Effects of Altered Thyroid Function on Galactosyl Diacylglycerol Metabolism in Myelinating Rat Brain*

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The effects of neonatal thyroid status on the metabolism of the myelin metabolite, galactosyl diacylglycerol, were examined in developing rat brain. There was a 40\% reduction in the concentration of monogalactosyl diacylglycerol in the brains of 20- and 23-day-old rats which were made hypothyroid at birth. These same animals had a retarded developmental pattern for the galactosyltransferase activities that synthesize monogalactosyl- and digalactosyl diacylglycerol. Replacement therapy with triiodothyronine restored both enzyme activities to control levels. In rats made neonatally hyperthyroid by daily administration of 2.0 \( \mu \)g of triiodothyronine, there was a premature rise in monogalactosyl diacylglycerol concentration. This increase in monogalactosyl diacylglycerol concentration was reflected in a premature appearance in the triiodothyronine-treated animals of the galactosyltransferase activities which synthesize galactosyl diacylglycerols. A number of other enzymes involved in galactosyl diacylglycerol metabolism were studied. These included: mitochondrial and cytosolic glycerol-3-phosphate dehydrogenase; glycerol-3-phosphate: fatty acyl-CoA acyltransferase; phosphatidic acid phosphohydrolase; \( \beta \)-galactosidase; and galactolipase. The developmental patterns of these enzymes were not significantly altered by neonatal hyperthyroidism. It was concluded from these studies that neonatal thyroid status can influence the deposition of myelin marker metabolite galactosyl diacylglycerols in developing rat brain, and that this influence is exerted at the enzymatic step committed to synthesizing a relatively specific myelin constituent.

The deposition of myelin in mammalian brain takes place during the so-called "critical period" (1). The onset of active myelination is abrupt, and the period of active myelination is relatively short and well defined. As yet, the biological factors responsible for this rather specific timing of myelination are unknown.

Neonatal thyroid status is known to affect the course of myelination (2-10). There is a decrease in the amount of myelin deposited in the brains of rats made thyroid-deficient at birth (2-8). In contrast, neonatal administration of thyroid hormone has been reported to advance the deposition of myelin in intact rats (9) and in cultured brain tissue obtained from newborn rats (10).

It has previously been shown that galactosyl diacylglycerol metabolism in developing rat brain is closely associated with the process of myelination (11-18). The availability of assay techniques for the galactosyltransferase responsible for the final step in the biosynthesis of monogalactosyl diacylglycerol and digalactosyl diacylglycerol (11, 12), as well as a sensitive gas chromatographic technique for quantitation of monogalactosyl diacylglycerol (15), has made these compounds useful as "markers" for myelination.

This present report examines the effects of neonatal thyroid status on various aspects of galactosyl diacylglycerol metabolism in developing rat brain in the hope of gaining insight into possible mechanisms regulating myelination. Part of this work has been presented in preliminary form.¹

**EXPERIMENTAL PROCEDURES

Materials - Chemicals were obtained from the following sources: 3,3',5-triiodo-L-thyronine, Cyclo Chemical Co.; UDP-[U-\(^{14}\)C]galactose and [U-\(^{33}\)H]dgalactose, New England Nuclear; Na\(^{131}\)I (carrier-free), Squibb Laboratories; \( \beta \)-galactosidase, phenazine methosulfate, 2-p-iodophenyl-3-p-nitrophenyl-5-phenylmonotetrazolium chloride, and 1,2-dipalmitoyl-sn-glycerol, Sigma Chemical Co.; NAD and NADH (dissodium salts) and UDP-galactose (dipotassium salt), Calbioche, Inc.; palmitoyl-CoA and ATP, P-L Biochemicals, Inc.; trimethylchlorosilane and hexamethyldisilazane, Pierce Chemical Co.

Hyper- and Hypothyroid Animals - Pregnant female Sprague-Dawley rats were obtained from Charles River Breeding Laboratory and fed Purina Rat Chow ad libitum. Radiothyroidectomy was carried out by the method of Goldberg and Chaikoff (19). Newborn rats were injected within 24 h after birth with 125 to 150 \( \mu \)Ci of Na\(^{131}\)I in 0.9% NaCl solution. Half of the \( ^{131}\)I-treated animals in each litter received daily injections of 2.5 \( \mu \)g of \( T_3 \) in 0.1 ml of 0.9% saline. Injections continued up to the day of death. Non-\( ^{131}\)I-treated animals served as littermate controls, and received daily injections of saline.


The abbreviation used is: \( T_3 \), 3,3',5-triiodothyronine.
only. The body and brain (wt) weights of control, 131I-treated, and T3
plus 131I-treated rats were determined at various ages and are listed in
the above order (brain weights are given in parentheses): 3 days of age;
10.4 g (0.45 g), 9.6 g (0.45 g), and 9.8 g (0.46 g); 5 days of age, 19.1
(0.82 g), 17.9 g (0.80 g), and 18.4 g (0.80 g); 10 days of age, 13.9 g
(0.96 g), 18.2 g (0.83 g), and 12.1 g (0.73 g); 14 days of age, 24.5 g
(1.03 g), 25.1 g (1.10 g), and 22.6 g (0.93 g); 20 days of age, 43.7 g
(1.23 g), 34.6 g (1.17 g), and 30.0 g (1.07 g); and 28 days of age, 51.4 g
(1.43 g), 77.8 g (1.30 g), and 86.6 g (1.10 g). In experiments where only
hyperthyroid effects were studied, litters of newborn rats were ad-
justed to 10 pups each. Five of these received daily injections of 2.0
\(\mu\)g of T3 in saline, while the other five served as controls and re-
ceived the saline only. Injections continued up to the day of death.

The body and brain (wt) weights of control and T3-treated rats were
recorded at different ages, and are listed in the above order (brain
weights are given in parentheses): 6 days of age, 14.5 g (0.52 g) and
13.0 g (0.53 g); 7 days of age, 15.4 g (0.55 g) and 13.1 g (0.52 g); 8 days of age, 17.9 g
(0.69 g) and 15.1 g (0.64 g); 9 days of age, 18.6 g (0.68 g) and 16.0 g
(0.64 g); 10 days of age, 21.2 g (0.82 g) and 18.5 g (0.78 g); 11 days of age,
23.7 g (0.92 g) and 20.0 g (0.81 g); 13 days of age, 30.5 g (1.16 g)
and 25.1 (1.10 g); 15 days of age, 37.1 g (1.21 g) and 30.4 (1.09 g); and 18
days of age, 42.2 g (1.32 g) and 34.9 g (1.17 g). The success of the treat-
ment used to induce hypo- and hyperthyroidism was assessed by
previously published anatomical criteria (20).

Quantitation of Enzymes — The rats were killed by decapitation.
The whole brain was quickly removed, weighed, and homogenized in
cold 0.2 M sucrose in a Potter-Elvehjem homogenizer. The homoge-
nate was fractionated according to the procedure of DeRobertis et al.
(21). Crude nuclear, mitochondrial, microsomal, and soluble frac-
tions were collected. The particulate fractions were lyophilized and
then stored at \(-20^\circ\)C with desiccant. The soluble fraction was frozen and
stored at \(-20^\circ\)C. Protein in each fraction was quantitated by the
method of Lowry et al. (22) using crystallized bovine serum albumin
(Sigma Chemical Co.) as a standard.

Mitoehondrial glycerol-3-phosphate dehydro-
genase was assayed according to the method of Lee and Lardy (23).
Soluble glycerol-3-phosphate dehydrogenase activity was measured
by the method of Laatsch (24) as modified by DeVellis et al. (13) as a
substrate [U-14C-galac-
tose].

Glycerol-3-phosphate:acyl-CoA acyltransferase activity was
measured by the method of Wanger et al. (11, 12).

Galecitolipase and \(\beta\)-galactosidase activities were determined by
the method of Subba Rao et al. (13) using as a substrate [\(\beta\)H-galac-
tose]monogalactosyl diacylglycerol which had been prepared with a
specific galecitolipase preparation (27).

Quantitation of Monogalactosyl Diacylglycerol — Whole brain lipid
was extracted by the method of Folch et al. (28). After deacylation
with mild alkali treatment, the water-soluble material was deriva-
tized with a triethylsilylating reagent and examined by gas-liquid
chromatography as described previously (15). Gas-liquid chromatog-
raphy was performed on a Glowsall model 310 gas chromatograph
equipped with a hydrogen flame detector and a coated glass column (6
\text{ft} \times \frac{1}{8} \text{in}) packed with 3\% SE-30 on 80 to 100 mesh Chromosorb W
(Supelco, Inc.).

Results

Effects of Neonatal Hypothyroidism on Monogalactosyl Diacyl-
glycerol Concentration in Developing Brain — The brains of 20- and
23-day-old hypothyroid rats and their litter-
mate controls were examined for monogalactosyl diacyl-
glycerol content. These ages were chosen because monogalac-
tosyl diacylglycerol concentration in rat brain is at its maxi-
mum at around 20 days of age (15). As Table I shows, neonatal
exposure to \(\alpha\) 40% decrease in monogalac-
tosyl diacylglycerol concentration. In contrast, \(\beta\) 40% of the
animals that had been supplemented with T3 had brain mono-
galactosyl diacylglycerol levels comparable to the controls.


<table>
<thead>
<tr>
<th>Age</th>
<th>No. of rats</th>
<th>Treatment</th>
<th>Monogalactosyl diacylglycerol</th>
<th>Decrease from control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>nmol/brain</td>
<td>%</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>1018 ± 165</td>
<td>986 ± 176</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td>131I</td>
<td>565 ± 178</td>
<td>565 ± 226</td>
<td>37</td>
</tr>
<tr>
<td>3</td>
<td>131I + T3</td>
<td>865 ± 96</td>
<td>1014 ± 163</td>
<td>37</td>
</tr>
</tbody>
</table>

Effects of Neonatal Hypothyroidism on Monogalactosyl Diacyl-
glycerol Biosynthesis — Galactosyltransferase activities for
both monogalactosyl diacylglycerol (11) and digalactosyl di-
acylglycerol (12) biosynthesis from 1,2-diacylglycerol and
UDP-galactose follow similar courses of development. Both
activities are low before 10 days of age, then rise sharply to a
maximum at about 18 to 19 days of age, and then decline to
adult values. Neonatal treatment with \(\alpha\) alters this pattern
of development (Figs. 1 and 2). At 18 days of age, both enzyme
activities are 30 to 40% lower in \(\beta\)-treated animals. Supple-
menting the \(\alpha\) -treated animals with T3 restored the develop-
mental pattern to near normal. However, one noteworthy
difference was the apparent premature rise in galactosyltrans-
ferase activities in the T3-supplemented animals.

Effects of Neonatal Hyperthyroidism on Monogalactosyl Diacyl-
glycerol Concentration in Developing Brain — The change in
concentration of monogalactosyl diacylglycerol in the
brains of hyperthyroid and control rats is shown in Fig. 3.

The developmental pattern for the controls is similar to that
reported previously (15). Monogalactosyl diacylglycerol concen-
tration is low before 10 days of age and then rises sharply
between 10 and 20 days of age. After 20 days of age, the
concentration of monogalactosyl diacylglycerol declines. At 7
days of age, monogalactosyl diacylglycerol levels in the T3-
treated animals are significantly higher than in the control
animals. By 10 days of age, there is over a 2-fold difference
in monogalactosyl diacylglycerol concentration between the
two groups. After 10 days of age, the difference between the
controls and the hyperthyroid animals becomes smaller, and after
15 days of age there is no longer any statistically significant
difference.

Effects of Neonatal Hyperthyroidism on Enzymes Involved in
Monogalactosyl Diacylglycerol Metabolism — Because it
was felt that the observed premature accumulation of monoga-
lactosyl diacylglycerol in the T3-treated animals should be
reflected in changes in enzyme activities involved in its syn-
thesis or degradation (or both), the effects of hyperthyroidism
on a number of enzyme activities in brain were studied.

The expected metabolic pathways for monogalactosyl diacyl-

\(\alpha\) indicates animals that were radiothyroidectomized at birth with a single intraperitoneal injec-
tion of about 150 \(\mu\)Ci of Na\(131I\). \(\beta\) indicates animals that were radiothyroidectomized at birth, but received replacement therapy
consisting of daily injections of 2.5 \(\mu\)g of T3 dissolved in 0.9% saline.

\(p < 0.05\)
Thyroxine and Myelin Galactosyl Diacylglycerol Metabolism

Fig. 1. The effects of neonatal thyroidectomy on monogalactosyl diacylglycerol (MGD)-synthesizing galactosyltransferase activity in developing rat brain. Hypothyroid animals (Ⅰ—I) were radiothyroidectomized shortly after birth with a single intraperitoneal injection of 100 to 200 μCi of carrier-free NaI125I in 0.9% saline. Half of the 125I-treated rats in each litter received daily supplementation with intraperitoneal injections of 2.5 μg of triiodothyronine in 0.9% saline, pH 8.2, beginning on the 2nd day of life (Ⅰ—Ⅰ). Non-125I-treated littermate controls (Ⅰ—not) received daily intraperitoneal injections of 0.9% saline, pH 8.2, beginning on the 2nd day of life. Galactosyltransferase activity was measured in crude microsomal preparations as described under "Experimental Procedures." Three brains were pooled for each determination.

Fig. 2. The effects of neonatal thyroidectomy on digalactosyl diacylglycerol (DGD)-synthesizing galactosyltransferase activity in developing rat brain. Thyroidectomized (Ⅰ—Ⅰ), thyroidectomized with T3 supplementation (Ⅰ—Ⅰ), and control animals (Ⅰ—not) are as described in the legend in Fig. 1. Galactosyltransferase activity was measured in crude microsomal preparations as described under "Experimental Procedures." Three to six brains were pooled for each determination.

Fig. 3. The effect of T3 treatment on monogalactosyl diacylglycerol (MGD) concentration in developing rat brain. T3-treated animals (Ⅰ—Ⅰ) received daily intraperitoneal injections of 2.0 μg of triiodothyronine in 0.9% saline, pH 8.2, beginning on day 2 of life and continuing up to the day of death. Control animals (Ⅰ—not) received a similar injection regimen of the 0.9% saline, pH 8.2, only. Whole lipid was extracted from brain and subjected to mild alkaline methanolysis as described under "Experimental Procedures." The water-soluble material after methanolysis was derivatized and quantitated on a gas chromatograph. Results are reported as nanomoles of monogalactosyl diacylglycerol (as monogalactosyl glycerol)/g. wet weight, of brain ± standard deviation. Standard deviation was less than the diameter of the dot at those points where it is not directly indicated. At least three litters of five or more rats were used for each point. Statistical significance was determined by the Student's t test. Brain and body weights of control and T3-treated rats of varying ages are given under "Experimental Procedures."

glycerol are shown in Fig. 4, and the particular enzymes examined are indicated.

Glycerol-3-Phosphate Dehydrogenase—The effects of neonatal hyperthyroidism on both soluble and mitochondrial glycerol-3-phosphate dehydrogenase activities are shown, respectively, in Figs. 5 and 6. The developmental pattern found for the soluble enzyme agrees well with that published previously (24). Activity remains relatively constant up to 15 days of age and then increases sharply. The mitochondrial enzyme activity shows a somewhat different course of development, increasing up to 20 days of age and then levelling off. For both enzymes, there is no significant difference in activity between the controls and the T3-treated animals at any age studied. The absence of an effect of neonatal hyperthyroidism on rat brain mitochondrial glycerol-3-phosphate dehydrogenase activity is in agreement with the findings of other authors (30).

Glycerol-3-Phosphate:Fatty Acyl-CoA Acyltransferase—Fig. 7 shows the brain acyltransferase activity which synthesizes phosphatidic acid as a function of age for both hyperthyroid and control rats. There is sharp increase in enzyme activity at 6 to 10 days of age and a more gradual increase in activity thereafter. This pattern of development is consistent with the role this enzyme would play in meeting the increased demand for glycerolipid synthesis during the period of active myelination. There are no significant differences in enzyme activity between the control and the experimental animals at any of the ages studied, indicating that neonatal hyperthy-
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Glucose

\[ \text{Glc-6-P} \rightarrow \text{Glc-1-P} \rightarrow \text{Glc-6-P} \]

\[ \text{Glc-1-P} \rightarrow \text{Glc-6-P} \rightarrow \text{Glc-1-P} \]

\[ \text{Dihydroxyacetone-P} \rightarrow \text{Glycerol-3-P} \rightarrow \text{Fatty Acyl-CoA} \]

FIG. 4. The metabolic pathways for galactosyl diacylglycerols in brain. The enzymatic reactions studied were the following: (1) glycerol-3-phosphate dehydrogenase; (2) fatty acyl CoA:glycerol-3-phosphate fatty acyltransferase; (3) phosphatidic acid phosphohydrolase; (4) UDP-galactose:1,2-diacylglycerol galactosyltransferase; (5) UDP-galactose:monogalactosyl diacylglycerol galactosyltransferase; (6) galactolipase; and (7) \( \beta \)-galactosidase. The enzymatic conversion of monogalactosyl diacylglycerol (MGG) to a sulfated derivative (MGG-SO₄) by rat brain with 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as sulfate donor has recently been reported (29). DGD, digalactosyl diacylglycerol; PA, fatty acid; PA, phosphatidic acid; MGG, monogalactosyl glycerol.

Phosphatidic Acid Phosphohydrolase: The next enzyme involved in the de novo synthesis of monogalactosyl diacylglycerol is phosphatidic acid phosphohydrolase. The effects of neonatal hyperthyroidism on the development of both the mitochondrial and microsomal phosphohydrolase activities are shown in Fig. 8, A and B. Both activities show a moderate increase between 6 and 18 days of age. Such an increase in activity for this enzyme in brain has been reported previously (31). Enzyme activity in the T₃-treated animals follows a course of development similar to that of the controls, and there were no significant differences between the two groups noted at any point studied.

UDP-Galactose:Diacylglycerol Galactosyltransferases—The final step involved in the biosynthesis of galactosyl diacylglycerols in brain is the transfer of galactose from UDP-galactose to diacylglycerol. This step is the first one depicted in Fig. 4 that is committed solely to the synthesis of a glycerolipid that is relatively myelin-specific (11-18). Because thyroid hormone is known to exert a direct effect on myelination (2-10), this reaction would be expected to be a key step in the metabolic control of myelination by thyroid hormone. As seen in Fig. 9, this appears to be the case for the monogalactosyl diacylglycerol-synthesizing galactosyltransferase. The control activities are similar to those published previously (11), and they follow a developmental pattern typical of that seen for...
enzymes associated with the process of myelination. Enzyme activity is low up to about 12 days of age, but then rises sharply to a peak at about 18 to 20 days of age. There is a 5- to 10-fold increase in activity during this period. In the T₃-treated animals, enzyme activity rises sharply at 7 days of age and by 10 days of age is twice that of the controls. The differences between the T₃-treated and control animals are statistically significant (p < 0.05) between 8 and 11 days of age.

Addition of deoxycholate to the assay mixture for monogalactosyl diacylglycerol-synthesizing galactosyltransferase activity causes a 10-fold stimulation of incorporation of radioactivity into product, but at the same time alters the reaction so that a digalactosyl diacylglycerol is the major product formed (12). Although digalactosyl diacylglycerol has only been tentatively identified in brain (32), digalactosyl diacylglycerol-synthesizing galactosyltransferase activity has been found to be a useful tool for studying myelination because it closely parallels the monogalactosyl diacylglycerol-synthesizing activity (12, 15-18) and the assay is about 10 times more sensitive. The effects of hyperthyroidism on digalactosyl diacylglycerol-synthesizing galactosyltransferase activity can be seen in Fig. 10 to be similar to those observed for monogalactosyl diacylglycerol synthesis. There is a premature rise in enzyme activity in the T₃-treated animals which results in a 2-fold increase over control levels by 10 days of age. By 15 days of age the activity in the two groups is once again similar.
The observed decrease in concentration of monogalactosyl diacylglycerol was reflected in the activities of the galactosyltransferases which synthesize both monogalactosyl diacylglycerol (Fig. 1) and digalactosyl diacylglycerol (Fig. 2). In the thyroidectomized animals, both enzymes showed a course of development with the peak of activity appearing at 18 days post partum, which is the same age of peak activity as was observed with the control animals. However, peak activity was reduced by thyroidectomy. Monogalactosyl diacylglycerol- and digalactosyl diacylglycerol-synthesizing galactosyltransferase activities were reduced, respectively, by about 30 and 40% in the ¹³¹I-treated animals relative to the controls. Although peak activity was reduced in the hypothyroid animals, both enzymes appeared to decline from peak activity at a slower rate than the enzymes in the control animals. This latter finding conforms with previous reports (2, 33–36) which stated that neonatal thyroidectomy extends the normal period of brain development which includes myelination.

Because of the apparent premature induction of galactosyltransferase activities in the thyroidectomized animals that received replacement therapy with a dose of T₃ which was in excess of that required to re-establish a euthyroid state (37), the effects of neonatal hyperthyroidism were examined. As Fig. 3 shows, neonatal treatment with thyroid hormone advances the deposition of monogalactosyl diacylglycerol in developing rat brain. This premature appearance of monogalactosyl diacylglycerol should be reflected in changes in activity of one or more of the enzymes involved in its biosynthesis or degradation. The enzymatic steps expected to be most intimately associated with galactosyl diacylglycerol metabolism in rat brain are depicted in the scheme of Fig. 4. The only enzymes in this scheme which showed any significant change in activity with T₃ treatment were the galactosyltransferases which are involved in the final step leading to monogalactosyl and digalactosyl diacylglycerol synthesis. There was no apparent effect on any of the enzymes leading to diacylglycerol synthesis. This finding is not unexpected since diacylglycerols are also the immediate precursors for phospholipid synthesis. A previous study (38) had indicated that there is neither a premature accumulation of phospholipids nor a significant induction of phospholipid biosynthesis in the brains of rats treated neonatally with thyroidine. Similarly, it has also been reported (39) that nucleotide sugar biosynthesis in rat brain is not affected by neonatal hypothyroidism. Thus, the observed effects of thyroid hormone on monogalactosyl and digalactosyl diacylglycerol synthesizing galactosyltransferase activities probably do not arise from changes in the availability of the substrates (i.e. diacylglycerol and UDP-galactose). Another factor that could contribute to the premature accumulation of monogalactosyl diacylglycerol in the brains of the T₃-treated rats as seen in Fig. 3 would be a decrease in degradative enzyme activities. However, the results presented in Figs. 11 and 12 seem to indicate that hyperthyroidism does not alter the activities of galactolipase or β-galactosidase.

The galactosyltransferases are the final step in the biosynthesis of galactosyl diacylglycerols, and they are the only enzymes depicted in Fig. 4 that are totally committed to the synthesis of myelin-associated lipids. This finding indicates that, in developing rat brain, thyroxine may have a specific effect with regard to myelination. The specific inductive effect of thyroxine on certain enzymes is probably better referred to as a "super induction," because neonatal administration of thyroxine has been reported to induce an overall increase in protein synthesis in developing rat brain (40). Other myelin-
related enzyme activities have been reported to undergo a premature induction in neonates made hyperthyroid (41-43). Although the data presented in this paper implicate thyroid hormones in myelogenesis at the molecular level, their precise role in this process is still unknown. One explanation that has been put forth is that thyroxine directs glial precursor cells from the proliferative phase into the differentiative stage thereby generating mature oligodendroglia which are capable of synthesizing myelin components (44). Conversely, oligodendroglia could mature at an early age, and then be induced to synthesize myelin components by the burst of circulating thyroid hormone at 10 to 12 days of age (45). Because galactosyl diacylglycerol metabolism in rat brain is associated with the thyroid hormone at 10 to 12 days of age (45). Because galactosyl diacylglycerol metabolism in rat brain is associated with the oligodendroglia (18), future studies will be directed toward using these molecular markers of myelin to pinpoint the effects of thyroxine at the cellular level.

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