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Stimulation of Brain Development in Chick Embryo by Elevated Temperature*

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Summary. Experimental chick embryos were incubated at 37.5° C till day 7 and after day 10, and at 40.5° C on days 7–10; their optic lobes and cerebral hemispheres at day 10 and at hatching were compared with controls incubated at 37.5° C only. Cell numbers at day 10 were directly counted by a new method involving formalin fixation and cell disaggregation by gentle sonication. At hatching, body weights, organ weights and organ DNA (cell numbers) were the same in experimentals and in controls, for both optic lobes and cerebral hemispheres, though the protein contents were significantly higher in experimentals. However, at 10 days (end of neuron proliferation) the weights and the cell numbers in experimentals were significantly higher. Two possible explanations have been offered: 1. Elevated neuron population in experimental animals at day 10 is followed by their elevated death rate, or 2. The increment in neuron number is permanent but at hatching it is overshadowed by the population of other cells.

Key words: Brain development – Brain, Chick embryo – Stimulation, brain development – Temperature, effect on brain development.

Cerebral cell numbers at birth (mammals) or at hatching (birds) tend to remain uniform within the species and the strain (Zamenhof and van Marthens, 1971); however, occasionally, they may undergo considerable deviations, presumably due to variations in the environmental conditions during the sensitive proliferation period of these cells. The deviations may be spontaneous or induced. Of the latter, the *decrease* in the number of cerebral cells in mammals, induced by prenatal or postnatal malnutrition, has been frequently reported. On the other hand, the *increase* has been demonstrated only in special cases (review in Zamenhof and van Marthens, 1974), and *a priori* one might suspect the presence of some regulatory mechanism that tends to limit this cell number.

* An abstract of this work has been presented (Zamenhof, 1975)

The aim of the present work was to obtain some information on the problem whether or not the *final* neuron number in optic lobes and cerebral hemispheres can be increased by a treatment during the sensitive period before the end of neuron proliferation (day 10).

The incubation of hen's eggs at temperatures above optimal (37.5° C) for a few days is known to produce temporary acceleration of embryonic growth (review in Romanoff and Romanoff, 1972); however, on further incubation, even at 37.5° C, the growth eventually slows down and those embryos that hatch may even have lower body weight. The growth of embryo's brain and of its parts has not been studied in these respects.

The present work is an attempt to determine whether such incubation at elevated temperature before the end of neuroblasts proliferation (day 10) can result in increases in final numbers of neurons in optic lobes and cerebral hemispheres. For this purpose, the eggs were incubated at elevated temperature at 7 to 10 days of embryonal age. The number of cells in 10 day old embryonal optic lobes and cerebral hemispheres was determined by a new direct count method, as well as from DNA content. Some eggs were subsequently incubated at optimal temperature until hatching, and weights, DNA and protein were determined in the optic lobes and cerebral hemispheres at that time.

Material and Methods

The eggs and their handling were essentially as described in our previous work (Zamenhof and van Marthens, 1971; Zamenhof, Grauel and van Marthens, 1971). Fertile eggs (White Leghorn strain K 137, from 10 month old hens) were supplied by the Kimber Farms, Pomona, California. All eggs were laid in the same month (September); this precaution is recommended to avoid seasonal variations (Zamenhof and van Marthens, 1971). The eggs were weighed and the experimental and control groups were matched for egg weight. The eggs were incubated in the Jamesway incubator, type 252, at 37.5° C ± 0.1° C during the first 10 days of embryonal life (controls), or at 37.5° C ± 0.1° C during the first 7 days and at 40.5° C ± 0.1° C during day 8 through 10 (experimentals)[1]. The humidity for both groups was 55%. After completion of 10 days of incubation, the eggs were opened, the embryos weighed and decapitated. In another series, the incubation of the controls and experimentals was continued beyond day 10, at 37.5° C, until hatching, which occurred after the same total incubation time for both groups. For DNA and protein determination (see below), the optic lobes and the cerebral hemispheres (without olfactory lobes) were separately dissected at day 10, or at hatching, weighed and stored at −15° C. For direct cell enumeration (see below), the heads were preserved at room temperature in 10% buffered formalin for a period of 10 days to one month.

DNA and Protein Determination. The optic lobes and the cerebral hemispheres were individually homogenized and the total DNA in each was determined by a modification (Zamenhof et al., 1972) of the Burton (1956) diphenylamine colorimetric method. From these data, the total cell number in the optic lobes or in the cerebral hemispheres was calculated, using the reported DNA content of one chick cell. Protein content was determined by the method of Lowry et al. (1951).

Direct Cell Enumeration. The optic lobes or cerebral hemispheres, preserved as above, were disintegrated in 0.2 ml water in a conical 15 ml centrifuge tube, using a glass rod tipped with rubber policeman. The coarse suspension was then transferred to a 19 mm dia. vial with a total of 2 ml water, and subjected to ultrasonication (Johnson and Erner, 1972) for 4 min at 55 watts, using

[1] This treatment did not reduce viability of embryos at day 10 and day 19, but reduced hatchability (at 21 days) by 25%

the "Sonifier Cell Disruptor", Model W185D, Heat Systems–Ultrasonics, Inc., Plainview, N.Y., with a microprobe of 3.5 mm dia. tip. The uniform fine suspension was then diluted with water to 4 ml; to an 0.5 ml aliquot of this suspension 0.005 ml of 1% aqueous thionine solution was added, and the stained suspension was further homogenized by moving up and down in a capillary pipette; the cells were then counted in a 0.1 mm deep Spencer Neubauer hemacytometer, using 43× objective and 10× eye-piece in the microscope. Total area in which the cells were counted was 0.2 mm^2. The counts were repeated in 4 such areas; subsequently the hemacytometer was emptied and refilled, and this procedure was repeated for the total of five times. The erythrocytes (approx. 1.5%), epithelial cells and blood vessel cells, easily identified under the microscope, were not counted.

Results

The comparison of the direct cell count obtained by the above method with the cell count calculated from DNA contents, is represented in Table 1 (controls

Table 1. Comparison of the direct cell counts with the cell counts calculated from DNA contents, in optic lobes and cerebral hemispheres of 10 day old chick embryos

Organ	Direct counts		DNA determinations		
	Actual counts[a] per 0.02 mm^3	Total count, cells per organ, $\times 10^{-7}$	DNA per organ	Corrected DNA[b]	Cells per organ[c], $\times 10^{-7}$
Optic lobes	104	2.08	74 µg	55.5 µg	2.37
Cerebral hemispheres	109.5	2.19	95 µg	71.25 µg	3.04

[a] Mean of 20 individual counts for each embryo; 6 embryos
[b] Corrected for 75% purity of DNA standard
[c] Calculated on the basis of 2.34×10^{-6} µg DNA/chick cell (Leslie, 1955)

Table 2. Organ weights[a] and direct cell counts (±standard deviation) in optic lobes and in cerebral hemispheres of 10 day old chick embryos

Incubation	Fresh body weight (g)	Optic lobes		Cerebral hemispheres	
		Weight[a] (mg)	Direct cell count[b] $\times 10^{-7}$	Weight[a] (mg)	Direct cell count[b] $\times 10^{-7}$
Control 37.5°	2.06 ±0.065	62.6 ±3.2	2.08 ±0.11	54.5 ±6.9	2.19 ±0.53
Experimental 40.5° days 7–10	2.625 ±0.184	69.5 ±5.7	2.63 ±0.25	65.8 ±7.5	3.23 ±0.70
Δ[c]	27	11	26	21	47
Probability[d]	$p < 0.0005$	$0.01 < p < 0.025$	$p < 0.0005$	$0.01 < p < 0.025$	$p < 0.01$

[a] After 20 days fixation in 10% buffered formalin
[b] Mean of 20 counts for each embryo; 6 embryos for each incubation. Erythrocytes and blood vessel cells not counted
[c] Difference, in % of control
[d] Student t test

Table 3. Parameters[a] of optic lobes and cerebral hemispheres of newly hatched chicks

Incubation	Body weight[b] (g)	Optic lobes					Cerebral hemispheres				
		Weight[b] (mg)	Protein (mg)	DNA[c] (mg)	Cell number[d] $\times 10^{-7}$	Protein/cell $\times 10^{-7}$ (mg)	Weight[b] (mg)	Protein (mg)	DNA[c] (mg)	Cell number[d] $\times 10^{-8}$	Protein/cell $\times 10^{-7}$ (mg)
Control 37.5°	37.39 ±1.31	139 ±6.2	8.61 ±0.56	0.160 ±0.008	6.84 ±0.34	1.26 ±0.10	429.8 ±19.8	30.5 ±1.4	0.422 ±0.029	1.80 ±0.12	1.69 ±0.15
Experimental 40.5°, days 7–10	36.99 ±1.28	139.6 ±10.6	9.21 ±0.85	0.159 ±0.009	6.80 ±0.38	1.35 ±0.14	444.3 ±19.6	33.1 ±2.3	0.423 ±0.034	1.81 ±0.14	1.83 ±0.247
Δ[e]	−1.0	+0.4	+7.0	−0.6	−0.5	+7.1	+3.3	+8.5	+0.2	+0.6	+7.7
Probability[f]	ns	ns	$p < 0.005$	ns	ns	$0.01 < p < 0.025$	ns	$p < 0.005$	ns	ns	$0.025 < p < 0.05$

[a] All values ± standard deviations. 15 animals for each incubation
[b] Fresh weights
[c] Corrected for 75% purity of DNA standard
[d] Calculated from DNA on the basis of 2.34×10^{-6} µg DNA/chick cell
[e] Difference, in % of control
[f] Student t test; ns = non significant

only; embryos 10 days old). It can be seen that the direct count gives cell numbers essentially similar to those calculated from DNA determinations. The bases for the calculations were: a. The amount of DNA per diploid chick cell is reported to be 2.34×10^{-6} µg (Leslie, 1955), and b. the purity of commercial DNA preparations, used as standards in DNA determination, was only 75% (Rosenbaum-Oliver and Zamenhof, 1972).

The comparisons of organ weights and direct cell counts for control and experimental 10 day old embryos are represented in Table 2. The organ weights are determined after fixation[2] because fresh organs in the embryo of this age could not be dissected with accuracy necessary for subsequent reliable determinations of cell numbers. It can be seen that the 3 days treatment at 40.5° C resulted in significant increases in organ weight and in cell number in both optic lobes and cerebral hemispheres. Shorter treatments (2 or 1 day, not shown in the table) gave less pronounced increases. The increases of cell numbers in the cerebral hemispheres were greater than in the optic lobes. However, all the increases were statistically significant.

The comparison of fresh organ weights[3], protein contents and cell numbers calculated from DNA contents, for newly hatched chicks are represented in Table 3. It can be seen that at this age the body weights, organ weights and organ DNA and cell number in controls and experimentals are essentially the same, both for the optic lobes and for the cerebral hemispheres. However, it is of interest that protein contents are significantly higher in experimental animals; consequently, protein contents per cell (index of cell size) are also higher in experimentals, both in the optic lobes and in the cerebral hemispheres, although these increments are only on the borderline of significance.

Discussion

The methods for dissociation of cells in fresh brain have been the subject of many publications. The purpose of these methods was mainly preparation for further fractionation, with an aim of obtaining viable cells for tissue culture (compare Varon and Rainborn, 1969). Quantitative recovery was usually not attempted. One method (Nurnberg and Gordon, 1957; Brizzee et al., 1964) aimed at cell enumeration by subjecting fresh tissue to the action of glass beads; this treatment resulted in considerable cell destruction which was taken into account by an elaborate correction factor. To avoid cell destruction, Johnson and Erner (1972) suggested the use of formalin-fixed, rather than fresh cells; such fixed cells were then dissociated by gentle sonication. The method was reported to be applicable to adult mice brains.

In our hands, adult brain tissue (rats, mice, chicks) could not be quantitatively dissociated by this method. After gentle sonication of such fixed tissue considerable proportion of cells remained undissociated, in forms of aggregates (clumps); more intense sonication resulted in considerable cell destruction. On the other

[2] We have found that the increase in organ weight due to formalin fixation of 10 day old embryo brain is of the order of 35%

[3] At this age there is no difficulty in accurate dissection of fresh organs

hand, embryonic tissue lent itself very well to such manipulations. Apparently in 10 day old chick brain (optic lobes and cerebral hemispheres) the connections between cells are not developed well enough to prevent cell dissociation: Clean cell separation can be achieved without residual aggregates and with no more than 5% of slightly injured cells; the latter are still integral enough to be counted.

The estimation of cell number from total DNA content is very convenient for fresh neonatal and older brains (Zamenhof et al., 1964; Zamenhof et al., 1972). On the other hand, reproducible *quantitative* dissection of specific brain parts in the very young fresh embryo is beset with great difficulties due to the consistency of the tissue. Formalin-fixed embryonic brains can be easily dissected free, but formalin fixation interferes with DNA determination (Zamenhof et al., 1972).

For this reason, in the present work it was decided to use for embryonic tissue direct cell enumeration, after fixation and gentle sonication. The method is quantitative and simple. The comparison of this method with DNA determination (Table 1) reveals that the results obtained by the two methods are essentially similar; whatever discrepancies remain may be due to a) imperfections in dissecting free fresh embryonal brain tissue, especially cerebral hemispheres, for DNA determination, and b) uncertainty in the value of the reported amount of DNA per cell, which is the factor used for calculation of number of cells from the known total DNA content; this includes uncertainty in the purity of DNA preparation that has been used for determination of the amount of DNA per cell.

The study of the effect of incubation (days 8 through 10) at elevated temperature (40.5° C) on parameters at hatching (Table 3) reveals that such short stimulation of growth had no effects on body weight, and on weight, DNA and cell number of both optic lobes and cerebral hemispheres. In contrast, there was a significant increase in total protein; this increase has occurred both in optic lobes and in cerebral hemispheres. It appears that protein synthesis, stimulated at days 8 through 10 of incubation, remained at higher level till end of incubation. Whether such increased cell protein content (cell size) has any consequences in brain development after hatching will be the subject of a later study.

The finding that the number of brain cells at hatching was normal does not preclude the possibility that the proportions of neuron and glia cells underwent a change. This possibility may be supported by the cell counts on day 10 (Table 2). At this age the mitoses in cells destined to become neurons are nearly terminated, in both optic lobes (Cowan et al., 1968) and in the cerebral hemispheres (Gordon and Nurnberg, 1956). Thus, the number of neurons at this age is nearly final. Further increase in DNA content (Gayet and Bonichon, 1960; Margolis, 1969) and in cell numbers of these organs is mostly due to proliferation of glial and vascular cells (Gordon and Nurnberg, 1956).

It can be seen from Table 2 that the incubation (days 8 through 10) at elevated temperature resulted in significantly higher body weight, and weight and cell number in optic lobes and cerebral hemispheres at the end of this

incubation[4]. The increases were different for different parameters, presumably due to differences in mitotic rates in various organs at this particular period of stimulation.

It thus appears that elevated temperature resulted in significant stimulation of brain cell divisions in the period when the dividing cells were preponderantly or exclusively neuroblasts, and that the resulting increment of cell numbers persisted until the cessation of neuroblast proliferation (mitoses) at day 10. This increment is not demonstrable at hatching. The results cannot be explained merely by assuming a transient acceleration of cell division in the experimentals, without the increase (over the controls) of the *total* aggregate number of cells generated: Since the cell count was purposely performed *after* cessation of neuroblast proliferation in the controls, they cannot "catch up" later on. Thus, the demonstrated increment in experimentals was indicative of the actual increase in their *total* number of neurons at that time (day 10).

At least two other possibilities could account for these results. One is that the elevated neuroblast population in experimental animals is followed by an increased cell death rate and prompt DNA depolymerization so that at hatching the DNA content and the total number of cells calculated from DNA appear normal. This may occur in the optic tectum, *if* the number of afferent fibers in the experimental animals has not increased to the same extent as the number of neurons (Cowan, 1971, 1973). However, it must be pointed out that the extensive prenatal neuron death in cerebral hemispheres of birds and mammals has not been demonstrated. Another possibility is that the number of neurons in experimental animals indeed remains elevated but this increment is easily overshowed by the proliferation of other cells; alternatively, the glioblast proliferation might have been subsequently depressed. In these cases, the experimental animals at hatching will have higher neuron/glia than the controls. This subject will be investigated further, as it bears on an important problem whether or not the *final* neuron number is dependent on the conditions (temperature) prevailing during the sensitive period before the end of neuroblast proliferation (day 10).

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[4] The stimulating effect of higher temperatures on brain development in *mammalian* fetus cannot be demonstrated: higher temperatures interfere with thermoregulation in the mother and the resulting stress has multiple teratogenic effect on the fetus, including inhibition of brain development (Edwards et al., 1974). The same would apply to *older* chick embryo in which thermoregulatory apparatus has already been established

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