The Metabolism of High-Molecular-Weight Ribonucleic Acid in Hypothalamic and Cortical Regions of the Developing Female Rat Brain

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The regional metabolism of high-molecular-weight RNA in the developing female rat brain was investigated after the intracranial injection of [32 P]P_i. The synthesis of polyadenylated RNA relative to high-molecular-weight RNA was determined after oligo(dT)–cellulose chromatography of total cellular high-molecular-weight RNA labelled after 4 h. In both hypothalamus and cortex this synthesis was significantly higher during the first 10 days *post partum* than at subsequent ages. In both regions apparently more mRNA is synthesized in the young. The ratio of the specific radioactivity of cytoplasmic high-molecular-weight RNA relative to that of the nucleus, measured after a 48 h period of labelling, was considered to be an index of the nucleo-cytoplasmic transport of newly synthesized RNA [Berthold & Lim (1976) *Biochem. J.* **154**, 529–539]. In the cortex, nucleo-cytoplasmic RNA transport in rats aged up to 20 days was significantly higher than in older rats, with the maximal value being attained between 16 and 19 days *post partum*. In contrast, in the hypothalamus, nucleo-cytoplasmic transport of RNA was low during the neonatal period and comparable with that of the mature animal. However, there were two periods of increased transport at later stages of development, the first between 15 and 19 days *post partum* and the second between 25 and 29 days *post partum*. These prepubertal changes in the nucleo-cytoplasmic transport of RNA in the female hypothalamus during weeks 3 and 4 *post partum* are coincident with other reported changes occurring during sexual differentiation. Differences in the timing of the maturational changes of the two brain regions thus appear to be reflected in developmental changes in RNA transport.

The development of the rat brain continues until well after birth (Davison & Dobbing, 1968). There is a difference in the timing of maturation of the various brain regions; for example, at birth the development of the cerebrum is at a more advanced stage than that of the cerebellum (Balázs, 1974). During this period of postnatal development the brain is susceptible to metabolic influences, and it is widely accepted that the hormonal milieu determines the pattern of differentiation (Balázs & Richter, 1973; Balázs *et al.*, 1977). This is particularly evident in the differentiation of the hypothalamus. In the adult, this brain region exhibits sexual dimorphism; it has been demonstrated that there are sex differences in the concentration of certain enzymes (Griffiths & Hooper, 1972; Libertun *et al.*, 1973) and in the morphology of specific areas (Raisman & Field, 1973; Dörner, 1976). Sex steroid hormones are intimately involved in the regulation of hypothalamic differentiation (Gorski, 1971; McEwen *et al.*, 1974). There is a 'critical' period, extending to 10 days *post partum*,

during which sex hormone imbalance leads to a permanent alteration of the expression of sexuality in the adult, in terms of gonadotropin release and sexual behaviour.

We have previously reported differences in the metabolism of high-molecular-weight RNA between newborn and adult rat forebrains (Berthold & Lim, 1976*a,b*). In the transition from the replicative developmental phase to the non-replicative fully differentiated state, the synthesis of mRNA relative to rRNA was decreased. There were also changes in the nucleo-cytoplasmic relationships of high-molecular-weight RNA that were suggestive of a decreased transport of the RNA into the cytoplasm in the adult. In the present paper we describe changes in these parameters of RNA metabolism throughout the period of postnatal development in two brain regions of the female rat, namely the cortex and the hypothalamus. The present study was undertaken to elucidate the patterns of RNA metabolism that might underlie sexual differentiation of the hypothalamic region during the 'critical' period and subsequent changes related to puberty.

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Experimental

Chemicals

A.R. grade chemicals were used in all cases. Glassware was treated with 0.05-M NaOH to destroy ribonuclease activity before being rinsed with water. Research, Waltham, MA, U.S.A. (supplied by Uniscience, Cambridge, U.K.). [^{32}P]P_i (sp. radioactivity 30–100 Ci/mg) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. as a sterile acidic solution (10 mCi/ml).

Animals

Female Wistar rats (Cobs strain), bred in our laboratory, were used at various ages between 3 and 70 days. The rats were sexed the day after birth by measurements of anal-genital distances. Males were culled and litters of 8 females kept with each mother. All animals were injected intracranially with a solution of iso-osmotic neutralized [^{32}P]P_i in 0.9% NaCl (0.1 ml/g wet wt. of brain) under ether anaesthesia. Rats under 21 days old were returned to their mothers. At periods of 4, 24 or 48 h after the injection, the rats were decapitated, the skulls opened and brains removed. Dissection and homogenization were carried out at 4°C. The brains were each dissected into a block of tissue containing the pre-optic area, hypothalamus and amygdala, i.e. the oestrogen-binding region described by McEwen & Pfaff (1970) and used in our studies on oestrogen receptors (White *et al.*, 1978). This block is hereafter referred to as the 'hypothalamus' and is accepted to be responsible for much of the sexual functions of the brain (Barraclough, 1973). The weight of each hypothalamic block was 0.18 g at 3 days *post partum*, increasing to 0.45 g at 30 days *post partum* and remaining constant thereafter. Blocks of cortical tissue were also dissected.

Preparation of subcellular fractions

The hypothalamic and cortical tissue from four litters was pooled separately and homogenized in 0.25 M-sucrose/50 mM-Tris/HCl (pH 7.6)/25 mM-KCl/1 mM-MgCl₂ at a 20% (w/v) tissue concentration. The homogenate was centrifuged at 1000g for 10 min in the MSE 4L refrigerated centrifuge at 4°C to obtain a crude nuclear pellet and a supernatant, cytoplasmic fraction. The nuclei from which RNA was isolated were purified by a modification (Berthold & Lim, 1976b) of the methods of Georgiev (1967) and Markov & Arion (1973), including a

phenol treatment and centrifugation. Briefly, the nuclear pellet, resuspended in homogenization medium, was treated with ice-cold phenol previously equilibrated to pH 6. After centrifugation the 'phenolic' nuclei formed a flocculent precipitate at the interphase between the aqueous and phenol phases. The aqueous phase was removed and replaced with a 1:1 (v/v) mixture of phenol and homogenization medium. After a second centrifugation, the aqueous phase was again removed and the nuclear preparation recovered from the interphase.

Isolation of high-molecular-weight RNA

High-molecular-weight RNA was isolated from nuclear and cytoplasmic fractions, and from homogenates in certain experiments, as previously described (Berthold & Lim, 1976a). Nucleic acids were first extracted by a modification of the phenol method of Kirby (1968). This involved the use of chloroform/3-methylbutan-1-ol in the phenol phase for facilitating the extraction of polyadenylated RNA (Perry *et al.*, 1972). Ethanol-precipitated and washed nucleic acid extracts were dissolved in 10 mM-Tris/HCl (pH 7.6)/0.2% (w/v) sodium dodecyl sulphate and the high-molecular-weight RNA selectively precipitated with a final concentration of 2 M-LiCl (Baltimore & Girard, 1966).

Isolation of polyadenylated RNA

The proportion of polyadenylated RNA in samples of ^{32}P -labelled high-molecular-weight RNA was determined after chromatography on oligo(dT)-cellulose, as previously described (Berthold & Lim, 1976b). This was a batchwise method involving the use of 90% formamide to elute polyadenylated RNA specifically bound to the oligo(dT)-cellulose.

Assay of radioactivity in purified RNA

Radioactivity in ^{32}P -labelled purified RNA was determined as described previously (Berthold & Lim, 1976a).

Results

Isolation of high-molecular-weight RNA from nuclear and cytoplasmic fractions of brain regional preparations

Our previous investigations on nucleocytoplasmic relationships of high-molecular-weight RNA in the rat forebrain were based on measurements of ^{32}P -labelled RNA isolated from subcellular fractions, the nuclear component being prepared by a combined centrifugation/phenol-treatment method. In this method the 'phenolic' nuclei were free of cytoplasmic

contamination and the RNA was recovered in an undegraded form (Berthold & Lim, 1976b), and high-molecular-weight constituents were isolated by the use of 2M-LiCl (Berthold & Lim, 1976a).

With preparations derived from the hypothalamus and the cerebral cortex, nucleic acids, i.e. RNA and DNA, were extracted from the various fractions generated by the procedure for isolating 'phenolic' nuclei. The contribution of each fraction to the total amount of nucleic acids extracted is shown in Table 1. In both regional preparations the nucleic acids were found essentially in three fractions: the post-nuclear supernatant (constituting the cytoplasmic fraction); the nuclei; and the first aqueous wash (W₁) of the original phenol-treated nuclear pellet. An electrophoretic analysis of these fractions showed that the supernatant contained 28S RNA, 18S RNA and tRNA, but no DNA, as did the fraction W₁ (Fig. 1). DNA was present in other fractions after removal of nuclei, but the contribution of this to the total is negligible (see Table 1). The DNA was present in the purified nuclear fraction and could be separated from high-molecular-weight RNA by selective precipitation of the RNA with 2.0M-LiCl (Fig. 2). DNA and tRNA were present in the LiCl-soluble fraction and the high-molecular-weight RNA was recovered in an undegraded form. The cytoplasmic fraction similarly analysed also showed the presence of only high-molecular-weight RNA species in the LiCl-precipitated fraction, but only

Fig. 1. Electrophoretic analysis of nucleic acids of fractions generated in preparation of 'phenolic' nuclei and of post-nuclear supernatant

The postnuclear supernatant from hypothalamic and cortical preparations of 27-day-old rats (see the Experimental section) was adjusted to contain TNS medium [1% (w/v) tri-isopropyl-naphthalene-sulphonic acid (sodium salt)/6% (w/v) *p*-amino-salicylic acid/8 mM-Tris/HCl, pH 8]. In (a) and (b), nucleic acids were phenol-extracted and precipitated with 2 vol. of ethanol overnight at -20°C. Precipitates were redissolved in Tris/sodium dodecyl sulphate buffer [10 mM-Tris/HCl (pH 7.6)/0.2% (w/v) sodium dodecyl sulphate] and electrophoresed on 2.5% (w/v) polyacrylamide gels at 5 mA per gel for 4.5 h. Nucleic acids were similarly isolated from other fractions generated in the preparation of 'phenolic' nuclei, except that the phenol phase was first extracted with TNS medium. The nucleic acids from fractions W₁ (c and d) and W₂ (e and f) and the 'phenol' phase (g and h) were electrophoresed on 2% (w/v) polyacrylamide gels at 5 mA per 4 h. The fractions for electrophoresis were from the cortex (a, c, e and f) and the hypothalamus (b, d, f and h).

Table 1. Proportional content of nucleic acids in the fractions derived from the nuclear-isolation procedure in both hypothalamus and cortex

Nucleic acids were extracted from all fractions generated in the preparation of purified nuclei, as well as from the postnuclear supernatant (cytoplasm). Precipitates from ethanol treatment of the extracts were washed twice in 70% ethanol/0.1 M-sodium acetate and dissolved in 10 mM-Tris/HCl (pH 7.6)/0.2% sodium dodecyl sulphate. The nucleic acid yield for each fraction was determined by A₂₆₀ measurements and the results are expressed as a percentage of the total nucleic acid recovered.

Fraction	Nucleic acid recovered (%)	
	Cortex	Hypothalamus
Cytoplasm (postnuclear supernatant)	40.9	33.8
'Phenolic' nuclei	35.8	33.9
First aqueous wash phase (W ₁)	20.3	24.5
Second aqueous wash phase (W ₂)	2.1	4.7
Phenol phase	0.6	0.8
Pellet	0.1	2.4

tRNA in the LiCl-soluble fraction. Thus it was possible to prepare undegraded high-molecular-weight RNA from the two subcellular fractions derived from the different brain regions. The post-nuclear supernatant and purified nuclei were used in subsequent experiments.

The radioactivity in the LiCl precipitate was found to be exclusively in RNA. As discussed previously

Fig. 2. Electrophoretic analysis of 2.0 M-LiCl-precipitated and LiCl-soluble nucleic acids from nuclei prepared from hypothalamus and cortex

Nuclei were prepared from both brain regions of 27-day-old rats as described in the Experimental section, and lysed in TNS medium. Nucleic acids were phenol-extracted and precipitated with 2 vol. of ethanol overnight at -20°C . Precipitates were dissolved in Tris/sodium dodecyl sulphate buffer and reprecipitated in 2.0 M-LiCl overnight at 4°C . Both the LiCl-soluble fraction and LiCl-precipitated material (which was first dissolved in the Tris/sodium dodecyl sulphate buffer and adjusted to contain 0.15 M-sodium acetate, pH 6.0) were again ethanol-precipitated. After redissolution in Tris/sodium dodecyl sulphate buffer, approx. 1 A_{260} unit each of high-molecular-weight RNA of the LiCl-precipitated fraction (a and c) and of the nucleic acids from the LiCl-soluble fractions (b and d) were electrophoresed on 2.5% (w/v) polyacrylamide gels at 5 mA per gel for 4 h. (a) and (b) Hypothalamus; (c) and (d) cortex. Similar profiles were obtained with RNA prepared from cytoplasm, except that the LiCl-soluble fractions contained large amounts of tRNA, but no DNA.

(Berthold & Lim, 1976a), ethanol-precipitated ^{32}P -labelled RNA from the phenol extract contained a high proportion of radioactive contaminants that were not precipitated with 5% trichloroacetic acid at 4°C . However, virtually all the radioactivity associ-

ated with high-molecular-weight ^{32}P -labelled RNA, prepared by precipitation with 2.0 M-LiCl, was precipitable in 5% trichloroacetic acid and solubilized after heating at 90°C for 15 min. The results shown in Table 2 are for hypothalamic preparations; similar results were obtained with cortical tissue.

Incorporation of ^{32}P into high-molecular-weight RNA

The incorporation of ^{32}P into high-molecular-weight RNA of the hypothalamus was studied over a 48 h period. Incorporation into RNA of both nuclear and cytoplasmic fractions from rats of two different ages is shown in Fig. 3 and is expressed as specific radioactivity (radioactivity/ A_{260}). After a single intracranial administration of ^{32}P , uptake of labelled precursor into RNA continued for 48 h in both cellular fractions, showing that there was continuing synthesis and nucleo-cytoplasmic transport of the RNA in this region during development.

The ratio of the specific radioactivity of RNA in the cytoplasm to that in the nucleus (C/N ratio) is a measure of the cellular distribution of the newly synthesized RNA. This ratio after 48 h of labelling has been used as an indication of the relative index of transport of newly synthesized RNA, and was shown to be high in the forebrains of newborn compared with the adult rat, reflecting a decreased transport in the adult (Berthold & Lim, 1976b). Similar studies were undertaken on the hypothalamus. The results shown in Fig. 4 are of changes in the C/N ratio throughout a 48 h period of labelling in the hypothalamus and in cortical tissue of neonatal rats. In

Fig. 3. Incorporation of ^{32}P into high-molecular-weight RNA of the hypothalamus

Female rats (12 in each age group) were injected intracranially with 1 mCi of ^{32}P /g of brain. Groups of four animals were killed after intervals of 4, 24 or 48 h. Measurements of radioactivity and A_{260} were made of the high-molecular-weight RNA prepared from the hypothalamic region of rats aged 3 days (b) and 18 days (a). ●, Nuclear RNA; ○, cytoplasmic RNA.

Table 2. Assay of radioactivity in hypothalamic RNA

Phenol-extracted, ethanol-precipitated and washed RNA (a) was prepared from the hypothalamic regions of 27-day-old rats injected intracranially 24 h previously with 500 μ Ci of [32 P]P_i. Trichloroacetic acid precipitation and assay of radioactivity in RNA was carried out as previously described (Berthold & Lim, 1976a). Samples (50 μ l) of 32 P-labelled RNA in Tris/sodium dodecyl sulphate buffer were dried on Whatman 3MM filters (2.5 cm) and radioactivity was determined in toluene/PBD scintillant (0.4% (w/v) butyl-PBD [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] in toluene). Filters were removed from the scintillant and soaked in 10% (w/v) trichloroacetic acid at 4°C for 30 min. After being washed successively in ice-cold 5% trichloroacetic acid, ethanol and finally in ether, filters were dried and returned to scintillant, to give values for radioactivity precipitated by trichloroacetic acid at 4°C (i). The filters were then removed and soaked in 5% trichloroacetic acid and heated at 90°C for 15 min in the same solution. The ethanol/ether washing procedure was repeated, and residual radioactivity in filters after removal of RNA was determined (ii). Radioactivity in LiCl-precipitated RNA (high-molecular-weight RNA), prepared from these total phenol-extracted nucleic acid fractions as described in the Experimental section and in Fig. 2, was similarly assayed (b).

Proportion of radioactivity recovered in precipitates (%)

Fraction	(a) Phenol-extracted ethanol-precipitated nucleic acid		(b) High-molecular-weight RNA, precipitated by 2.0M-LiCl	
	(i) Insoluble in trichloroacetic acid at 4°C	(ii) Insoluble after heating at 90°C	(i) Insoluble in trichloroacetic acid at 4°C	(ii) Insoluble after heating at 90°C
Total cellular fraction	5.4	0.75	99.5	4.3
Nucleus	8.2	0.73	95.9	2.1
Cytoplasm	6.2	0.49	99.7	2.1

both regions, the C/N ratio was low at 4 h, then subsequently increased. This can be interpreted as transport of newly synthesized RNA from nucleus to cytoplasm (Fig. 4). The initial increase in the C/N ratio was similar in the hypothalamus and cortex, but,

after 48 h of labelling, values for the neonatal hypothalamus were lower than for the cortical tissue.

Regional differences in nucleo-cytoplasmic relationships of RNA during brain development

We therefore studied the changes in the C/N ratio after 48 h of labelling in these two brain regions in the same groups of rats throughout development to compare alterations in transport in the hypothalamus with those in the cortex (Fig. 5). The quotient (C/N ratio in cortex)/(C/N ratio in hypothalamus) decreased gradually during the first 35 days from a value of 1.5 at 3 days to values of about 1 at 35 days. Thus there appeared to be a greater degree of nucleo-cytoplasmic transport of RNA in the neonatal cortex, reflecting a differential rate of development in terms of RNA metabolism in the cortex and hypothalamic regions. In the adult there was no regional difference, the quotient being 1.

An analysis of relative changes in this quotient, which fit a regression line ($P < 0.001$), represents a gross assessment of the differences in RNA metabolism between the two regions. An analysis of the C/N ratio in the separate regions was also undertaken to consider individual regional patterns of RNA metabolism during development. The results for the cortex (Fig. 6) show that the ratio was high in the neonatal animal, increasing to a maximum between 16 and 19 days and then decreasing to a constant low adult value by week 4. When divided into two groups, the values for the first 19 days were signi-

Fig. 4. Nucleo-cytoplasmic relationship of 32 P-labelled high-molecular-weight RNA in neonatal cortex and hypothalamus

Cytoplasmic and nuclear high-molecular-weight RNA was prepared from cortex (a) and hypothalamus (b) of 3-, 5- and 10-day-old rats at various times after injection of [32 P]P_i, and specific-radioactivity measurements were made as described in Fig. 3. Results are expressed as the ratio (specific radioactivity in cytoplasmic RNA)/(specific radioactivity in nuclear RNA) (C/N ratio). $\circ$, 3 days; Δ , 5 days; $\square$, 10 days.

Fig. 5. Comparison of the nucleo-cytoplasmic relationship of high-molecular-weight RNA in the cortex and in the hypothalamus during development

Nuclear and cytoplasmic high-molecular-weight RNA was prepared from hypothalamic and cortical tissue from female rats aged between 3 and 35 days (four animals per age group), as well as from sexually mature females, injected 48 h previously with the appropriate dose of [32 P]P_i. The C/N ratios at 48 h after labelling (see Fig. 4) were determined in both brain regions from the same groups of animals, and data for each age group are plotted as the quotient of (C/N ratio after 48 h labelling in the cortex)/(C/N ratio after 48 h labelling in the hypothalamus). Linear-regression analysis ($n = 25$; $r = -0.73$) gave a significant correlation ($P < 0.001$). The average value for this quotient in the sexually mature female was 1.08. Measurements for cortex and hypothalamus from which these quotients were derived are separately shown in Figs. 6 and 7.

significantly higher than those of older animals ($P < 0.001$). By analysis of variance of the values assigned to seven consecutive groups according to age, values derived from animals aged between 15 and 19 days showed the greatest statistical differences from those of rats older than 20 days.

In the hypothalamus (Fig. 7) the C/N ratio was low immediately after birth and similar to the adult value. This ratio increased at two periods of development in the prepubertal female rat. These were between 17 and 20 days and between 26 and 30 days. An analysis by Student's t test of these changes showed that the values in these two groups (B and C) were significantly higher than those of animals in the first 12 days *post partum* (group A). By analysis of variance, similar findings were obtained; the values derived from rats aged between 15 and 19 days and between 25 and 29 days were significantly higher than those for the first 14 days *post partum* as well as those of mature females. Values of the C/N ratio in

Fig. 6. Changes in nucleo-cytoplasmic RNA transport during development of the cortex

Nuclear and cytoplasmic high-molecular-weight RNA was prepared from cortical tissue dissected from rats (aged between 3 and 70 days) killed 48 h after the administration of [32 P]P_i. The results show the ratio of the specific radioactivities of the RNA from the cytoplasmic to that of the nuclear fractions (48 h C/N ratio) ($\bullet$). Statistical analysis of data by Student's t test showed values derived from group (i) (animals less than 20 days of age) were significantly higher than those for group (ii) (animals 20 days and older) ($P < 0.001$). For analysis of variance the data were regularly grouped into seven consecutive sets as follows: a, 1–5 days; b, 6–10 days; c, 11–14 days; d, 15–19 days; e, 20–24 days; f, 25–29 days; g, 30 days and older. The values derived from groups (b), (c) and (d) were found to be significantly the highest [group b greater than groups e and g ($P < 0.05$); group c greater than groups e, f and g ($P < 0.05$); group d greater than groups e, f and g ($P < 0.002$)]. Group means ($\square$) are also shown.

the intervening period between these two periods were statistically similar to those of the neonatal animal and the adult. There are apparently two post-natal periods of increased transport to RNA.

The measurements for the early 'quiescent' period were found to show little variation, but at the later periods of increased activity the measurements showed a greater fluctuation, possibly owing to differences in the exact timing of developmental changes in individual animals. This was quite marked at the end of week 4 and may be related to variability in the onset of puberty (with vaginal opening in our rats occurring between 35 and 42 days, the majority opening at 40 days).

Metabolism of polyadenylated RNA

The metabolism of polyadenylated RNA was previously shown to alter during development of the cortex (Berthold & Lim, 1976b), the proportion of 32 P-labelled polyadenylated RNA in the high-molecular-weight RNA being used as an index of the relative synthesis of the polyadenylated RNA. We

Fig. 7. Developmental changes in the C/N ratio in the hypothalamus

The C/N ratio (●) of hypothalamic high-molecular-weight RNA was measured after a 48 h labelling period in groups of animals aged between 3 and 70 days. Data were regularly grouped according to age for analysis of variance as follows: neonatal group a (0–14 days); groups b, c and d (5-day intervals up to 30 days) and group e (30+ days). Means of groups (□) are also plotted. Values for groups b and d were significantly greater than groups a and e ($P<0.02$). A different statistical analysis, using Student's t test, of the data derived from three separate groupings of animals, showed that the values for groups A (2–12-day-old rats) was significantly less than those for both group B (17–20-day-old rats, $P<0.001$) and group C (26–30-day-old rats; $P<0.05$).

first compared the values for this index obtained after 4 h of labelling with those after 24 h of labelling in the different brain regions (Table 3). At the early time period, the proportion of ^{32}P -labelled polyadenylated RNA in high-molecular-weight RNA isolated from the unfractionated homogenate (i.e. total cellular) was similar to that in the purified

nuclear fraction, in both brain regions. The proportion of polyadenylated RNA in the cytoplasmic fractions was found to be lower. After a 24 h labelling period the proportion of polyadenylated RNA in nuclear high-molecular-weight RNA was higher than that in total cellular high-molecular-weight RNA. Values for the latter corresponded more closely to those of the cytoplasmic fraction. These results for both cortex and hypothalamus are in agreement with previous results for the forebrain (Berthold & Lim, 1976b), showing that the proportion of polyadenylated RNA in total cellular RNA approximated to that of the nucleus after short labelling periods.

The metabolism of polyadenylated RNA in the hypothalamus was next investigated over a 48 h period. Results for animals aged 3 and 10 days are shown in Fig. 8. The proportion of ^{32}P -labelled polyadenylated RNA decreased in the cytoplasm from 20% at 4 h to 5% at 48 h. As with the forebrain of young animals, the initially high value probably reflects an early transfer of newly synthesized polyadenylated RNA into the cytoplasm, where it forms a significant proportion of transported ^{32}P -labelled RNA; the lowered value at the later time period is indicative of the continuing transport and accumulation of rRNA, which constitutes over 95% of the high-molecular-weight RNA. The proportion of ^{32}P -labelled polyadenylated RNA in the nucleus fell much more gradually during this period.

The proportion of polyadenylated RNA in total cellular ^{32}P -labelled high-molecular-weight RNA after 4 h of labelling was then determined both in the hypothalamus and in the cortex throughout the period of development. This value, which is equivalent to that of the nucleus at this early time period of labelling (see Table 3), was used to provide an indication of the relative synthesis of polyadenylated RNA within the nucleus. The basis for the use of this

Table 3. Proportion of ^{32}P -labelled polyadenylated RNA in high-molecular-weight RNA from subcellular fractions Female rats (aged 27 days), injected intracranially with $500\text{ }\mu\text{Ci}$ of $[^{32}\text{P}]\text{P}_i$, were killed after intervals of either 4 or 24 h. High-molecular-weight RNA was prepared from nuclear and cytoplasmic fractions, as well as from total homogenates, from both hypothalamus and cortex. The proportion of ^{32}P -labelled polyadenylated RNA in the various fractions was determined by oligo(dT)-cellulose chromatography as described previously (Berthold & Lim, 1967b). Briefly, high-molecular-weight RNA was allowed to interact with oligo(dT)-cellulose in high-salt buffer [0.4M-NaCl/10mM-Tris/HCl (pH7.6)/0.1% (w/v) sodium dodecyl sulphate/0.1% (v/v) dodecylsarcosine/mM-EDTA/10% (v/v) formamide]. Unbound RNA was eluted by the same buffer. The polyadenylated RNA was eluted with modified buffer in which the formamide concentration was raised to 19% (v/v) and NaCl omitted.

Labelling period (h)	Radioactivity in polyadenylated RNA (%)					
	Cortex			Hypothalamus		
	Total cellular fraction	Nucleus	Cytoplasm	Total cellular fraction	Nucleus	Cytoplasm
4	15.7	18.3	10.0	22.1	20.2	4.2
24	8.0	20.7	7.6	11.5	17.0	6.2

Fig. 8. Metabolism of ^{32}P -labelled polyadenylated RNA in the neonatal hypothalamus

Nuclear and cytoplasmic high-molecular-weight RNA was prepared from the hypothalamus of 3- and 10-day-old female rats killed at 4, 24 or 48 h after the injection of $[\text{}^{32}\text{P}]\text{P}_i$ at the appropriate dose. The proportion of ^{32}P -labelled polyadenylated RNA was determined by oligo(dT)-cellulose chromatography. Radioactivity was monitored by Čerenkov radiation. ●, Nucleus (3-day-old); ■, nucleus (10-day-old); ○, cytoplasm (3-day-old); □, cytoplasm (10-day-old).

value as an index of mRNA synthesis, which obviates the need for a prior fractionation of the nucleus, has been rationalized previously (Berthold & Lim, 1976a).

Electrophoretic analysis of the polyadenylated RNA recovered after oligo(dT)-cellulose chromatography of total cellular RNA from the hypothalamus showed it to consist of a heterogeneous population of large species of RNA. The analysis in Fig. 9 is of polyadenylated RNA derived from 46-day-old animals; it shows little contamination with rRNA and is comparable with that of forebrain polyadenylated RNA, with no evidence of degradation.

In three different experimental series, the proportion of the ^{32}P -labelled polyadenylated RNA decreased with age in both the cortex and the hypothalamus. There was some variability in the actual values for the proportions of ^{32}P -labelled polyadenylated RNA determined for the same age group in the three separate experimental series. Chloroform/3-methylbutan-1-ol was used in phenol extraction to minimize the selective loss of polyadenylated RNA (Perry *et al.*, 1972). However, some variability in the recovery of polyadenylated RNA by this method has been reported (Morrison *et al.*, 1977), and this could explain the variability observed between separate experimental series. The proportion of ^{32}P -labelled polyadenylated RNA in 46-day-old sexually mature females varied between 17.5 and 22% in different

Fig. 9. Electrophoretic analysis of ^{32}P -labelled polyadenylated RNA from hypothalamus

High-molecular-weight RNA was prepared from the whole homogenate (total cellular fraction) of hypothalamic tissue of 46-day-old female rats, killed 4 h after the administration of $600\mu\text{Ci}$ of $[\text{}^{32}\text{P}]\text{P}_i$. The polyadenylated RNA fraction, after chromatography of the RNA on oligo(dT)-cellulose, was adjusted to 0.15M-sodium acetate (pH 6.0) and precipitated with 2.5 vol. of ethanol overnight at -20°C . The polyadenylated RNA in Tris/sodium dodecyl sulphate buffer containing 70% (v/v) formamide, 0.1% (v/v) dodecylsarcosine and 1 mM-EDTA was electrophoresed on composite gels of 1.5% polyacrylamide/0.5% agarose; the gels were frozen and cut into 2 mm serial sections with a Mickle gel slicer, dried and the radioactivity was determined as previously described (Berthold & Lim, 1976b). The positions of 28S and 18S RNA markers are shown for comparison.

experiments, with the values for other ages varying to the same extent.

The results from different experiments were normalized by taking the value for the mature 46-day-old animal in each experimental series to be 100% and expressing all other values for that series as a percentage of this (Fig. 10). This enabled us to combine results from the different series for a clearer assess-

Fig. 10. Developmental changes in the proportion of polyadenylated RNA synthesized in the cortex and hypothalamus

The proportion of ^{32}P -labelled polyadenylated RNA in total cellular high-molecular-weight RNA, after 4h of labelling *in vivo*, was determined in both hypothalamic and cortical regions at various ages (as described in Table 3). Three experiments are represented, each one involving the use of groups of animals of a similar range of ages, with values in each experiment being expressed as a percentage of the 46-day value for that experiment [46-day-old value = 100% (■)]. Horizontal bars represent the spread of ages that were grouped together, and the vertical bars indicate the standard deviation. (a) Hypothalamus; (b) cortex. Statistical analysis by Student's *t* test showed values for the first 10 days to be higher than those from animals older than 25 days in both regions (cortex, $P < 0.01$; hypothalamus, $P < 0.001$).

increases in cell size and cellular accumulation of rRNA (Adams, 1966; Lim, 1977). The development of the hypothalamus is especially interesting, since sex hormones are directly involved in its sexual differentiation, which can be interfered with by the administration of inhibitors of RNA metabolism at certain critical perinatal periods (Gorski, 1971; Salaman, 1974). Because of our interest in the role of oestrogen receptors in hypothalamic sexual functions (White *et al.*, 1978), we have extended our investigations to include a comparison of RNA metabolism in the hypothalamus with the cortex, not only in the newborn and the adult, but throughout the postnatal period of development.

Measurements of C/N ratio

Among the several factors that contribute to the final value of the C/N ratio, which is a reflection of the nucleocytoplasmic relationship of high-molecular-weight RNA, are: (1) rate of uptake of labelled precursor into RNA; (2) the intracellular processing and turnover of the labelled RNA; (3) transport of newly synthesized RNA from nucleus to cytoplasm; (4) the stability of RNA in the cytoplasm; (5) the total amounts of RNA in both compartments. The relative importance of each of these factors varies with the time of labelling. At short labelling periods (4h) the C/N ratio is small (Fig. 4), since labelled RNA that is transported into the cytoplasm forms only a small proportion of the RNA in this fraction, and is thus influenced to a large extent by the rate of synthesis and processing and the extent of nucleocytoplasmic transport. The stability of the cytoplasmic RNA is relatively unimportant, as the half-life of most RNA species is generally much greater than 4h. The C/N ratio increases with longer labelling periods in both brain regions. In the adult forebrain, the rate of increase of this C/N ratio has been shown to be lower than in young forebrain, reflecting a restriction of nucleocytoplasmic transport as well as intranuclear turnover of newly synthesized RNA (Berthold & Lim, 1976b; Lim, 1977).

Since nucleocytoplasmic transport of labelled RNA contributes to a major extent to the C/N ratio after longer labelling periods, we have used the value for the C/N ratio after 48h of labelling as an index of nucleocytoplasmic RNA transport. The ratio is also influenced to a lesser extent by other factors, including RNA stability. (Absolute rates of RNA synthesis and degradation have not been measured in brain as this requires a detailed knowledge of precursor pool relationship.) The lower values of the C/N ratio found in the neonatal hypothalamus compared with the cortex was not due to a selective depletion of labelled precursor in the hypothalamus, which would limit increases in the specific radioactivity of nuclear RNA. The specific radioactivities of neither the

ment of developmental changes, which were based on larger numbers of rats. The proportion of polyadenylated RNA in the hypothalamus of rats under 10 days old was significantly higher than in animals older than 25 days. A significant decrease in the values was also obtained in the developing cortex. Thus there appeared to be no regional difference in the developmental decrease in the relative synthesis of polyadenylated RNA, with adult values being reached by week 4.

Discussion

Our previous studies (Berthold & Lim, 1976a,b) have shown that changes in RNA metabolism accompany the development of the rat forebrain. For example, in comparison with the adult there is increased transport of RNA into the cytoplasm of the neonatal animal, possibly to accommodate

nuclear nor the cytoplasmic RNA decreased substantially over a 48h labelling period (see Fig. 3).

Regional RNA metabolism in relation to brain development

(a) *Cortex*. The higher values of the C/N ratio in the cortex of animals less than 20 days old compared with those of older animals can be interpreted as being representative of the extensive transport and cytoplasmic accumulation of newly synthesized RNA during this phase of active cell growth. In the first week after birth, the average cell size in the rat cerebrum increases 4-fold, on the basis of tissue weight per unit DNA (Balázs & Richter, 1973), and there is an accumulation of rRNA (Adams, 1966) such that the RNA/DNA ratio increases rapidly during the first week, reaching a peak at around 14 days. The DNA content of the cerebrum reaches the adult value at 20 days.

The developmental events occurring in the first 3 weeks *post partum* include biochemical maturation in terms of an efficient conversion of glucose into amino acids, and the attainment of adult patterns of metabolic compartmentation; increases in axonal density, number of cortical synapses and neurotransmitter enzyme activities (Balázs, 1974). The changes in the nucleocytoplasmic transport of RNA that may underlie these other metabolic changes are complete at about the same time, adult values being almost achieved by 20 days. Similar changes (Fig. 10) occur in the relative synthesis of polyadenylated RNA, i.e. mRNA and its precursor forms (Lim & Canellakis, 1970; Lim *et al.*, 1970, 1974; Brawerman, 1974), suggesting that in the cortex the regulation of mRNA synthesis as well as transport of both rRNA and mRNA are closely integrated.

(b) *Hypothalamus*. As with the cortex, the relative synthesis of presumptive mRNA is greater in the neonatal animal than in the adult. The developmental decrease occurs gradually over a 3-week period. It appears that brain cells, in common with other mammalian cells, synthesize more mRNA during the active phase of growth (Johnson *et al.*, 1974).

However, unlike the cortex, where extensive transport of newly synthesized RNA is associated with extensive mRNA synthesis, in the hypothalamus these two parameters of RNA metabolism are dissociable. In the developing female hypothalamus a selective increase in the cell nuclear volume occurs in some areas. During the last 2 days of foetal life, there is a 65% increase in the cell nuclear volume in the medial preoptic area and a 30% increase in the ventro-medial nucleus of the female rat. In these and other areas the nuclear volumes continue to increase up to the end of week 2 (Dörner, 1976). The nucleocytoplasmic relationships of ³²P-labelled RNA may accordingly be influenced at early stages in develop-

ment by these morphological changes in cell nuclei. The expansion of nuclear volume coupled with an increase in the content of nuclear RNA may result in a lower C/N ratio than would otherwise be expected, the accumulation of labelled RNA within the nucleus counteracting transport into the cytoplasm. In the developing male, unlike the female, growth of the cell nucleus in the equivalent hypothalamic areas is restricted. It would therefore be informative to compare the masculine pattern of RNA transport during this early postnatal period, particularly since masculine differentiation of the brain is thought to be sensitive to the effects of testosterone at this time.

Increases in the transport of RNA in the female hypothalamus nevertheless could be detected at two prepubertal phases (Fig. 7). The maximal increase in the first phase (at around 19 days *post partum*) is coincident with a period of greatest changes in the cortex. The accompanying metabolic changes in the hypothalamus include maturation of the hypothalamic-pituitary system regulating thyrotropin release (Dussault & Labrie, 1975); the maximal prepubertal concentration of hypothalamic thyroliberin being attained at this period and reflected directly by pituitary concentration of thyrotropin. Cortical and hypothalamic activities at this time may be associated with the process of weaning that occurs at 21 days. However, it is possible that the changes in RNA transport in the hypothalamus may also be involved with regulation of gonadotropin metabolism, since the content of pituitary follitropin also gradually increases after birth, reaching a maximum at 20 days *post partum*. The content subsequently declines [see review by Ramirez (1973)].

The maximal increase in RNA transport at the second phase occurred at 29 days. Interestingly, increases in the pituitary content of the other gonadotropin, lutropin, also occur later with maximal concentrations being attained at around 30 days *post partum*. The concentrations then decrease to reach low values immediately before the initial preovulatory surge of lutropin (at puberty).

In the male, increases in pituitary follitropin occur later than in the female, with maximal concentrations being attained at 40 days; puberty also occurs later, at about 50 days (Ramirez, 1973). Since the time course of sexual differentiation is different, a comparison of RNA metabolism in the hypothalamus of both sexes should provide interesting information on certain aspects of the regulation of hypothalamic differentiation, particularly as 'masculinization' of the female rat brain can be achieved by the perinatal administration of testosterone. Conversely, castration of neonatal males results in 'feminization' of the brain (Gorski, 1971). The expression of sexuality in the mature rat includes a cyclic release of gonadotropins in the female contrasted with a tonic release of gonadotropins in males.

Corresponding morphological differences have also been observed, with the female hypothalamus having an increased incidence of certain synapses in the pre-optic area (Raisman & Field, 1973), as well as having a greater neuronal nuclear size in the preoptic area and ventro-medial nucleus (Dörner, 1976). Studies on RNA metabolism in the different sexes during development should lead to a better understanding of the regulation of brain differentiation and the role of sex hormones.

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