Maternal hypothyroxinemia disrupts neurotransmitter metabolic enzymes in developing brain

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Abstract

Maternal thyroid status influences early brain development and, consequently, cognitive and motor function in humans and rats. The biochemical targets of maternal thyroid hormone (TH) action in fetal brain remain poorly defined. A partially thyroidectomized rat dam model was therefore used to investigate the influence of maternal hypothyroxinemia on the specific activities of cholinergic and monoaminergic neurotransmitter metabolic enzymes in the developing brain.

Maternal hypothyroxinemia was associated with reduced monoamine oxidase (MAO) activity in fetal whole brain at 16 and 19 days gestation (dg). A similar trend was observed for choline acetyltransferase (ChAT) activity. In contrast, DOPA decarboxylase (DDC) activity was markedly elevated at 21 dg. Further study of these enzymes at 14 dg showed no differences between normal and experimental progeny – suggesting they become TH sensitive after this age. Tyrosine hydroxylase (TyrH) and acetylcholinesterase (AChE) activities were unaffected prenatally. During postnatal development, the activities of TyrH, MAO, DDC and, to a lesser extent, AChE were increased in a brain region- and age-specific manner in experimental progeny.

The prenatal disturbances noted in this study may have wide-ranging consequences since they occur when neurotransmitters have putative neurotropic roles in brain development. Furthermore, the chronic disturbances in enzyme activity observed during postnatal life may affect neurotransmission, thereby contributing to the behavioural dysfunction seen in adult progeny of hypothyroxinemic dams.

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Introduction

Maternal thyroid hormone (TH), notably thyroxine (T4), is transferred to the fetus prior to the onset of fetal thyroid function in both humans and rats. The transferred TH accumulates within the fetal brain – coincident with the expression of TH metabolic enzyme activities and 3,5,3′-triiodothyronine (T3) nuclear receptors (Porterfield & Hendrich 1993, Pickard *et al.* 1997). It is possible therefore, that the critical period of TH-dependency of brain development begins prior to fetal thyroid function, when an adequate maternal TH contribution is crucial. Indeed, field studies in iodine-deficient endemias have shown that maternal serum T4 levels in hypothyroxinemic pregnancies correlate with subsequent motor and cognitive function in the children (Pharoah & Connolly 1989). Furthermore, impaired intellectual and motor function are also apparent in children born to hypothyroxinemic women in iodine-sufficient environments (Man *et al.* 1991).

Previous studies utilizing thyroidectomized (Tx) rat dam models have shown that adult progeny exhibit impaired motor performance, cognition and learning ability (Hendrich *et al.* 1984, Attree *et al.* 1992, Sinha *et al.* 1994), suggesting underlying neurotransmitter dysfunction. Furthermore, brain region-specific alterations in acetylcholinesterase (AChE), choline acetyl transferase (ChAT) and several myelin metabolic enzyme activities have been described in adult experimental progeny (Pickard *et al.* 1997). It is thought that the compromise in adult brain function stems from the insult incurred during fetal life. Indeed, the brains of fetal and postnatal Tx dam progeny exhibit a range of biochemical abnormalities, including changes in cellular protein and DNA concentrations, and ornithine decarboxylase activity (Morreale de Escobar *et al.* 1985, Pickard *et al.* 1993, Porterfield & Hendrich 1993).

During postnatal development in the rat, neurotransmitter systems and synaptogenesis constitute major targets for TH action (Porterfield & Hendrich 1993). Cholinergic and aminergic neurotransmitter systems in rat brain are particularly susceptible to alterations in postnatal thyroid status, disruption occurring in neurotransmitter levels (Rastogi & Singhal 1976), metabolic enzyme activities

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(Geel & Timiras 1967, Ladinsky *et al.* 1972, Rastogi & Singhal 1974, Gripois & Fernandez 1977*a*,*b*, Kalaria & Prince 1985, Virgili *et al.* 1991) and receptor number (Patel *et al.* 1980, Smith *et al.* 1980). Similar findings have been reported in rat neural cell culture models (Honegger & Lenoir 1980, Safaei & Timiras 1985, Garza *et al.* 1988). Several of the affected neurotransmitter systems are expressed in early fetal brain, before the onset of electrical activity (Saito *et al.* 1991, Schambra *et al.* 1994, Thomas *et al.* 1995, Zhou *et al.* 1995) when they are thought to have neurotropic activities (Lauder 1993, Leslie 1993). Whether such TH-responsive neurotransmitter systems are also targets for maternal TH action during early fetal brain development has not been investigated.

In this study we have therefore employed a partially Tx rat dam model to investigate the effects of maternal hypothyroxinemia on the pre- and postnatal ontogeny of several cholinergic and monoaminergic metabolic enzymes in brain. A preliminary report of these data has appeared elsewhere (Evans *et al.* 1995).

Materials and Methods

Materials

General reagents were obtained from Sigma Chemical Co. (Poole, Dorset, UK) and BDH-Merck Ltd (Lutterworth, Herts, UK). R-(−)-deprenyl hydrochloride was obtained from ICN Biomedicals Ltd (Thame, Oxon, UK), and acetylcoenzyme A and 6,7-dimethyl-5,6,7,8-tetrahydropterine hydrochloride from Calbiochem–Novabiochem Ltd (Beeston, Notts, UK). [^{3}H]Acetylcoenzyme A was purchased from Amersham International (Amersham, Bucks, UK), D,L-3,4-[alanine-1-^{14}C] dihydroxyphenylalanine and L-[1-^{14}C]tyrosine from NEN Dupont Ltd (Hounslow, Herts, UK) and Picofluor scintillant from Canberra-Packard Ltd (Pangbourne, Berks, UK). Total T4 and T3 radioimmunoassay kits were obtained from North East Thames Regional Immunoassay Service (London, UK) and rat thyroid stimulating hormone (TSH) radioimmunoassay kit from Biocode Biotechnology (Liege, Belgium).

Animals

Sprague–Dawley rat dams, partially Tx by surgical removal of the left lobe and isthmus (parathyroid-spared), were mated with normal males when circulating T4 levels were <20 nM. The control group constituted euthyroid (N) dams mated with normal males. All animals were maintained at 22 °C on a cycle of 14 h light: 10 h darkness, with free access to an iodine-replete diet. The drinking water of the Tx dams was supplemented with calcium lactate (0·1% w/v). When pregnancy was allowed to continue to term, litters were standardized to seven pups on the day of birth.

Sample preparation

Pregnant dams and postnatal progeny were stunned and killed by cervical dislocation. A cardiac blood sample was taken from the dams immediately after killing for serum TH determination. Brains were dissected from fetal and postnatal progeny, placed on ice and cleaned of meninges and blood vessels. Postnatal brains were dissected into four gross anatomic regions – cerebellum, brain stem (comprising pons, medulla and midbrain), subcortex and cerebral cortex – whereas prenatal brain was used whole. Tissue was homogenized in 9 vol ice-cold 0·32 M sucrose and aliquots stored at −20 °C.

Enzyme assays

Monoamine oxidase (MAO) activity was assayed by the fluorimetric method of Krajl (1965), using kynuramine as substrate. At several age points, 1 μM clorgyline or 1 μM deprenyl hydrochloride was included to assess the activity of the MAO-A and -B isoforms respectively (Squires 1972). These concentrations of inhibitor were determined at 19 days gestation (dg) in preliminary experiments (data not shown). DOPA decarboxylase (DDC), tyrosine hydroxylase (TyrH) and ChAT activities were assayed radiometrically according to the methods of Okuno & Fujisawa (1982), Waymire *et al.* (1971) and Fonnum (1969, 1975) respectively. AChE activity was measured using the colorimetric procedure of Ellman *et al.* (1961).

Protein determination

Protein was assayed in tissue homogenates using Folin–Ciocalteau reagent (Lowry *et al.* 1951) with bovine serum albumin as standard.

Thyroid function

Total T3, total T4 and TSH were determined in maternal serum by radioimmunoassay using commercial kits.

Statistical analysis

All values are expressed as mean ± S.E.M. Statistical significance was determined either by two-way analysis of variance (ANOVA) with *post-hoc* analysis by Fisher's PLSD test, or Student's *t*-test, as indicated. In all cases, values of $P<0{\cdot}05$ were taken to be significant. Where necessary, data was logarithmically transformed prior to analysis to satisfy the criteria for ANOVA (Snedecor & Cochran 1980).

Results

Initially, the study was restricted to samples from 16 dg to 30 postnatal days (pnd) and these constitute the main

Table 1 Maternal serum TH levels, litter number and fetal body weight in normal (N) and partially Tx rat dam pregnancies. Values are means ± S.E.M., $n \geq 5$. Treatment effects (two-way ANOVA) were observed for T4 ($P<0{\cdot}0005$) T3 ($P<0{\cdot}001$), fetal body weight ($P<0{\cdot}001$), and litter number ($P<0{\cdot}05$)

	Dam status	T4 (nM)	T3 (nM)	Litter number	Body weight (g)
Days gestation					
16	N	40·86 ± 2·21	1·19 ± 0·12	14·73 ± 0·94	0·500 ± 0·011
	Tx	14·46 ± 1·22***	0·76 ± 0·14*	13·00 ± 1·11	0·424 ± 0·022**
19	N	29·50 ± 2·55	1·88 ± 0·34	15·12 ± 1·32	2·440 ± 0·080
	Tx	16·93 ± 2·35***	1·03 ± 0·08*	12·14 ± 0·67	2·180 ± 0·075*
21	N	25·53 ± 2·64	2·12 ± 0·38	14·00 ± 1·28	5·008 ± 0·248
	Tx	9·87 ± 1·29***	1·43 ± 0·31*	12·89 ± 1·02	4·613 ± 0·177

*$P<0{\cdot}05$, **$P<0{\cdot}01$ and ***$P<0{\cdot}005$ compared with N dam progeny (Fisher's PLSD).

section of the results below. Additional samples were later collected at 14 dg to assay those enzyme activities showing disturbed prenatal ontogeny in Tx dam progeny and these are presented separately.

Animal model

The experimental dams in this study were moderately hypothyroid (Table 1); total T4 levels were reduced to 40–60% of controls, while total T3 was less severely deficient. A further series of dams ($n \geq 4$) with comparable total T4 levels (Tx vs N dam: 11·68 ± 2·50 vs 51·41 ± 6·14 nM at 16 dg; 15·74 ± 2·61 vs 29·00 ± 2·61 nM at 19 dg; and 11·51 ± 2·28 vs 22·88 ± 4·68 nM at 21 dg) were also evaluated for maternal serum TSH levels. These were found to be markedly elevated at all stages of pregnancy studied ($P<0{\cdot}0005$; Fisher's PLSD test). Values (Tx vs N dam) were: 18·94 ± 2·84 vs 2·82 ± 0·21 ng/ml at 16 dg; 22·99 ± 5·34 vs 5·59 ± 1·18 ng/ml at 19 dg; and 27·10 ± 3·89 vs 2·09 ± 0·33 ng/ml at 21 dg. Litter sizes were marginally lower throughout gestation in Tx dams relative to controls (Table 1). Fetal body weight was reduced in Tx dam progeny at 16 and 19 dg but normal near term (Table 1). Brain weight and protein concentration (in terms of whole brain and by region) were normal at all stages of development (data not shown).

Monoaminergic metabolic enzyme activities

The ontogenic profiles of TyrH specific activity in fetal brain from N and Tx dam progeny were very similar, both displaying a 1·4-fold increase between 16 and 21 dg (data not shown). During postnatal development however, TyrH specific activity was 32% higher in the cerebral cortex of Tx dam progeny at 10 pnd and although the overall treatment effect was significant, the difference had disappeared by 20 pnd (Fig. 1). A similar increase (by 28%) was observed in the subcortex at 20 pnd, although no overall treatment effect was found for this region (Fig. 1).

In normal fetal brain, the specific activity of DDC declined linearly by 58% between 16 and 21 dg, whereas in experimental progeny this decline was absent (Fig. 2). Consequently, although the specific activity of DDC in Tx dam progeny at 16 dg was slightly lower than in controls, by 21 dg it was 58% higher (Fig. 2). Enhanced levels of DDC specific activity persisted in a region-specific manner during postnatal development in Tx dam progeny, with significant treatment effects apparent in cerebral cortex and brain stem (Fig. 3). *Post-hoc* analysis confirmed the statistical significance of the higher activities

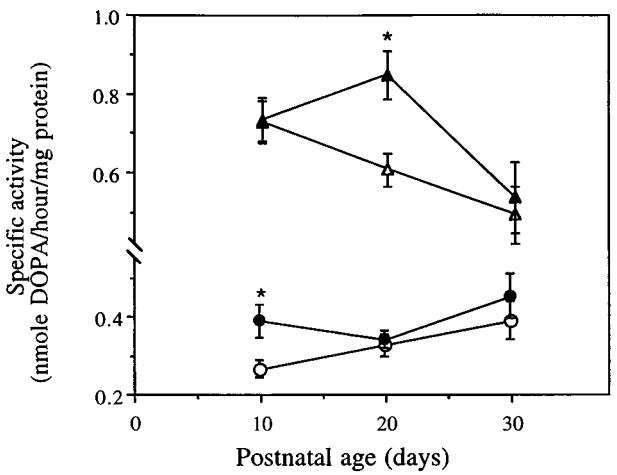

Figure 1 Postnatal ontogeny of TyrH specific activity in cerebral cortex (circles) and subcortex (triangles) of normal (open symbols) and Tx (closed symbols) dam progeny. Values are means ± S.E.M., $n \geq 5$. Two-way ANOVA indicates an overall treatment effect in cerebral cortex ($P<0{\cdot}05$). *$P<0{\cdot}05$ compared with N dam progeny by Fisher's PLSD.

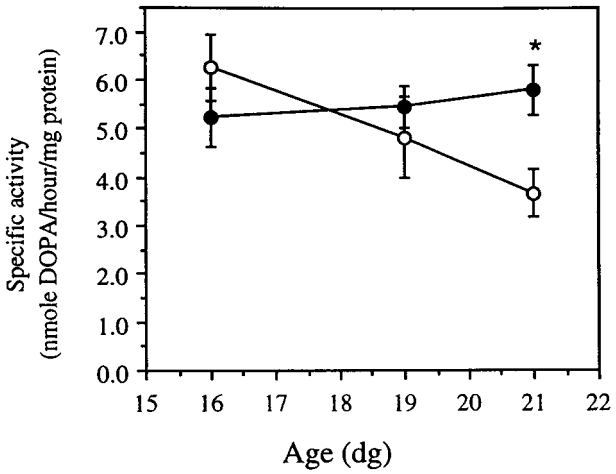

Figure 2 Prenatal ontogeny of DDC specific activity in whole brains of normal (○) and Tx (●) dam progeny. Values are means ± S.E.M., $n \geq 6$. Two-way ANOVA indicates an overall age-treatment interaction ($P<0{\cdot}05$). *$P \leq 0{\cdot}05$ compared with N dam progeny by Fisher's PLSD.

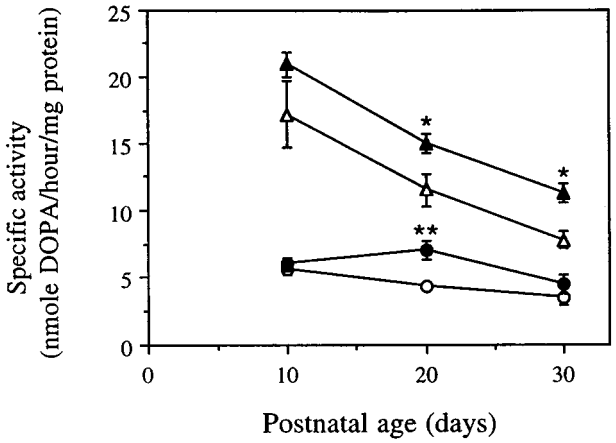

Figure 3 Postnatal ontogeny of DDC specific activity in cerebral cortex (circles) and brain stem (triangles) of normal (open symbols) and Tx (closed symbols) dam progeny. Values are means ± S.E.M., $n \geq 5$. Two-way ANOVA indicates overall treatment effects in cerebral cortex ($P<0{\cdot}01$) and brain stem ($P<0{\cdot}001$). *$P<0{\cdot}05$ and **$P<0{\cdot}01$ compared with N dam progeny by Fisher's PLSD.

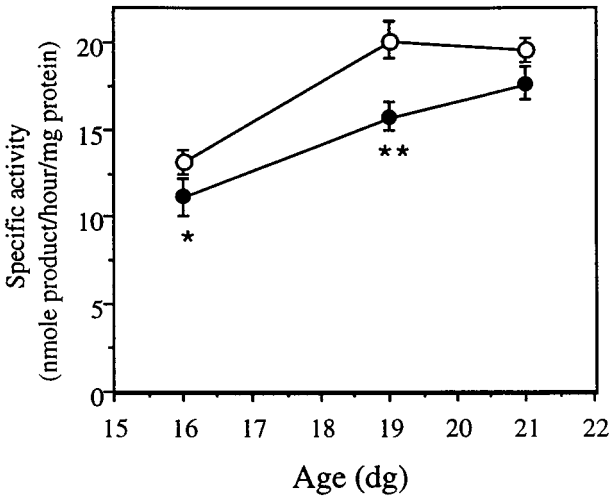

Figure 4 Prenatal ontogenic profile of MAO specific activity in whole brain of normal (○) and Tx (●) dam progeny. Values are means ± S.E.M., $n \geq 5$. Two-way ANOVA indicates an overall treatment effect ($P<0{\cdot}001$). *$P<0{\cdot}01$, **$P<0{\cdot}005$ compared with N dam progeny by Fisher's PLSD.

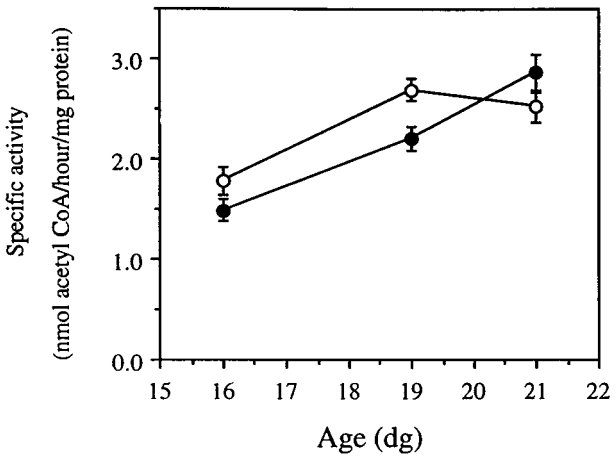

Figure 5 Prenatal ontogeny of ChAT specific activity in whole brain of normal (○) and Tx (●) dam progeny. Values are means ± S.E.M., $n \geq 7$.

in experimental progeny at 20 pnd in the cerebral cortex and at 20 and 30 pnd in the brain stem (Fig. 3).

In normal fetal brain, total MAO specific activity increased 1·5-fold between 16 and 19 dg, then remained stable (Fig. 4). The initial phase of induction was blunted in Tx dam progeny, with total MAO activity being reduced by *ca.* 25% at 16 and 19 dg. Using isoform-specific inhibitors no treatment-related effect on MAO-A or -B specific activity (nmol product/h/mg protein) was apparent at 16 dg (data not shown), whereas MAO-A specific activity was significantly reduced ($P<0{\cdot}05$ by Fisher's PLSD) at 19 dg ($13{\cdot}34 \pm 0{\cdot}64$ vs $15{\cdot}80 \pm 0{\cdot}65$; n=5). Despite the apparent normalization of total MAO activity by 21 dg (Fig. 5), differences in activity were seen in brain stem ($P<0{\cdot}05$ treatment effect for two-way ANOVA) in postnatal Tx dam progeny. Specific activity was *ca.* 20% higher in the experimental progeny at 10 ($76{\cdot}83 \pm 8{\cdot}10$ vs $64{\cdot}77 \pm 3{\cdot}37$; $n \geq 6$) and 20 pnd ($78{\cdot}23 \pm 5{\cdot}11$ vs $65{\cdot}11 \pm 4{\cdot}40$; $n \geq 7$), but only 7% higher at 30 pnd ($84{\cdot}52 \pm 5{\cdot}77$ vs $78{\cdot}69 \pm 6{\cdot}37$; $n \geq 8$).

Cholinergic metabolic enzyme activities

Following a very similar pattern to MAO, ChAT specific activity in normal fetal brain increased 1·5-fold between

Table 2 Whole brain DDC, MAO and ChAT specific activities in 14 dg normal (N) and partially Tx rat dam progeny. Values are means ± S.E.M., $n \geq 5$. No significant differences were observed as determined by Student's *t*-test

	DDC (nmol DOPA/h/mg protein)	MAO (nmol product/h/mg protein)	ChAT (nmol acetylCoA/h/mg protein)
Dam status			
N	0·958 ± 0·223	9·963 ± 0·701	0·702 ± 0·053
Tx	0·694 ± 0·112	9·068 ± 0·354	0·635 ± 0·054

16 and 19 dg and then remained constant through to 21 dg (Fig. 5). In Tx dam progeny the profile was different in that the initial rise in activity continued from 19 to 21 dg ($P<0·05$, Fisher's PLSD), although there was no overall treatment effect. After birth, ChAT activity was normal in all brain regions between 10 and 30 pnd (data not shown).

AChE specific activity increased 4-fold from 16 to 21 dg in normal fetal brain and this profile was unaffected by maternal thyroidectomy (data not shown). Postnatal development was also largely unaffected in Tx dam progeny, the only exception occurring in cerebral cortex at 10 pnd where the specific activity was significantly ($P<0·05$ by Fisher's PLSD) raised (56·0 ± 9·9 vs 40·7 ± 2·4 μmol thiocholine/min/mg protein; $n=5$); this normalized by 20 pnd however and was not associated with any overall treatment effect.

Defining the onset of TH sensitivity

Having demonstrated that the prenatal ontogeny of certain enzymes, namely DDC, MAO and to a lesser extent ChAT, were disrupted as a consequence of maternal hypothyroxinemia, it was decided to investigate whether their ontogeny was disturbed even earlier in development. Subsequent experiments were therefore performed at 14 dg. As with the previous results (Table 1), serum total T4 and T3 levels were reduced ($P<0·05$ by Student's *t*-test) in the Tx dams relative to controls (13·50 ± 1·86 vs 39·69 ± 1·70 nM and 0·71 ± 0·16 vs 0·89 ± 0·06 nM for T4 and T3 respectively; $n \geq 5$). Litter sizes were not significantly reduced (13·50 ± 1·34 vs 16·00 ± 1·10; $n \geq 5$), but followed the same trend as previously (Table 1). Similarly, brain weight and protein concentration did not differ from control values (data not shown).

In contrast to the 16 dg data, body weight was not affected (0·164 ± 0·003 vs 0·157 ± 0·005 g; $n \geq 5$ for N and Tx dam progeny respectively). Furthermore, no significant differences were apparent in any of the enzyme activities measured at this age (Table 2).

Discussion

In this Tx rat dam model, fetal TH deprivation will be greatest prior to – but mitigated by – the onset of fetal TH secretion at 17·5 dg, since intrauterine TH is predominantly maternally derived before this time. At 16 dg, maternal serum total T4 in Tx dams was reduced by 65% relative to controls. Total T3 was less severely affected, therefore these dams were considered 'hypothyroxinemic'. Serum TSH levels were significantly elevated in Tx dams, confirming thyroid dysfunction and the expected pituitary feedback response.

Maternal hypothyroxinemia disrupted the ontogeny of several neurotransmitter metabolic enzymes in fetal brain. Total MAO activity was reduced at 16 and 19 dg, but normal at 21 dg; ChAT activity exhibited a similar trend. These changes are consistent with the effects of TH insufficiency at later stages of brain development, as studied in *in vivo* and cell culture models. For example, total MAO activity is reduced by combined materno-fetal (Gripois & Fernandez 1977*a*) or neonatal hypothyroidism (Gripois & Fernandez 1977*b*). Furthermore, MAO-A activity is TH sensitive in neuroblastoma cell lines (Safaei & Timiras 1985) and was reduced by maternal hypothyroxinemia in this study. ChAT activity is also regulated by postnatal thyroid status *in vivo* (Ladinsky *et al.* 1972, Kalaria & Prince 1985) and induced by T3 in neuronal cultures (Honegger & Lenoir 1980, Garza *et al.* 1988).

These results suggest that TH may regulate MAO and ChAT activities through common mechanisms both before and after the onset of fetal TH synthesis. In postnatal brain, TH is considered to act via nuclear TH receptors (TR). TRs are present at detectable levels in rat fetal brain from 14 dg (Perez-Castillo *et al.* 1985, Falcone *et al.* 1994), rising 3-fold by 16 dg (Perez-Castillo *et al.* 1985). Thus, MAO activity is disrupted after 14 dg, coincident with the rise in TR number. Further work is required therefore to examine TH-mediated transcriptional regulation of neurotransmitter metabolic enzymes.

TyrH and AChE are also regulated by postnatal thyroid status (Geel & Timiras 1967, Rastogi & Singhal 1974) but were unaffected prenatally by maternal hypothyroxinemia. TyrH activity appears less TH-sensitive however than ChAT in an *in vivo* model (Kalaria & Prince 1985) or MAO-A in neuroblastoma cells (Safaei & Timiras 1985), while AChE is less responsive than ChAT to T3 in neuronal cultures (Honegger & Lenoir 1980, Garza *et al.* 1988). Prenatal changes in brain AChE and TyrH

activities may have occurred if more severe maternal hypothyroxinemia had been induced. The interpretation of findings from overtly hypothyroid rat dam models is however confounded by factors such as severe maternal metabolic compromise and placental maldevelopment (Bonet & Herrera 1991). Thus severe maternal hypothyroidism produces permanent deficits in body and brain weight, and brain protein concentration (Hendrich *et al.* 1997). The influence of such confounding factors in the present model appears to be minimal, since in Tx dam pregnancies: fetal body weight normalized near term; fetal brain weight and protein concentration were unaffected; effects on neurotransmitter metabolic enzyme activities were specific rather than general; and both MAO and ChAT normalized by 21 dg (i.e. appear to be corrected by fetal TH synthesis).

Maternal hypothyroxinemia also disrupted the prenatal ontogeny of DDC, but only near term when the intrauterine TH environment is determined largely by the fetus. This effect may be a progressive consequence of the earlier TH deficit. Indeed, postnatal dysthyroidism has little effect on DDC activity (Virgili *et al.* 1991). DDC activity remained elevated postnatally in brain stem and cerebral cortex, indicative of long term or, in the case of brain stem, permanent compromise to monoaminergic neurons. Chronic changes were also apparent for other enzyme activities, in particular TyrH in cerebral cortex and MAO in brainstem. Interestingly, all the enzymes affected postnatally showed increased activity, and disturbances were confined to brain regions in which neurogenesis occurs during early gestation. No changes were seen in cerebellum, possibly because this region develops largely after the onset of fetal TH synthesis. These results confirm and extend previous observations in postnatal Tx dam progeny (Pickard *et al.* 1993), and strongly support a prenatal origin for the postnatal disturbances. Neurotransmitters have putative neurotropic roles in early gestation (Lauder 1993, Leslie 1993) and it is therefore possible that neuronal differentiation is disturbed by maternal hypothyroxinemia thus leading to chronic brain maldevelopment and perhaps the observed postnatal effects. Alternatively, the early TH deficit may impact on other signals which regulate the development of monoaminergic neural pathways, such as polyamines (Slotkin & Bartolome 1986). Indeed, ornithine decarboxylase, which regulates polyamine biosynthesis, is sensitive to maternal hypothyroxinemia in fetal and postnatal rat brain (Pickard *et al.* 1993).

Earlier work in adult progeny of Tx dams showed disturbances in brain ChAT and AChE activities (Sinha *et al.* 1994). These results seem to be at variance with the present study, however they may reflect covert disturbances which become apparent over time. A similar effect was noted regarding TyrH activity in this study. Alternatively, the more severe maternal hypothyroxinemia induced in the study of adult progeny (T4 levels were depressed to only 10% of controls (Sinha *et al.* 1994)), may be responsible.

In summary, this study demonstrates that maternal thyroid status regulates the ontogeny of monoaminergic and, to a lesser extent, cholinergic neurotransmitter metabolic enzyme activities in rat brain. These changes are evident during fetal life (from 16 dg) when the neurotransmitters concerned have putative neurotropic roles, and may therefore have long term repercussions for brain development. Compromise to monoaminergic metabolic enzymes during postnatal life, may impinge upon neurotransmission and contribute to the behavioural dysfunction seen in young adult progeny of Tx dams (Attree *et al.* 1992, Sinha *et al.* 1994). These findings may be pertinent to humans, since maldevelopment of cholinergic and monoaminergic nerve pathways during the first half of gestation, when the intrauterine TH environment is determined by the mother, may contribute to the impaired cognitive and motor development reported in offspring of hypothyroxinemic women (Man *et al.* 1991).

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