 20 Ci/mmol, UVVVR, Prague). The offsprings (3 to 4 animals per age group randomly chosen from both litters) were killed by cervical dislocation on postnatal day 25 or at ages 3, 6 or 9 months. After weaning (the 28th day), the animals were fed Altromin diet (Altrogge, Lage/Lippe). The brains were fixed in Carnoy solution (6:3:1) and embedded in paraffin.

Autoradiography.

Five micrometers thick sections cut at the middle sagittal plane were covered with Stripping Film

Kodak AR10 (stretched in 0.05 % KBr solution) and exposed for 60 days in the dark at 4 °C. The autoradiograms were stained by toluidine blue. Some slides were autoradiographed after treatment with 0.03 % DNase I (Serva, Heidelberg) in 0.1 M phosphate buffer, pH 7, at 37 °C for 5 h. Sections from animals which did not receive ^{3}H-thymidine were autoradiographed in parallel. The silver grains were counted above the whole nuclei and then, separately above the nucleolar region, involving the nucleolus and the perinucleolar chromatin. The background was evaluated in the nuclei-free neuropil of the molecular layer. In order to diminish the sampling error, the background was always determined in the largest area of nuclei-free neuropil of the molecular layer near the Purkinje cells. The background values (grain counts per 100 μm^2) were adjusted to the area of the nearby Purkinje cells nucleus and subtracted from the crude grain counts observed above the nuclei by an on-line computer program. A Leitz Ortholux microscope, with a graphic tablet on line with an Apple IIc microcomputer was used at 1000x magnification. Purkinje cells were randomly selected, i.e. non-labelled cells were also enclosed in the counting.

Nuclear size and dry mass density measurements.

Areas (in square arbitrary units, a.u.) of PC nuclei were calculated from the diameters (d_1, d_2) determined with a graphic tablet by the formula:

$$A = d_1 \times d_2 \times K^2 \times \pi/4,$$

where K is a constant depending on the instrumental setting. The dry mass concentrations of PC nuclei and nucleolar region were measured in deparaffinized sections mounted in distilled water. The optical path difference was determined using a Leitz double-beam microinterferometer, in the "homogeneous monochromatic light field" (Hg green light, lambda = 5461 A). The mass density was calculated according to the formula:

mass density = optical path density x lambda/alpha,

where lambda = 5461 A and alpha is the specific light increment (0.170 cm^3/g for proteins and nucleic acids, Vohringer and Maurer 1971).

Statistics.

The data are given as means ±S.E.M. Statistical differences in grain counts were evaluated by the Mann-Whitney and χ^2 tests; for dry mass concentration and nuclear size data Student's t test and analysis of variance were used.

Results

Autoradiography

The average labelling in the neuropil free of cell nuclei was 1.00±0.04 silver grains per 100 μm^2 and did not significantly differ in the autoradiograms of particular age groups. In slides treated with DNAse, the average grain count was 0.87±0.03 above PC nuclei and 0.98±0.04 above the adjacent nuclei-free neuropil. Similar values (0.88±0.04) were measured in animals which did not receive ^{3}H-thymidine.

Table 2
Silver grain counts in Purkinje cell nuclei

Age	No.nuclei	Whole nucleus	Signif.	Nucleolar region	Signif.
25 days	361	14.8±0.60		3.64±0.19	
3 months	261	13.4±0.89	*	3.00±0.19	**
6 months	288	12.8±0.75* (−13.6 %)	**	2.82±0.17** (−22.6 %)	**
9 months	284	11.9±0.70** (−19.6 %)		2.58±0.15** (−29.1 %)	

*p < 0.01, **p < 0.001 Significance according to Mann-Whitney test. The values measured in 25 days age group were always taken as 100 % for calculation of the decrease in ^{3}H DNA indicated in brackets.

The labelling intensity of PC nuclei (after subtraction of the background) is given in Table 2. The data concerning whole nuclei indicate a progressive decrease in the grain counts with the age of animals. The decrease became first significant at 6 months (−13.6 %, $p<0.01$) and reached −19.6 % by 9 months ($p<0.001$).

More profound changes in grain count densities were found above the nucleolar region of PCs (Table 2). The decline in radioactivity became highly significant between 25 days and 6 months (−22.6 %; $p<0.001$). At 9 months, the drop in the labelling had reached -29.1 % ($p<0.001$). The same level of significance was obtained when the χ^2 test was applied to evaluate the differences in the nuclear and nucleolar labelling between the age groups studied.

The frequency distribution of the grain counts above the PC nuclei of the individual age groups is shown in Fig. 1. A high scatter of DNA labelling, from 0 to about 60 silver grains per nucleus, was found. This is likely due to the phase of the cell cycle (G_1, S, G_2) in which the cells were at the time of isotope injection,

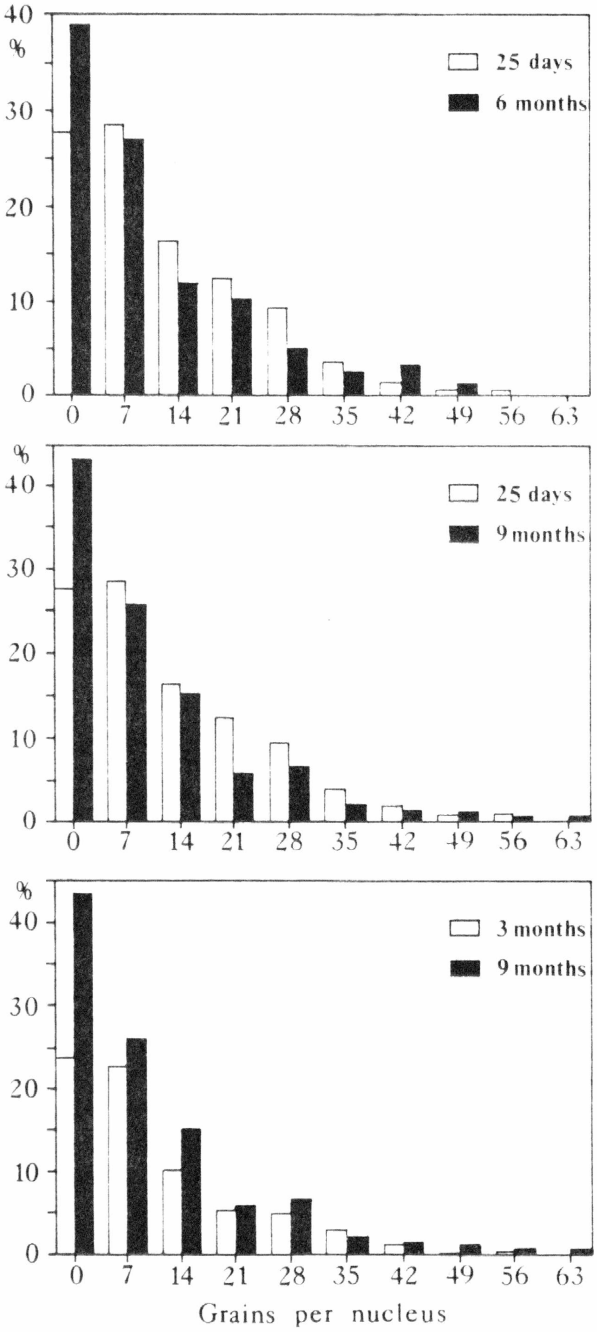

Fig. 1
The frequency distribution of grain counts (abscissa) in Purkinje cell nuclei of mice of various age.
(a) 25 days vs. 6 months (b) 25 days vs. 9 months (c) 3 months vs. 9 months. Data corrected for background.

and/or the number of mitotic divisions the cell had passed after the injection of ^{3}H-thymidine. In any case, the range of silver grain counts was the same for all age groups, so that even the oldest animals still showed densely labelled cells. This may indicate that the loss of radioactivity found in 6- or 9-month-old mice does not depend on the intensity of the initial labelling.

Nuclear size and dry mass concentration

The average area of nuclei of Purkinje cells in 25-day-old and 9-month-old animals did not differ (Table 3). The values measured in 3- and 6-month-old groups ranged insignificantly within these limits.

The average dry mass concentration in the whole nuclei and nucleoli of 25-day-old and 9-month-old animals was also not significantly different (Table 3).

Table 3
Area and dry mass concentration in Purkinje cell nuclei

Age	Area[1]	Dry-mass concentration[2]	
		Nucleus	Nucleolar region
25 days	165 ± 1.23 (361)	0.128 ± 0.004 (115)	0.181 ± 0.021 (73)
9 months	161 ± 1.57 (284)	0.125 ± 0.004 (78)	0.190 ± 0.007 (45)
	(−2.4 %) n.s.	(−2.3 %) n.s.	(+4.9 %) n.s.

[1] in square arbitrary units (a.u.); [2] in mg/cm^2, the number of measured nuclei in parenthesis, n.s.: non-significant difference (Student's t-test)

Discussion

The changes in grain counts in follow-up autoradiographic studies may be caused by an exchange of the labelled parts of a molecule, different efficacy of autoradiographic detection of radioactivity in the individual age groups or a loss of the more heavily labelled cells due to radiation. The efficacy of ^{3}H-thymidine autoradiography depends on the dry mass concentration, and/or beta-self-absorption, as well as on the volume of examined nuclei (Maurer and Primbsch 1964, Mareš *et al.* 1974).

As shown in Table 3, none of these parameters changed significantly in Purkinje cells (PCs) within the age-span studied. A similar incidence of heavily labelled nuclei in the youngest and oldest age groups

of animals (Fig. 1) excludes a loss of more heavily labelled PCs due to radiation. Therefore, the grain counts reported in our study can be considered as a reliable indicator of the relative amount of ^{3}H-DNA and their drop in PCs of 6 to 9-month-old animals as evidence of the nucleoside replacement and/or renewal of the labelled parts of the DNA molecule.

The study also revealed a higher rate of DNA renewal in the nucleolar region (Table 2), which is characterized by a distinct structure of chromatin and an intense transcription of rRNA genes (Stoykova *et al.* 1985). This fits well with earlier observations of a more intense incorporation of ^{3}H-thymidine in this part of the nucleus of PCs or some other types of neurones (Dropp and Sodetz 1971, Mareš *et al.* 1973, Winzerith *et al.* 1977, Cameron and Adrian 1979, Brodsky *et al.* 1984) as well as an uneven distribution of ^{3}H-thymidine incorporation, and/or DNA repair, in the genome of some non-neuronal cells (for a review see Rattan 1989) and PCs with damaged DNA by bleomycin (Scherini *et al.* 1988)

The average rate of DNA renewal revealed in PCs in this study is rather low but exceeds that reported for some other neuronal populations. For instance, no change in ^{3}H-DNA content occurred in the rat retina photoreceptor cells until the 730th day of postnatal life (Ishikawa 1986). Similarly, Bennett *et al.* (1960) did not find any change in ^{14}C-DNA activity in the brain of mice surviving one year after a prenatal injection of ^{14}C-adenine. Neither has a reasonable DNA turnover been found in studies of the human cerebellum. This follows from the unchanged carbon isotope ratio (^{13}C/^{12}C), acquired from the maternal diet during embryonic development, in adult Europe-born Americans, Europe-born Europeans and American-born Americans during the whole of postnatal life (Slatkin 1985). A significant change of ^{3}H-DNA content was also not found in our earlier study on mouse PCs monitored for the first trimester of life (Mares *et al.* 1974). Some of these studies have, however, not performed at cellular level (Bennett *et al.* 1960, Slatkin 1985) and only whole nerve cell nuclei were evaluated by Mares *et al.* (1974). Similarly, a low rate of DNA turnover might have been masked by crude adjustment of DNA radioactivity data for postnatal growth of brain (Bennett *et al.* 1960). Finally, a difference in the phenotype of retinal neurons and PCs (Ishikawa 1986), or insufficient duration of the earlier experiment on PCs (less than 3 months, Mares *et al.* 1974) may explain some differences in the present and earlier findings.

On the other hand, the rate of DNA turnover in PCs observed in this study is much lower than in non-dividing neurones labelled postnatally i.e. due to unscheduled DNA synthesis. For instance, the half-life time of ^{3}H-DNA in nerve cells of the mouse hypoglossal nucleus and brain stem neurones was only about 20 days (Cameron and Adrian 1979, Williams *et al.* 1982). In the cortical neurone-enriched fractions of young adult rats, the biochemically measured half-life of DNA was less than 13 h (Perrone-Capano *et al.* 1982). These comparisons suggest that repair of DNA in postmitotic neurones may not be error-free and the newly synthesized DNA prone to a faster renewal. If so, many of the discrepancies in the earlier studies may be attributed to different biological models and experimental conditions. It is, however, to be pointed out that indications of the very short half-life time of DNA reported in some biochemical studies (Perrone-Capano *et al.* 1982) may also be due to the contamination of bulk isolated neurones by glial cells, particularly those dying soon after mitosis as a consequence of the physiological renewal of the population (Mareš and Lisý 1983).

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