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SEROTONIN AS A GROWTH FACTOR FOR CHICK EMBRYO BRAIN[1]

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SUMMARY

It has been suggested that biogenic amines, in addition to their role as neurotransmitters, may also act as growth factors in the embryo. In the present work the concentrations of serotonin (5-HT), norepinephrine (NE) and dopamine (DA) were first determined in cerebral hemispheres of chick embryo at 10, 12 and 14 days of continuous incubation at 37.5°C (controls). When the incubation on days 7-10 was at 40°C (experimentals), a procedure known to increase brain weight, brain protein content and brain cell number, the concentration of 5-HT in cerebral hemispheres at day 10 (end of neuron proliferation) was significantly increased; this increase persisted at days 12 and 14 but ceased to be significant. No such increases were observed in the concentrations of NE and DA in experimentals at either day 10, 12 or 14. When 5-HT was injected into albumen of eggs at day 7 (37.5°C), cerebral weights, optic lobe weights and cerebral concentrations of 5-HT at day 10 were significantly increased over non-injected controls. Elevated temperature of incubation (40°C) further increased cerebral weight and 5-HT concentration. Cerebral protein contents and the ratios of cerebral protein/cerebral DNA at day 10 were also significantly increased but cerebral DNA and body weights were unchanged. The optimal doses have been determined. It is concluded that 5-HT may be a growth promoting or regulating factor for embryonal brain.

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[1]A short abstract of this work has appeared (1).

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The appearance of neurotransmitters in early embryonal life, long before the development of synapses, has been puzzling. In the rat, catecholamines and possibly serotonin (5-hydroxytryptamine; (5-HT) were detected as early as in the ovum prior to and during cleavage (2); this finding is compatible with similar findings in invertebrates (3,4). In the chick brain, norepinephrine (NE) and dopamine (DA) were detected at days 8-10 of incubation, and 5-HT at days 7-13 (5-9). High affinity uptake of 5-HT and DA by homogenates of various regions of chick brain was found after day 6 (10). It was suggested (9,11) that neurotransmitters are also involved in cellular processes during early embryogenesis, possibly by regulating protein synthesis (3) and/or DNA and RNA synthesis (reviews in 12,13).

The purpose of the present work was to obtain some evidence on this subject. We have first determined the concentrations of 5-HT, NE and DA in the normal chick embryo brains of various ages, starting with day 10 of incubation at 37.5°C. When the incubation on days 7-10 was at 40°C, a procedure known to increase weight, protein content, protein per cell and cell number in the brain (14,15), the concentrations of 5-HT (but not of NE and DA) in cerebral hemispheres at days 10, 12 and 14 were increased. Conversely, when 5-HT was introduced into eggs at day 7 (37.5°C), the weights and protein content of cerebral hemispheres and the weights of optic lobes at day 10 have also increased. The effects of higher temperature of incubation and of 5-HT introduction were synergetic. We have concluded that 5-HT may be a growth promoting or regulating factor for embryonal brain.

MATERIALS AND METHODS

The eggs and their handling were essentially as described in our previous work (14-16). Fertile eggs (White Leghorn strain K137, from 10 month old hens) were supplied by Pace Setter Products (Altaluma, California). All eggs were laid in the same month; this precaution is recommended to avoid seasonal variations (17). 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 only (control and Experiment B) or at 37.5°C ±0.1°C during the first 7 days, but at 40.5°C ± 0.1°C during day 8 through 10 (Experiment A). The humidity for both groups was 55%. After completion of 10 days of incubation, the eggs were opened, the embryos weighted and decapitated. In another series, the incubation of the controls and experimentals was continued beyond day 10, at 37.5°C, until day 12 or 14. The procedures for injections of eggs with 5-HT (Experiment B) were essentially similar to those used for glucose (16). After 7 days of incubation at 37.5°C each experimental egg was punched, injected (into albumen) with 0.05 to 0.2 ml of aqueous solution containing 10 mg 5-HT per ml, the holes sealed with paraffin, and the incubation continued at 37.5°C or 40°C as indicated. Since in previous work (14,15,18) it was established that injection of eggs with up to 0.2 ml of saline has no effect on embryonal development, such control was not used in the present work.

Another method of introduction of 5-HT was that of "dropped membrane". On the 7th day of incubation, the eggs were candled to locate the embryo. Two holes, one over the air space and other over the embryo, were made in the shell. The air from the air

space was then aspirated by holding a rubber tubing under vacuum against the hole. A new air space was thus created over the embryo. 0.02 ml to 0.1 ml of an aqueous solution of 5-HT, containing 5 mg per ml., was then carefully injected trough the hole above the embryo, care being taken that the needle does not injure the embryo. Both holes were then sealed with wax.

The optic lobes and the cerebral hemispheres (without olfactory lobes) were separately dissected at day 10, 12 or 14, weighed and stored at -15°C.

DNA and Protein Determination. The optic lobes and the cerebral hemispheres (separately) were individually homogenized and the total DNA in each was determined by a modification (19) of the Burton (20) diphenylamine colorimetric method. Protein content was determined by the method of Lowry et al (21).

Determination of Neurotransmitters. The extraction of neurotransmitters from the tissue was performed by the method of Butterworth et al (22). A 0.25 ml sample of the extract was used for the determination of 5-HT and 1 ml for the isolation (23) and determination of NE and DA.

5-HT. O-Phthaldialdehyde (OPT) reaction of Maickel and Miller (24) with reagent modification of Schlumpf et al (25) was used for the fluorometric determination of 5-HT. 0.30 ml of the OPT reagent (20 mg/100 ml conc. HCl) was added to 0.25 ml of the extract. The fluorescence was developed by heating in boiling water bath for 10 minutes. The mixture was washed with chloroform as described by Thompson et al (26) and the fluorescence was measured in an Aminco-Bowman Spectrophotofluormeter at an activation wavelength of 360 mμ and emission wavelength of 470 mμ, using quartz microcuvettes.

NE and DA. The modification of the hydroxyindole method described by Laverty and Taylor (27) was used for the determination of NE and DA. 0.5 ml of iodine solution (0.02 N I_2 in 5% aqueous NaI) was added to 0.5 ml of extract and immediately mixed. After 3 minutes 0.2 ml of alkaline sulfite solution (2.5% Na_2SO_3, 1% Na_2EDTA in 2.5 N NaOH) was added again and immediately mixed. The reaction was stopped exactly 5 minutes later by addition of 0.1 ml of glacial acetic acid. Fluorescence for NE was determined after 25 minutes at activation and emission wavelengths of 385 and 485 mμ, respectively. The tubes were then heated for 5 minutes and the fluorescence of oxidation product of DA was determined at activation wavelength 320 mμ and emission wavelength 375 mμ.

Chemicals. 5-HT (as creatinine sulfate complex), NE (HCl salt) and DA (HCl salt) used as standards in determination of these neurotransmitters were supplied by Sigma Chemical Co. (St. Louis, Mo.).

TABLE 1

The Effect of Raised Temperature during Incubation on the Levels of Neurotransmitters in Chick Embryo Cerebral Hemispheres

Group	Days of Incubation	Fresh wt. (g)	Neurotransmitters: Serotonin total (ng)	Serotonin conc. (ng/g)	Norepinephrine total (ng)	Norepinephrine conc. (ng/g)	Dopamine total (ng)	Dopamine conc. (ng/g)
Control (37.5°C)	10	0.059±0.007	26.39±3.45	443.6±46.2	16.26±2.20	273.6±32.8	10.43±1.91	174.6±24.9
	12	0.100±0.011	62.31±8.75	619.3±69.5	29.35±3.07	291.8±8.9	17.68±3.30	175.0±21.2
	14	0.179±0.012	84.14±9.4	471.7±56.1	24.71±2.5	137.9±7.2	12.69±2.47	71.24±14.5
Experimental (40°C)	10	0.065±0.006	*41.13±3.88	*633.1±52.7	14.79±1.85	227.6±24.7	8.96±1.39	137.4±17.2
	12	0.097±0.011	69.37±7.51	733.7±72.7	27.86±3.35	283.4±33.2	18.08±3.81	187.6±35.5
	14	0.166±0.016	83.60±6.11	503.7±36.5	22.80±1.50	144.4±22.7	9.69±2.10	57.87±8.51

* = Difference to control Δ=+56% significant at P< 0.001 level
† = Difference to control Δ=+43% significant at P< 0.001 level

TABLE 2

The Effect of Serotonin Injected on Day 7 of Incubation on Embryonic Development at Day 10

Dose of serotonin per egg (mg)	Body weight (g)	Cerebral hemispheres: Fresh wt. (mg)	DNA (mg)	Protein (mg)	Protein/DNA	Optic lobes: Fresh wt. (mg)	DNA (mg)	Protein (mg)
0 (control)	1.73±0.16	49.8±6.3	0.117±0.010	2.86±0.29	23.7±2.5	52.7±8.7	0.109±0.007	2.97±0.25
0.5	1.66±0.16	47.9±6.6	0.118±0.007	3.05±0.24	25.9±1.8	51.9±6.4		
1.0	1.78±0.15	§54.6±5.7	0.123±0.008	*3.42±0.40	†28.0±0.29	55.5±7.3		
1.5	1.75±0.15	50.2±3.8				58.2±6.2		
2.0	1.82±0.15	50.1±5.6	0.115±0.007	2.87±0.38	20.9±2.4	‡61.3±7.8	0.115±0.008	2.97±0.29

* = Difference to control Δ=+19.6% significant at P< 0.005 level
† = Difference to control Δ=+18% significant at P< 0.005 level
‡ = Difference to control Δ=+16.3% significant at 0.005< P< 0.01 level
§ = Difference to control Δ=+9.4% significant at 0.01< P< 0.025 level

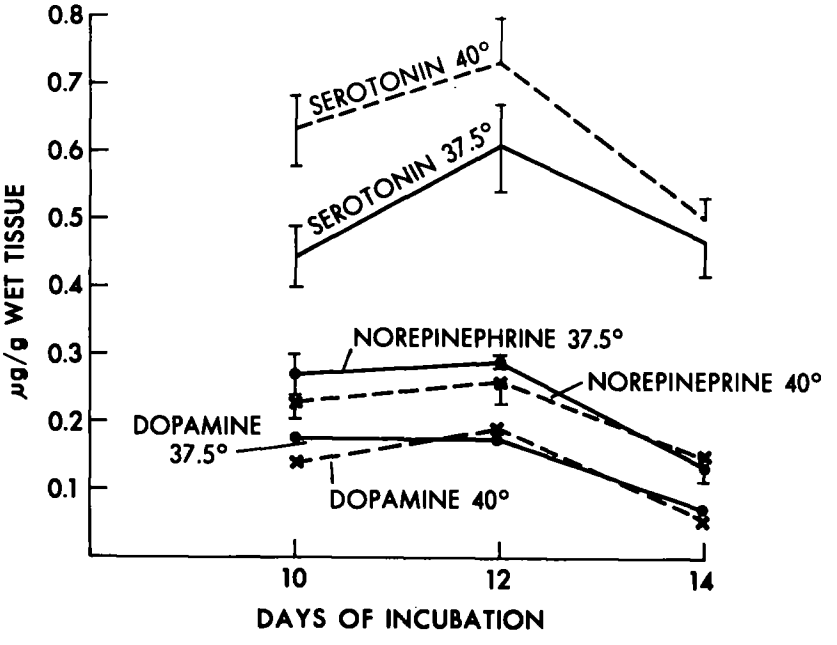

Figure 1 The effect of raised temperature of incubation on the levels of neurotransmitters in chick cerebral hemispheres.

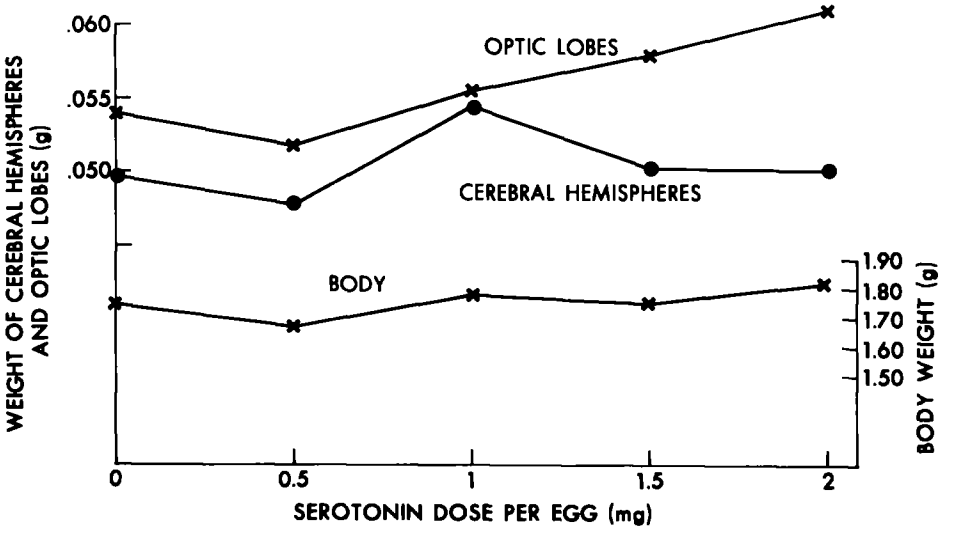

Figure 2 The effect of serotonin injected on day 7 on embryonic development at day 10.

RESULTS

The effects of incubation temperature on the levels of neurotransmitters are represented on Fig. 1 and in Table 1. It can be seen that higher temperature of incubation on days 7-10, which is known to stimulate brain cell proliferation (see introduction), results also in highly significant increases in amounts of 5-HT per cerebrum or per gram wet weight of cerebrum. The increases are most pronounced on day 10 (end of incubation at higher temperature) and become less pronounced and not statistically significant while the incubation is changed to control temperature (days 12 and 14).

In contrast to 5-HT, the total amount and the concentration of NE and DA was not significantly changed by incubation at higher temperature.

When 5-HT was injected into the egg on day 7 (preliminary experiment; total dose 0.3 to 1.2 mg), the normal 5-HT level in cerebral hemispheres (day 10) of 448.81 $\pm$ 33.5 ng/g tissue raised to 1087.82 $\pm$ 158.8 ng/g, cerebral weight 50.4 mg. to 54.8 mg., and cerebral protein 2.47 mg to 2.68 mg. If, in addition, the incubation on days 7-10 was at elevated temperature, 5-HT level raised to 1201.35 $\pm$ 225.6 ng/g, an increase of 168% over control, the weight of cerebral hemispheres raised to 58.4 mg., and protein to 2.81 mg.

The effect of injection into the egg of various doses of 5-HT on the brain and body parameters of the 10 day old[2] embryo are represented on Fig. 2 and in Table 2. It can be seen that embryonal body weight is unaffected by injection of 5-HT. On the other hand, cerebral weight, cerebral protein content and the ratio cerebral protein/cerebral DNA (index of cerebral neuron or neuroblast size), as well as optic lobes weight were significantly increased. The increase in cerebral protein signifies that the increase in cerebral weight is not just due to increase in water content but is indicative of actual cerebral growth. For cerebral hemispheres the optimal dose appears to be 1 mg per egg; higher doses may be harmful. For optic lobes the highest increase was for the highest dose tested (2 mg/egg; Fig. 2). The effect of introduction of 5-HT by the method of "dropped membrane" were essentially the same. However, since here 5-HT was delivered directly over the embryo (rather than into the egg), the access to the embryo was more direct and the optimal dose was lower (0.3 mg per embryo).

DISCUSSION

The results (Fig. 1 and Table 1) indicate first (Experiment A) that the treatment known to stimulate neuron (neuroblast) proliferation (incubation at elevated temperature before the end of neuronal proliferative period, and before the onset of glial proliferation (14,15)), also results in a significant increase in 5-HT

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[2]At hatching, the cerebral parameters of experimental animals were not different from the controls.

concentration in cerebral hemispheres of the chick embryo. This increment over the control is most pronounced right after the termination of the treatment (day 10) and decreases afterwards, even though the concentration of 5-HT as such is higher at day 12. These results are compatible with the hypotheses that increased concentrations of 5-HT are causally correlated with the stimulation of cerebral growth. The alternate hypothesis would be that higher incubation temperatures have a general (unspecific) stimulatory effect, on neurotransmitters and on cerebral growth, without the two being causally correlated. Our results (Fig. 1 and Table 1) do not support this alternate hypothesis: the higher incubation temperature did not significantly increase the concentration of the other two neurotransmitters NE and DA. Thus, 5-HT, but not NE and DA are correlated with stimulation of cerebral growth. Within this concept, one may consider the possibility that the increased concentration of 5-HT indeed stimulates cerebral growth. This possibility is supported by the results of Experiment B (Fig. 2 and Table 2): the introduction of 5-HT into egg at day 7, i.e. before the end of neuronal proliferation, results in a significantly increased weight of optic lobes, protein content and protein/DNA ratio of cerebral hemispheres. DNA contents (i.e. cell numbers) do not appear to be affected. It is conceivable that in this case the increased cell size (as evidenced by increased cell protein content or protein/DNA ratio) does not lead to the stimulation of cell division. As expected, the concentration of 5-HT in cerebral hemispheres was also elevated; the two treatments: elevated temperature of incubation (days 7-10) and introduction of 5-HT (day 7) act in the same direction in increasing 5-HT concentration and the weight of cerebral hemispheres (see Results).

Vernadakis and Gibson (12) found that NE and DA had a stimulating effect on RNA synthesis in neural explants from 14-day chick embryos. NE had also a stimulating effect in glial cells in tissue culture on enzymatic activities of acetylcholinesterase and butyrylcholinesterase (12) and lactose dehydrogenase (28).

The analysis of maturation patterns of NE, 5-HT and DNA contents in chick embryos has led to the suggestion that high levels of NE and 5-HT during early embryonic development signify another function involving cellular growth process in addition to their role in synaptic transmission (9,12). These authors have also suggested that NE is predominantly involved in the cerebral hemispheres, and 5-HT in the cerebellum and optic lobes. With respect to 5-HT, these suggestions appear to be strengthened by the evidence in the present paper, although cerebral hemispheres, rather than optic lobes, seem to be predominantly involved. Thus not only earliest cleavage divisions (see Introduction), but also much latter processes (cerebral growth) may be stimulated by 5-HT. Obviously, further studies will be needed to determine whether 5-HT acts by direct or indirect stimulation of protein synthesis, or whether it requires an intermediate substance(s) or systems.

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REFERENCES

1. G. AHMAD and S. ZAMENHOF, Federation Proc. 36, 925 (1977).
2. H. W. BURDEN and I. E. LAWRENCE, Amer. J. Anat. 136, 251-257 (1973).
3. G. A. BUZNIKOV, N. D. ZVEZDINA, and R. G. MAKEEVA, Dokl. Akad. Nauk. SSSR, Otd. Biol. 166, 1252-1255 (1966).
4. T. GUSTAFSON and M. TONEBY, Exper. Cell Res. 62, 102-117 (1970).
5. B. B. BOURNE, Life Sci. 4, 583-591 (1965).
6. S. EIDUSON, J. Neurochem. 13, 923-932 (1966).
7. C. KELLOG, A. VERNADAKIS and C. O. RUTLEDGE, J. Neurochem. 18, 1931-1938 (1971).
8. O. SUZUKI, F. NAGASE, and K. YAGI, Brain Res. 93, 455-462 (1975).
9. A. VERNADAKIS, Progr. Brain Res. 40, 231-243 (1973).
10. S. C. BONDY and J. L. PURDY, Brain Res. 119, 403-416 (1977).
11. J. RENSON, In Chemistry and Brain Development (R. Paoletti and A. N. DAVISON, eds) pp. 175-184. Plenum Press, New York (1971).
12. A. VERNADAKIS and D. A. GIBSON, In Perinatal Pharmacology Problems and Priorities. (J. DANCIS and J. C. HWANG, eds.) pp. 65-76, Raven Press, New York (1974).
13. L. P. LANIER, A. J. DUNN, and C. VAN HARTESVELDT, In Reviews of Neuroscience (L. P. EHRENPREIS AND I. J. KOPIN, eds.) Vol. 2, pp. 195-256. Raven Press, New York (1976).
14. S. ZAMENHOF, Wilhelm Roux's Arch. 180, 1-8 (1976).
15. S. ZAMENHOF, Brain Res. 109, 392-394 (1976).
16. S. ZAMENHOF and D. KLIMUSZKO, Brain Res. 128, 385-388 (1977).
17. S. ZAMENHOF and E. VAN MARTHENS, In Cellular Aspects of Neural Growth and Differentiation (D. C. PEASE, ed.) pp. 329-359. University of California Press, Berkeley (1971).
18. S. ZAMENHOF, L. GRAUEL, and E. VAN MARTHENS, Res. Commun. Chem. Path. Pharmacol. 2, 261-270 (1971).
19. S. ZAMENHOF, L. GRAUEL, E. VAN MARTHENS, and R. A. STILLINGER, J. Neurochem. 19, 61-68 (1972).
20. K. BURTON, Biochem. J. 62, 315-323 (1956).
21. O. M. LOWRY, N. J. ROSENBROUGH, A. L. FARR, and R. J. RANDALL, J. Biol. Chem. 193, 265-275 (1951).
22. R. F. BUTTERWORTH, F. LANDREVILLE, M. GUITARD, and A. BARBEAU, Clin. Biochem. 8, 298-302 (1975).
23. G. METCOLF, Anal. Biochem. 57, 316-320 (1974).
24. R. P. MAICKEL and F. P. MILLER, Anal. Chem. 38, 1937-1938 (1966).
25. M. SCHLUMPF, W. LICHTENSTEIGER, H. LANGEMANN, P. G. WASER, and F. HEFTI, Biochem. Pharmacol. 23, 2337-2446 (1974).
26. J. H. THOMPSON, CH. A. SPEZIA, and M. ANGULO, Experientia 26, 327-329 (1970).
27. R. LAVERTY and K. M. TAYLOR, Anal. Biochem. 22, 269-279 (1968).
28. J. DE VELLIS, D. INGLISH, and F. GALEY, In Cellular Aspects of Neural Growth and Differentiation (D. C. PEASE, ed.) UCLA Forum in Medical Sciences. No. 14, pp. 23-32, University of California Press, Berkeley (1971).