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CEREBELLAR tRNA METHYLTRANSFERASES: A DEVELOPMENTAL STUDY

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SUMMARY

Developmental patterns of homologous and heterologous tRNA methylation by cerebellar tRNA methyltransferases are described. The study revealed that: (a) homologous tRNA methylation results in the predominant formation of N^2-methylguanine and 1-methyladenine; (b) tRNA methyltransferases of bulk-isolated Purkinje and granule cells methylate *E. coli* $tRNA^{glu}_2$ in vitro in a characteristic manner, and (c) the methylation of 8-day-old cerebellar, cortical and hepatic tRNA in vivo yields tRNAs containing different proportions of methylated bases. The findings suggest that the presumably cell-specific populations of cerebellar tRNA methyltransferases continue to alter their substrate recognition characteristics up to and beyond the first month of post-natal life.

INTRODUCTION

Since methylation is the principal post-translational modification undergone by tRNA precursors during their maturation[1,14,15] and since tRNA maturation accompanies cell differentiation and tissue growth[23], special significance attaches to studies of tRNA methylation in growing tissues. Several reports have shown that the immature brain is an active tRNA methylating organ[2,7,9,16,22] in which the homologous methylation of its own tRNAs remains active for up to 3 weeks[16]. Moreover, work from our laboratory has shown that significant differences in residue and site specificity patterns exist among the tRNA methyltransferases of rat brain[4,5,17].

It appeared of interest therefore, to examine the methylation of tRNA and the activity of several base-specific tRNA methyltransferases during the early post-natal

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period of cerebellar development in the rat. Preliminary reports have appeared[4,20].

MATERIALS AND METHODS

Animals and chemicals

Male rats of the Sprague–Dawley strain were used. S-adenosyl-L-[Me^{14}C]-methionine (spec. radioact. 46 mCi/mmol) was obtained from Research Products International, Elk Grove Village, Ill., [Me-^{3}H]L-methionine (5 Ci/mmol) was from Amersham, Arlington Heights, Ill., the methylated purines and pyrimidines were from Sigma, St. Louis, Mo. or Cyclo Chemical, Los Angeles, Calif., bulk *E. coli* tRNA and the purified individual tRNAs were from Miles Laboratories, Elkhart, Ind. or from Boehringer-Mannheim Biochemicals, Indianapolis, Ind. All other reagents were of analytical grade.

The preparation of the cerebellar tRNA methyltransferases

The cerebella were obtained from rats 3, 6, 8, (Table IV), 10, 14, (Table III), 18, (Fig. 1), 21 and 43 days old. The tissue was homogenized in 3.5 vol. of ice-cold 10 mM Tris·HCl buffer pH 7.0, containing 1 mM dithiothreitol (DTT) and 0.1 mM sodium EDTA. All subsequent operations were at 4 °C. The homogenates were centrifuged at 165,000 $\times$ *g* for 150 min and the clear supernatants were used as the source of the tRNA methyltransferases. The use of this relatively crude enzyme source was intentional as it was of interest to assess the total cerebellar tRNA methylating capacity.

The bulk-isolation of cerebellar Purkinje and granule cells

The procedure of Sellinger et al.[21] was used. The neuronal cell bodies were suspended in the buffer used to homogenize the whole tissue (see above), and were then vigorously homogenized. The homogenate was centrifuged at 165,000 $\times$ *g* for 150 min and the high-speed soluble supernatant used as the source of the 'cell-specific' tRNA methyltransferases.

The methylation of cerebellar tRNA in vivo

[Me-^{3}H]L-methionine (4 μCi/g, 25 μl) was injected intracranially 45 min before sacrifice.

Analytical

tRNA was extracted, purified and characterized by the procedure of Muramatsu and Fujisawa[13]. Protein was determined by the method of Lowry et al.[12].

High performance liquid chromatography (HPLC)

The conditions for the total hydrolysis of the methylated tRNA were described previously[16,18]. HPLC of the methylated bases was performed by the procedure of Salas and Sellinger[18]. The effluence from the HPLC run was collected in plastic 3 ml mini-vials (3–5 drops/vial) and its radioactivity determined.

Enzyme assay

The incubation mixture designed to estimate heterologous tRNA methylation contained in a total vol. of 0.6 ml/60 μmol of Tris·HCl buffer, pH 8.6, 600 nmol of DTT, 12 nmol of S-adenosyl-L-[Me-^{14} C]methionine (0.2 μCi), 2.5–3 mg of cerebellar supernatant protein, *E. coli* tRNA and spermidine, as desired. Homologous tRNA methylation was estimated in the presence of 0.01 mM spermidine and 0.2–0.3 mg of cerebellar supernatant protein. Incubation was at 37 °C. The reaction was stopped by the addition of 1 vol. of 88% (v/v) phenol (distilled before use) in 0.1 M sodium acetate, pH 5.1. After extraction[13], and the addition of 0.1 mg of carrier bulk *E. coli* tRNA, the tRNA was precipitated by 2.5 vol. of absolute ethyl alcohol and the suspension allowed to stand at —20 °C overnight. The tRNA pellet obtained by centrifugation was suspended in water, an aliquot withdrawn for radioactivity determination and the remainder hydrolyzed[17,18]. The [Me-^{14}C] bases were separated by HPLC[18].

RESULTS

The kinetics of methylation of tRNA^{phe} by cerebellar tRNA methyltransferases

Methylation of tRNA^{phe} (Fig. 1) resulted in the formation of N^2-methylguanine, 5-methylcytosine and 1-methyladenine as the principal methylated bases. The linear formation of N^2-methylguanine became arrested after 45 min, while 5-methylcytosine

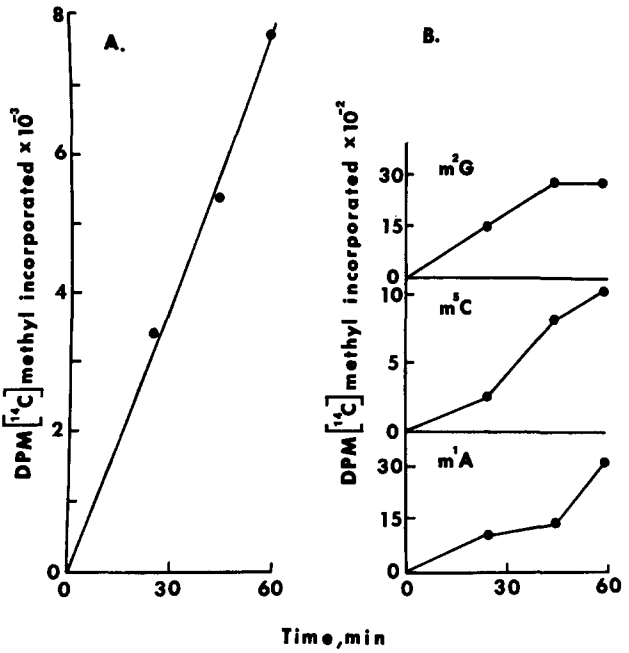

Fig. 1. The time course of methylation by cerebellar tRNA methyltransferases of 18-day-old rats. A: methylation of 1.6A_{260} tRNA^{phe}. B: formation of N^2-methylguanine (m^2G), 5-methylcytosine (m^5C) and 1-methyladenine (m^1A). Spermidine: 2.5 mM. The results are the means of two experiments with less than 15% variation between experiments.

TABLE I

Homologous methylation of the principal tRNA bases in the developing rat cerebellum

The values are expressed as percentages of the total radioactivity recovered after HPLC. Incubation, 60 min; for details see Methods. The values are means from 3 separate experiments, with less than 15% variation between experiments.

[Me-^{14}C]base	*Age (days)*					
	3	*6*	*10*	*14*	*21*	*43*
	%	%	%	%	%	%
1-methylguanine	7.1	4.4	3.4	2.7	2.7	3.3
N^2-methylguanine	53.9	31.9	46.6	44.2	43.1	35.4
5-methylcytosine	5.6	16.7	12.5	20.4	17.9	17.8
1-methyladenine	7.8	20.1	15.1	8.3	12.0	18.0
Sum*	74.4	73.1	77.6	75.6	75.7	74.5
Total dpm	1005	1404	980	1009	1066	577

* Does not include 7-methylguanine, $N^2{}_2$-dimethylguanine, 5-methyluradil, 2-methyladenine, N^6-methyladenine and 1-methylhypoxanthine.

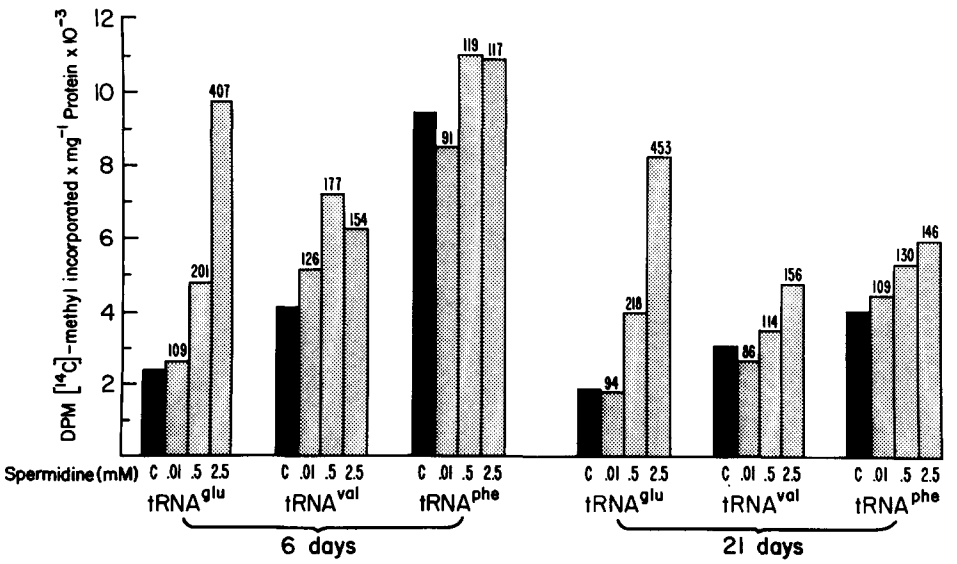

Fig. 2. Effect of spermidine on the methylation of individual *E. coli* tRNAs at 6 and 21 days. Cerebellar tRNA methyltransferases (2.8 mg of protein) were incubated for 60 min in the presence of 30 μg of the respective tRNA. The numbers on top of the bars refer to per cent control values (C). C, incubations from which spermidine was omitted. The results are the means of 3 experiments with $< 15\%$ variation between experiments.

TABLE II

The heterologous formation of [Me-^{14}C]N^2-methylguanine, 1-methyladenine and 5-methylcytosine by tRNA methyltransferases of 6- and 21-day-old cerebella

The values represent the radioactivity contained in the HPLC peak. To convert to pmol, 100 dpm = 1 pmol. The conditions of incubation are as outlined in Methods. All tubes contained 2.5 mM spermidine.

	t-RNA $^{glu}_2$		*tRNA ^{val}*		*tRNA ^{phe}*		*tRNA ^{fmet}*	
	Age (days)							
[Me-^{14}C]base	*6 (dpm)*	*21 (dpm)*	*6 (dpm)*	*21 (dpm)*	*6 (dpm)*	*21 (dpm)*	*6 (dpm)*	*21 (dpm)*
N^2-methylguanine	397	188	1950	1670	3320	1985	1010	2210
1-methyladenine	4583	3384	1341	1202	1273	1515	330	455
5-methylcytosine	2222	1090	222	358	292	381	n.d.	n.d.

and 1-methyladenine continued to be formed, after an initial lag period, at increased rates, particularly beyond 30–45 min of incubation.

Developmental effects

Table I follows the homologous methylation of cerebellar tRNA bases over the 3–43 days postnatal period. Fig. 2 illustrates the effects of spermidine on the 'specific radioactivity' (dpm/mg of protein) of 3 individual *E. coli* tRNAs following incubation with tRNA methyltransferases obtained from cerebella of 6- and 21-day-old rats. At 21 days the methylations of tRNA$^{glu}_2$, tRNA^{phe}, and tRNA^{val} were highest at 2.5 mM spermidine; at 6 days methylation of tRNA^{val} was highest at 0.5 mM spermidine. The formation of N^2-methylguanine, 1-methyladenine and 5-methylcytosine in tRNA$^{glu}_2$, tRNA^{val}, tRNA^{phe} and tRNA^{fmet} at 6 and 21 days is shown in Table II. These experiments revealed, in general, somewhat lower radioactivity values in the [Me-^{14}C] bases at 21, relative to 6 days. This was particularly noticeable with 1-methyladenine and 5-methylcytosine in tRNA$^{glu}_2$ and N^2-methylguanine in tRNA^{phe}, yet N^2-methylguanine in tRNA^{fmet} was twice as radioactive at 21 as compared to 6 days. Similar results (not shown) were obtained when spermidine was omitted from the incubation medium, indicating that the observed tRNA- and base-specific differences in methylation with age were not due to effects of the polyamine on the relevant tRNA methyltransferases, but, rather, were the consequence of intrinsic alterations in the ability of these tRNA-modulating enzymes to recognize a heterologous substrate.

The cell-specific methylation of tRNA$^{glu}_2$

To compare the tRNA methylating capacity of different neuronal cell types, tRNA methyltransferases prepared from Purkinje and granule cells were incubated with tRNA$^{glu}_2$, a tRNA species particularly well suited as a substrate of the predominantly neuronal tRNA (adenine-1-methyltransferase[19]. Table III shows that there was

TABLE III

The methylation of E. coli $tRNA^{glu}_2$ by tRNA methyltransferases of Purkinje and granule cell neurons

The Purkinje and granule cell neurons were isolated by the procedure of Sellinger et al. The rats were 14 days old. Incubation, 60 min; spermidine, 2.5 mM; $tRNA^{glu}_2$, 20 μg. The values are expressed as percentages of the radioactivity recovered after HPLC and corrected to 100%. The actual recoveries (as per cent of the dpm injected onto the HPLC column) were 79–80%. The results are the averages of 2 consecutive experiments with $< 10\%$ difference between individual values.

[Me-^{14}C]base	*Purkinje cell neurons*	*Granule cell neurons*
1-methylguanine	13.9	8.9
N^2-methylguanine	5.0	2.5
5-methylcytosine	28.3	20.5
1-methyladenine	40.5	43.7
1-methylhypoxanthine	4.5	9.0
5-methyluracil	7.8	15.4

TABLE IV

The methylation of tRNA in vivo

[Me-^{3}H]L-methionine (4μCi/g, 25 μl) was injected intracranially into 8-day-old rats 45 min before sacrifice. tRNA was isolated, purified and hydrolyzed to the constituent bases and the methylated bases separated by HPLC (see Methods). Between 8000 and 13,000 dpm were subjected to hydrolysis. The values are expressed as dpm $\pm$ S.D. in each [Me^{3}H]base and as percentages of the radioactivity recovered after HPLC and corrected to 100%. To convert to pmol, 100 dpm = 1 pmol. The actual recoveries (as per cent of the dpm injected onto the HPLC column) were in excess of 90%. The results are means of 3 separate determinations. Dash, not detected.

	Cerebellum		*Cerebral Cortex*		*Liver*	
[Me-^{3}H]base	*(dpm)*	*%*	*(dpm)*	*%*	*(dpm)*	*%*
1-methylguanine	1048 $\pm$ 138	13.1	696 $\pm$ 88	6.9	1064 $\pm$ 360	8.6
N^2-methylguanine	1504 $\pm$ 240	18.8	1634 $\pm$ 67	16.2	1776 $\pm$ 105	14.3
5-methylcytosine	1324 $\pm$ 187	16.5	1504 $\pm$ 167	14.8	2289 $\pm$ 309	18.4
1-methyladenine	1557 $\pm$ 79	19.4	2949 $\pm$ 533	29.1	3037 $\pm$ 342	24.4
1-methylhypoxanthine	777 $\pm$ 156	9.7	1150 $\pm$ 39	11.4	1300 $\pm$ 310	10.4
7-methylguanine	—	—	331 $\pm$ 64	3.3	517 $\pm$ 88	4.2
$N^2{}_2$-dimethylguanine	1036 $\pm$ 210	12.9	1253 $\pm$ 138	12.4	958 $\pm$ 104	7.7
5-methyluracil	360 $\pm$ 59	4.5	344 $\pm$ 39	3.4	882 $\pm$ 288	7.1
N^6-methyladenine	414 $\pm$ 30	5.1	253 $\pm$ 55	2.5	612 $\pm$ 100	4.9
Total dpm	8020 $\pm$ 1040	100.0	10,114 $\pm$ 1186	100.0	12,435 $\pm$ 2006	100.0

half as much 1-methylhypoxanthine and twice as much 5-methyluracil in the $tRNA^{glu}_2$ following its methylation by the Purkinje and granule cell tRNA methyltransferases, respectively.

The methylation of tRNA in vivo

Following the intracranial administration of [Me-^{3}H]L-methionine (4-μCi/g) to 8-day-old rats, tRNA fractions were purified from the cerebellum, the cerebral cortex

and the liver and their [Me-^{3}H]base distribution was determined. The results (Table IV) show that N^2-methylguanine and 1-methyladenine accounted for about 40% of the radioactivity in each organ.

DISCUSSION

The present study extends our knowledge concerning the development of neural tRNA methylation systems to the cerebellum of the growing rat. Although previous studies found tRNA methylation more active in immature than in adult tissues, in most of these only heterologous tRNA methylation was monitored, i.e. the capacity of neural tRNA methyltransferases to methylate non-neural tRNA. It is known, however, that in the cerebral cortex of the rat, homologous tRNA methylation also diminishes with age[16].

Changes with age in the distribution of the principal cerebellar [Me-^{14}C] tRNA methyl bases are shown in Table I. The Table also shows that the 4 [Me-^{14}C] bases listed are the principal constituents of cerebellar tRNA, for the percentage represented by their sum remained constant (73–77%) from 3 to 43 days. This observation implies that cerebellar tRNA methylation is principally mediated by tRNA-(1-methylguanine)-, (N^2-methylguanine)-, (5-methylcytosine)- and (1-methyladenine)-methyltransferases with the resulting formation of tRNAs which contain 1- and N^2-methylguanine, 5-methylcytosine and 1-methyladenine as their principal methylated bases. The results in Table I also suggest that cerebellar tRNAs contain from 2 to 3 times less 5-methylcytosine and 1-methyladenine at 3 than at 43 days. This observation infers that processing and/or methylation of tRNA is an active process in the developing cerebellum.

Support for the above inference is provided by the results shown in Table II in which the activites and substrate specificities of 3 of the 4 tRNA methyltransferases, predicted by the findings in Table I as among the most active in the developing cerebellum, are compared. All 3 enzymes were found to exhibit appreciable activity changes between days 6 and 21; these changes were at times in opposite directions with different substrates as, for instance, in the case of tRNA-(N^2-methylguanine)-methyltransferase whose activity toward $tRNA^{phe}$ decreased by about 40% while that toward $tRNA^{fmet}$ increased by about 100%. tRNA-(5-methylcytosine)-methyltransferase behaved similarly toward $tRNA^{glu}_2$ and $tRNA^{val}$, respectively. In agreement with our recent results obtained using cerebellar tRNA methyltransferases of 6- and 21-day-old Gunn rats[3], the activity of tRNA-(1-methyladenine)-tRNA-methyltransferase fluctuated very little under all conditions tested.

The small, but consistent differences in the methyl base distribution of $tRNA^{glu}_2$ methylated by the tRNA methyltransferases of Purkinje and granule cells (Table III) are of interest because they underscore the possibility that intercellular differences in tRNA methylation capacity and tRNA content might involve the minor bases in addition to the 4 principal methyl bases listed in Table II.

Finally, the results shown in Table IV emphasize the appreciable qualitative and quantitative differences in the in vivo methylation patterns of cortical, cerebellar and

hepatic tRNAs in the 8-day-old animal. They point out, for example, that cerebellar tRNAs do not contain detectable amounts of 7-methylguanine and, furthermore, that cerebellar tRNAs are highest in 1- and N^2-methylguanine and lowest in 1-methyladenine. Work is in progress to characterize the methyl base composition of individual brain tRNAs and to identify developmental changes in their methylation. It is hopeful that this information will help understanding of some of the mechanisms responsible for the manufacture of brain specific proteins and of the rigid timetable governing this process.

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