Ontogeny, regional distribution and properties of thyroid-hormone receptors in the developing chick brain

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Studies on the thyroid-hormone receptors in the nuclei of developing chick brain revealed a single class of binding sites for tri-iodothyronine (T_3) and thyroxine (T_4) at all embryonic and adult ages. High-affinity [$K_a = (1.85\text{--}3.3) \times 10^9 \text{ M}^{-1}$ and $(0.3\text{--}0.6) \times 10^9 \text{ M}^{-1}$ for T_3 and T_4 respectively] receptors were detected in the brain as early as day 7 of embryonic development; their level increased progressively rapidly until day 13, and thereafter the value remained essentially constant during development. Occupancy of the receptor site with endogenous hormone was 75–90% at 7–11 days, 50–60% during the late phase of embryogenesis (13–17 days), and 80% after hatching. Comparison of the binding properties of the receptors with T_3 and T_4 indicates that, although the binding capacities per nucleus are almost identical, T_4 has four to five times less binding affinity than T_3 . The half-lives of dissociation of solubilized T_3 –receptor complexes were 20–30 h between 0° and 7°C, about 4 h at 20°C and less than 15 min at 37°C. Studies of the regional distribution of receptors in the brain indicate that cerebrum has the highest concentration of T_3 receptors (4000–7000 sites per nucleus); this concentration is 2–4-fold higher than that in the cerebellum, optic lobe or medulla oblongata. The overall results indicate that between 7 and 13 days of embryonic development the thyroid-hormone receptors in the embryonic chick brain, particularly in the cerebrum, assume a very high level and appear to be mostly saturated with endogenous hormone. This, and the temporal correspondence of the phenomenon with the period of neuronal growth and synaptogenesis, strongly indicate the influence of the hormone in the maturation of the developing brain.

It is well known that thyroid hormones play a crucial role in the development and maturation of the central nervous system (Eayrs & Taylor, 1951; Eayrs & Horn, 1955; Hamburg *et al.*, 1971; Bass *et al.*, 1977). However, little is known concerning the biochemical basis of thyroid-hormone function in the developing brain. Interaction of thyroid hormones with specific chromatin-associated receptors represent one of the first and essential steps of hormone action (Oppenheimer, 1979). Recently, we have reported that thyroid-hormone receptors of embryonic-chick brain are localized predominantly in the neurons (Haidar *et al.*, 1983). In the present paper we report the ontogeny of the thyroid-hormone receptors, their regional distribu-

tion in different parts of the chick brain, developmental changes in the occupancy of the receptor sites with endogenous hormone and some aspects of their physico-chemical properties.

Materials and methods

Fertile White-Leghorn-chicken eggs were purchased from a local poultry farm and incubated in the laboratory. $L\text{--}[^{125}\text{I}]\text{T}_3$ and $L\text{--}[^{125}\text{I}]\text{T}_4$ with specific radioactivities of 70–80 Ci/mmol were purchased from Bhaba Atomic Research Centre, Bombay, India. Unlabelled T_3 and T_4 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Brain nuclei were prepared at 0–4°C as described previously (Haidar *et al.*, 1983). The protein/DNA ratios of the purified nuclei ranged between 4.3 and 4.92:1 at different ages. The average DNA content per nucleus was found to be

Abbreviations used: T_3 , tri-iodothyronine; T_4 , thyroxine; MBC, maximum binding capacity.

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2.5 pg. Nuclear pellets were washed once with 0.25% Triton X-100 containing homogenizing buffer (0.32M-sucrose/3mM-MgCl₂/1mM-sodium phosphate, pH 6.5) and thrice with the same buffer without Triton X-100. Thyroid-hormone receptors were extracted at 0°C as described previously (Silva *et al.*, 1977) by vortex-mixing nuclei for 15s at a time at 5min intervals for 45min in TEM buffer [20mM-Tris/HCl (pH 8.0)/2mM-EDTA/5mM-MgCl₂/5mM-mercaptoethanol/10% (v/v) glycerol] and finally centrifuging the suspension at 20000g for 20min. A 1ml portion of extraction buffer was used per 250 × 10⁶ nuclei.

Portions of nuclear extracts were incubated in duplicate with increasing concentrations (0.1–6.0nM) of [¹²⁵I]T₃ or [¹²⁵I]T₄. Incubations were carried out at 30°C for 40min to allow dissociation of endogenously bound hormone (Samuels *et al.*, 1974), followed by 17–18h at 0°C (separate experiments showed that such incubations do not lead to any detectable loss of receptor activity). Bound and free hormones were separated in columns of Sephadex G-25 of bed volume 2.5ml. Non-specific binding was determined in the presence of 1000-fold excess of unlabelled hormone. To determine the extent of occupancy with endogenous hormone, parallel sets of tubes were incubated directly at 0°C for 17–18h. All data were analysed by the method of Scatchard (1949). Receptor-concentration data (maximum binding capacity, MBC) were corrected for the efficiency of extraction of nuclear receptors as determined by the extractability of receptor-hormone complexes from nuclei of different ages preincubated with [¹²⁵I]T₃ (Bellabarba *et al.*, 1983). The extraction efficiency increased with age and was found to be 25 ± 3% for 7–13-day embryos, 36 ± 4% for 17-day embryos and 40 ± 3% for nuclei from brains of newly hatched or adult chicks. Protein and DNA were estimated by the methods of Lowry *et al.* (1951) and Giles & Myers (1965) respectively, with bovine serum albumin and calf thymus DNA as standards.

Results and discussion

Ontogeny of T₃ and T₄ receptors during development

When extracts from brain nuclei, containing solubilized receptors for thyroid hormones, were incubated with increasing concentrations of [¹²⁵I]-T₃ or [¹²⁵I]T₄ and the data analysed by the method of Scatchard (1949), a single class of binding sites was observed for all ages studied. A representative Scatchard (1949) plot showing the data for 13-day embryonic brain is shown in Fig. 1. Age-related changes in the binding affinity and MBC of the

receptor sites for T₃ as derived from the Scatchard (1949) plots of the binding data are shown in Table 1. The equilibrium association constants (*K_a*) of the nuclear receptors for T₃ are very high [1.85–3.3) × 10⁹M⁻¹] and remain essentially constant throughout the embryonic and adult life.

The binding capacity of these receptor sites, expressed as the number of sites/nucleus, increased rapidly from 1120 to 4580 between 7 and 13 days of embryogenesis and thereafter shows a slow increase until 17 day of embryogenesis (5200 sites/nucleus). This value declines by about 20% in the newly hatched chick (4312 sites/nucleus) and increased again during adulthood (5136 sites/nucleus). The reason for the fall and the subsequent increase is not clear. Since the receptor concentration in the glial nuclei is very low in comparison with that in the neuronal nuclei, and the neuron/glia (N/G) ratio decreases with age (Haidar *et al.*, 1983; in that study, no correction for extraction efficiency was made), the decline in receptor concentration in the newly hatched chick may be due to a decrease in the N/G ratio. The subsequent increase in the receptor concentration in the adult could probably be due to a very high concentration of receptors in the neuron compensating the large fall in N/G ratio with age.

These results differ significantly from those reported recently by Bellabarba *et al.* (1983);

Fig. 1. Scatchard plot of [¹²⁵I]T₃ binding to nuclear extract of 13-day embryonic brain

Nuclear extracts (corresponding to 60 × 10⁶ nuclei) were incubated in duplicate with increasing concentrations of [¹²⁵I]T₃ in the presence or absence of 1000-fold unlabelled T₃, and the amount of free and bound hormone was determined as described in the Materials and methods section.

Table 1. Age-related changes in the MBC of receptors per nucleus, their equilibrium association constant and endogenous saturation with T₃

Maximum binding capacity, expressed as number of T₃-binding sites/nucleus and the equilibrium association constants, were obtained from the Scatchard plots of the binding data (extracts equivalent to 60 × 10⁶ nuclei were used for each point of the plot; depending on the age, different numbers of chick brains were used for each experiment, ranging from 15 for adult to 100 for 7-day embryo). Endogenous saturation was determined as described in the Materials and methods section. Data represent means ± s.d. from three to four experiments with different batches of chicks for all ages. Probabilities of age-related changes (MBC) are: 7 days versus 9, 11, 13, 17, hatched and adult, *P* < 0.001; 9 days versus 11 days, 0.002 < *P* < 0.01; 9 days versus 13 days, 17 days and adult, *P* < 0.001; 9 days versus hatched and adult are 0.001 < *P* < 0.002 and *P* < 0.001 respectively; 11 days versus 13 days, 0.02 < *P* < 0.05; 11 days versus 17 days, 0.002 < *P* < 0.01; 11 days versus hatched, 0.01 < *P* < 0.05; 11 days versus adult 0.001 < *P* < 0.002; 13 days versus 17 days, 0.01 < *P* < 0.02; 13 days versus hatched, not significant; 13 days versus adult, 17 days versus hatched and hatched versus adult, 0.002 < *P* < 0.01; 17 days versus adult, not significant.

Age (days) ...	Embryo					Newly hatched	Adult
	7	9	11	13	17		
MBC (no. of receptor sites/nucleus)	1120 ± 92	3004 ± 206	4016 ± 198	4580 ± 190	5200 ± 287	4312 ± 234	5135 ± 207
Equilibrium association constant (10 ⁹ M ⁻¹)	2.31 ± 0.19	2.27 ± 0.12	2.59 ± 0.05	1.85 ± 0.16	1.93 ± 0.17	3.17 ± 0.16	3.33 ± 0.14
Endogenous saturation (%)	85 ± 2.14	91 ± 1.5	75 ± 3.08	51 ± 3.93	62 ± 2.06	81 ± 3.82	83 ± 2.54

whereas those workers reported an overall decline in the level of nuclear T₃ receptors in brains from embryonic ages after day 12 (4667 sites per nucleus*) of development to 10 days after hatching (527 sites/nucleus), we did not find a decline in receptor concentration during the corresponding period. The exact reason for this discrepancy is not clear; however, it appears that at least one of the reasons for the low yield of receptors at higher embryonic ages might be due to the isolation of nuclei for all ages by using the same sucrose gradient under identical sedimentation conditions. Since the density of the neurons declines with age (Gozes *et al.*, 1977; Haidar *et al.*, 1983), this might lead to incomplete recovery of neurons at higher ages, resulting in a lower yield of receptors, which are present predominantly in the neurons.

Of greater importance is the fact that our findings agree with those of Bellabarba *et al.* (1983) in that the major rise in the level of T₃ receptors occurs in the early phase of embryonic development (7–13 days), temporally coinciding with the period of rapid neuronal differentiation and maturation of the brain (Oppenheim & Foelix, 1972).

As in the case of T₃, a single class of binding site was also observed when nuclear extracts from 13-day-embryo, newly hatched or adult chick brains were incubated with [¹²⁵I]T₄ and the data subjected to Scatchard (1949) analysis. A representative Scatchard plot for 13-day embryonic brain is

* The data were converted from fmol/μg of DNA to the number of sites per nucleus by assuming that there are 2.5pg of DNA per nucleus.

Fig. 2. Scatchard plot of [¹²⁵I]T₄ binding to a nuclear extract of 13-day embryonic brain. Nuclear extracts (corresponding to 60 × 10⁶ nuclei) were incubated in duplicate with increasing concentrations of [¹²⁵I]T₄ as described in the legend to Fig. 1.

shown in Fig. 2. Although the binding affinities for T₄ [(0.3–0.5) × 10⁹ M⁻¹] was found to be four to five times less than that for T₃, the binding capacities of T₄ per nucleus were nearly identical with that of T₃ for all three ages examined, suggesting that T₃ and T₄ probably bind to the same receptor molecule.

Developmental changes in receptor occupancy with endogenous T_3

Table 1 further shows the age-related alterations in the level of nuclear T_3 receptors endogenously saturated with T_3 . The endogenous receptor occupancy is extremely high (75–90%) between 7 and 11 days of embryogenesis, declines to 50–60% in the late phase of embryogenesis and finally rises again to about 80% in the brain of the newly hatched or adult chick. These developmental changes in the endogenous saturation pattern are generally parallel with the plasma T_3 concentrations (Thomas & Hylka, 1977). The high-level endogenous saturation of the T_3 receptor in early embryonic brain is compatible with a natural role of the hormone in the process of brain development. To our knowledge, this is the first report on alterations in endogenous nuclear occupancy of the hormone during development and its coincidence with the period of neuronal maturation of the brain.

Stability of the embryonic and adult solubilized receptor-hormone (T_3) complexes

To assess the relative stability of the T_3 -receptor complexes of embryonic newly hatched and adult brain, we studied the kinetics of dissociation of these complexes at different temperatures (0°, 7°, 20° and 37°C). Nuclear extracts from 13-day embryos and from hatched- and adult-chicken brains were incubated with 1 nM-[125 I] T_3 ; the resulting complexes were separated from free T_3 on a column of Sephadex G-25, and their dissociation rates were studied in the presence of 1 μ M- T_3 by measuring the amount of undissociated complexes left after various time intervals at 0°, 7°, 20° or 37°C; the time required for dissociation of 50% of the complex ($t_{1/2}$) was computed. Fig. 3 shows the representative dissociation pattern of the T_3 -receptor complex for 13-day brain at 0°, 7° and 20°C. In both embryonic and adult brain, the receptor-hormone complex was found to be very stable at 0°C ($t_{1/2}$ values for the complexes from brain of 13-day embryo and hatched and adult chickens are 25, 29 and 31 h respectively), 7°C ($t_{1/2}$ values for 13-day embryo and hatched and adult chickens are 20, 23 and 24 h respectively) and 20°C ($t_{1/2}$ of 13-day embryo and hatched and adult chickens are 4.5, 5 and 5.5 h respectively). At 37°C, $t_{1/2}$ values for complexes for all the above ages were found to be less than 15 min.

Distribution of thyroid-hormone receptors in different regions of the brain

To determine if the effect of thyroid hormones on the developing brain is preferential in any particular area of the brain, we investigated the distribution of receptors in different parts of the

Fig. 3. Dissociation kinetics of solubilized T_3 -receptor complexes of 13-day embryonic brain

Nuclear extracts were incubated with 1 nM-[125 I] T_3 at 0°C for 17–18 h. Receptor-hormone complexes were separated from free [125 I] T_3 on Sephadex G-25 columns. The complexes were then incubated at 0°C, 7°C and 20°C with 1 μ M-unlabelled T_3 . At different times, portions of the incubation mixtures ($\circ$, 0°C; $\bullet$, 7°C; Δ , 20°C) were passed through Sephadex G-25 columns to determine the undissociated [125 I] T_3 -receptor complexes.

Fig. 4. Scatchard plot of [125 I] T_3 binding to nuclear extracts of cerebrum, cerebellum, optic lobe and combined pons + medulla oblongata

Nuclear extract corresponding to 75×10^6 nuclei of cerebrum (a), cerebellum (b), optic lobe (c) and combined pons + medulla oblongata (d) were incubated with [125 I] T_3 as described in the Materials and methods section and in the legend to Fig. 1.

brain (cerebrum, cerebellum, optic lobe and combined pons and medulla oblongata), by using brains from 13-day embryo and newly hatched and adult chickens. Scatchard plots for binding of T_3 to

Table 2. *Regional distribution of T₃ receptors and their binding affinities for T₃ in different parts of the brain* MBC/nucleus and binding affinities were determined from Scatchard plots (extracts equivalent to 75×10^6 nuclei were used for each point of the plot). For each experiment with different regions of the brain of 13-day embryo, hatched chick and adult, 250, 125 and 75 chick brains respectively were used. Data represent means $\pm$ S.D. from three experiments with different batches of chicks of the indicated ages. Probabilities of age-related changes are: MBC (cerebrum) 13-day embryo versus hatched chick and adult, $P < 0.001$ and $0.01 < P < 0.02$ respectively; hatched versus adult, $0.002 < P < 0.01$; MBC (cerebellum) 13-day embryo versus hatched, not significant; 13-day embryo versus adult and hatched versus adult, $0.002 < P < 0.01$; MBC (optic lobe) 13-day embryo versus hatched and adult, not significant; hatched versus adult, not significant; MBC (combined pons + medulla oblongata) 13-day embryo versus hatched and adult, $0.002 < P < 0.01$ and $0.02 < P < 0.05$ respectively; hatched versus adult, $P < 0.001$.

	13-Day embryo		Newly hatched		Adult	
	MBC (no. of sites/nucleus)	Affinity (10^9 M^{-1})	MBC (no. of sites/nucleus)	Affinity (10^9 M^{-1})	MBC (no. of sites/nucleus)	Affinity (10^9 M^{-1})
Cerebrum	7129 $\pm$ 450	2.08 $\pm$ 0.23	3913 $\pm$ 375	3.04 $\pm$ 0.34	5679 $\pm$ 399	3.42 $\pm$ 0.26
Cerebellum	3443 $\pm$ 361	1.70 $\pm$ 0.38	3758 $\pm$ 254	2.53 $\pm$ 0.27	2709 $\pm$ 256	3.05 $\pm$ 0.33
Optic lobe	2059 $\pm$ 257	1.94 $\pm$ 0.21	2302 $\pm$ 213	2.88 $\pm$ 0.32	1927 $\pm$ 255	3.20 $\pm$ 0.29
Combined pons + medulla oblongata	2050 $\pm$ 307	0.65 $\pm$ 0.20	1129 $\pm$ 141	1.62 $\pm$ 0.24	2690 $\pm$ 231	2.99 $\pm$ 0.32

nuclear receptors of these regions of brains from 13-day embryo are shown in Fig. 4. T₃ binding affinities (Table 2) of these receptors for cerebrum, cerebellum, optic lobe and combined pons and medulla oblongata were found to be in the ranges $(2.08\text{--}3.42) \times 10^9 \text{ M}^{-1}$, $(1.70\text{--}3.05) \times 10^9 \text{ M}^{-1}$, $(1.94\text{--}3.20) \times 10^9 \text{ M}^{-1}$ and $(0.65\text{--}2.29) \times 10^9 \text{ M}^{-1}$ respectively at different ages.

Developmental changes in the distribution of T₃ receptors in different parts of the brain are also shown in Table 2. The MBC is 2–4-fold higher in the cerebrum of the brain for all the ages examined. The most interesting observation is that within 13 days of embryonic development, cerebrum (7129 sites/nucleus) and cerebellum (3443 sites/nucleus) assumed a high concentration of T₃ receptor, exceeding that in the newly-hatched-chick or adult-chicken brain. The cerebral receptor concentration/nucleus shows a fall at hatching (3913 sites/nucleus) and increased again in adulthood (5679 sites/nucleus). The receptor concentration in the cerebellum and optic lobe appeared to be relatively less variable during development. Combined pons and medulla oblongata show a fall in receptor concentration from 2050 to 1129 sites/nucleus between 13-day embryo and hatched chick and thereafter again increased to 2690 in adulthood.

In conclusion, our results on the developmental profile of T₃ receptors in brain during early embryonic development generally agree with those recently reported by Bellabarba *et al.* (1983). Furthermore, we have looked into two other important aspects of the problem related to the action of T₃ in brain, namely the endogenous occupancy of the receptors by T₃ and the regional distribution of these receptors. It is interesting to

note that, as in the case of developing rat brain (Schwartz & Oppenheimer, 1978), the concentration of T₃ receptors in the cerebrum greatly exceeds that in other regions during brain maturation, suggesting the cerebrum as the major site of action of T₃ in the developing brain. Finally, the temporal correspondence of the period (7–13 days of embryogenesis) of maturation, as evidenced by intense neuronal differentiation (Oppenheim & Foelix, 1972), with the sharp rise in the level of T₃ receptors and the high level of endogenous T₃ in brain conforms with the involvement of T₃ in the normal development of the brain.

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