

Maternal thyroid status regulates the expression of neuronal and astrocytic cytoskeletal proteins in the fetal brain

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Abstract

Maternal thyroid hormone (TH) crosses the placenta and is postulated to regulate fetal brain development. However, TH-dependent stages of fetal brain development remain to be characterised. We have therefore compared the levels of several neuronal and glial cytoskeletal proteins in fetal brains from normal (N) and partially thyroidectomised (TX) rat dams by immunoblotting. Pregnancies were studied both before and after the onset of fetal TH secretion, which occurs at 17.5 days gestation (dg) in the rat.

Maternal hypothyroidism disrupted fetal growth, so that fetal body and brain weights were reduced near term. Vimentin expression was unaffected, however, indicating normal acquisition of neuronal and glial precursor cells. Fetal brain levels of glial fibrillary acidic protein (GFAP) were reduced at 21 dg, suggesting delayed astrocytic

differentiation, although regression analysis demonstrated appropriate GFAP levels for brain weight. Levels of α -internexin, the earliest neurofilament protein expressed in fetal brain were reduced at 16 dg in TX dams, but increased at 21 dg. The ontogeny of neurofilament-L was also perturbed in these pregnancies, with deficient levels apparent at both 16 and 21 dg. These effects on neuronal cytoskeletal proteins were unrelated to fetal brain growth retardation.

These findings confirm that maternal hypothyroidism disrupts early fetal brain development. Early disturbances in neuronal differentiation are not corrected by the onset of fetal TH secretion. Such disturbances may contribute to the neurological damage observed in children born to hypothyroxinaemic mothers.

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Introduction

Maternal thyroid hormone (TH) is transferred to the fetus throughout pregnancy and is postulated to regulate early fetal brain development (Porterfield & Hendrich 1991, Morreale de Escobar *et al.* 1997, Pickard *et al.* 1997, Sinha *et al.* 1997). In humans, maternal hypothyroidism during pregnancy is associated with impaired cognitive and motor function in children (Man *et al.* 1991, Haddow *et al.* 1999). Even in healthy pregnancies, infant neurodevelopment correlates directly with maternal plasma free thyroxine (T_4) levels during the first trimester (Pop *et al.* 1999). Field studies in the iodine-deficient endemias have also emphasised the importance of maternal thyroid status before the onset of fetal TH synthesis with respect to neurological and intellectual development of the offspring (Connolly & Pharoah 1989).

The influence of thyroid hormone on brain development has been extensively investigated in cell culture and *in vivo* models of congenital hypothyroidism (Bernal & Nunez 1995). Thyroid hormone regulates most – if not all – stages of neuronal and glial cell development. Consequently, congenital hypothyroidism is associated

with disturbed expression of neuronal and glial cell-specific structural proteins, including glial fibrillary acidic protein (GFAP) and the neurofilament triplet proteins (Rami & Rabie 1988, Ghosh *et al.* 1999).

In contrast, the influence of maternal TH on fetal brain development is poorly characterised. Adult progeny of thyroidectomised rat dams exhibit locomotor and learning dysfunction (Hendrich *et al.* 1984, Attree *et al.* 1992) and fetal brain growth is impaired in such models (Morreale de Escobar *et al.* 1985, Porterfield & Hendrich 1991, Pickard *et al.* 1993). Although brain weight normalises after the onset of fetal TH synthesis in moderately hypothyroid dams (Pickard *et al.* 2000), neuronal maturation is impaired (Ruiz de Elvira *et al.* 1988, Pickard *et al.* 1993, Evans *et al.* 1999), and long-term changes occur to oligodendroglial and myelin metabolic markers (Hadjzadeh *et al.* 1990). In addition, iodine deficiency during pregnancy decreases GFAP expression in radial glia of the fetal hippocampus near term (Martinez-Galan *et al.* 1997).

In order better to understand the influence of maternal thyroid dysfunction on early fetal brain development, a partially thyroidectomised rat dam model was utilised to

Table 1 Maternal serum thyroid hormone levels, litter size and fetal growth in euthyroid (N) and partially thyroidectomized (TX) rat dam pregnancies

Days of gestation	Dam	Maternal T ₄ (nM)		Maternal T ₃ (nM)	Litter size	Fetal tissue weight (mg)		Fetal brain protein (mg/g tissue)
		Pre-mating	Post-mortem			Body	Brain	
16	N	56.5 ± 3.9	38.1 ± 2.4	0.85 ± 0.11	14.4 ± 1.5	482 ± 11	52.9 ± 1.4	64.3 ± 2.3
	TX	14.5 ± 1.3 ^c	16.3 ± 2.4 ^c	0.40 ± 0.07 ^b	10.4 ± 1.2 ^a	456 ± 10	49.5 ± 2.0	67.6 ± 1.3
19	N	67.3 ± 6.4	27.2 ± 1.0	0.62 ± 0.12	15.9 ± 1.1	2359 ± 54	134.4 ± 3.5	65.8 ± 1.3
	TX	12.8 ± 2.3 ^c	9.1 ± 2.3 ^c	0.25 ± 0.12 ^a	13.2 ± 0.7	2075 ± 42 ^a	125.8 ± 5.8	64.5 ± 2.2
21	N	58.9 ± 3.7	15.1 ± 2.3	0.57 ± 0.15	18.6 ± 0.8	4740 ± 184	187.6 ± 4.7	62.7 ± 1.7
	TX	11.9 ± 1.7 ^c	9.84 ± 2.5	0.27 ± 0.08 ^a	10.6 ± 1.2 ^b	3483 ± 198 ^b	168.3 ± 5.0 ^a	67.5 ± 1.8

^a*P* < 0.05, ^b*P* < 0.01, ^c*P* < 0.001, TX compared with N dam pregnancy (Fisher's PLSD test; *n* ≥ 7 different pregnancies).

evaluate the expression of cytoskeletal proteins immediately before and after the onset of fetal TH secretion, which occurs at 17.5 days gestation (dg) in the rat (Morreale de Escobar *et al.* 1985). The cytoskeletal proteins investigated included the intermediate filament (IF) protein vimentin, expressed in early immature neurons and glia (Dahl *et al.* 1981, Bignami *et al.* 1982); GFAP, the predominant IF protein of mature astrocytes (Eng 1985); α -internexin, an early axonal type IV IF protein expressed in developing CNS neurons, and the low molecular weight neurofilament protein (NF-L), expressed in developing neurons subsequent to α -internexin (Kaplan *et al.* 1990).

Materials and Methods

Materials

Monoclonal antibodies against vimentin (clone V9), GFAP (clone G-A-5) and NF-L (clone NR-4) were from Sigma Chemical Co. Ltd (Poole, Dorset, UK); the α -internexin monoclonal antibody was from Chemicon International (Harrow, UK). Rainbow protein molecular weight markers, Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane, secondary antibody (anti-mouse immunoglobulin, horseradish peroxidase linked whole antibody from sheep; NA 931) and ECL Western blotting detection reagents were from Amersham Pharmacia Biotech UK Ltd (Little Chalfont, Bucks, UK). Coomassie Protein Plus reagent and bovine serum albumin standard were from Pierce (Chester, Cheshire, UK). The total T₄ RIA kit was from NETRIA (London, UK) and the total 3,5,3'-tri-iodothyronine (T₃) RIA kit was from Brownes UK Ltd (Calcot, Reading, Berks, UK).

Animal model

Sprague-Dawley rats were housed at 22 °C on a 14 h light and 10 h darkness cycle. Female rats were partially surgically thyroidectomized (parathyroid-spared; TX) and allowed to recover for 2 weeks, then mated with normal

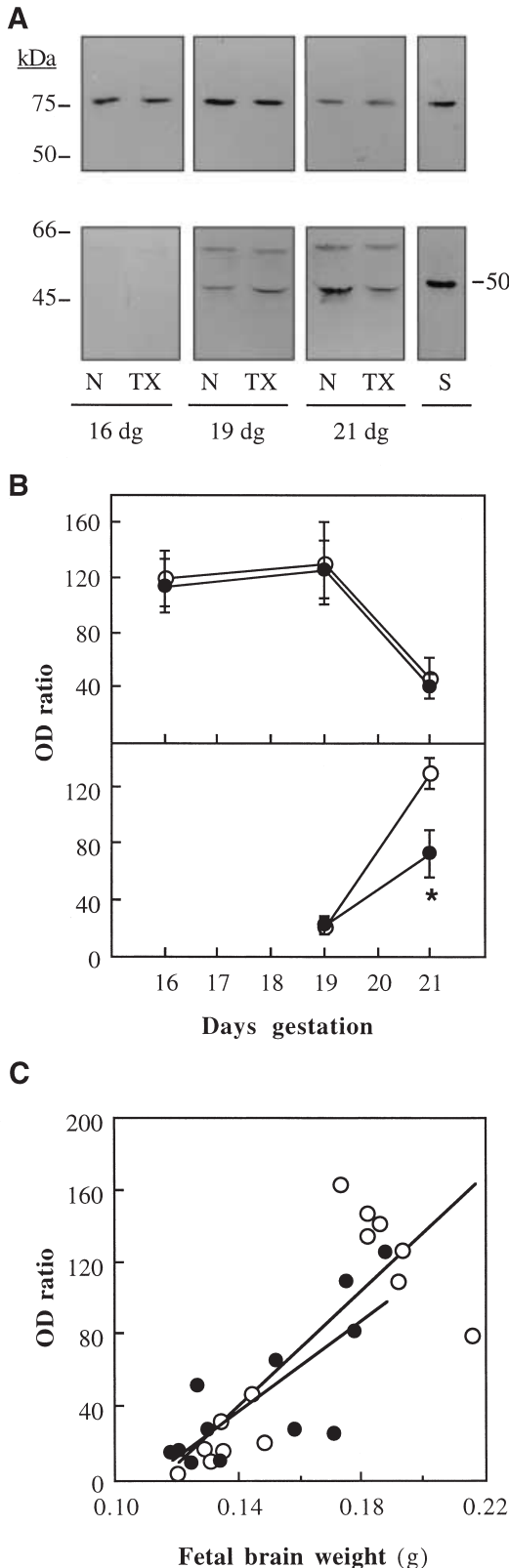
males when total serum T₄ levels were ≤ 25 nM. Age-matched pregnant normal (N) females were the control group. Animals were maintained on an iodine replete (13 µg/g) standard small laboratory animal diet and drinking water for TX dams was supplemented with 0.1% (w/v) calcium lactate. Pregnant animals were stunned then killed by cervical dislocation at 16, 19 or 21 dg and maternal blood collected by cardiac puncture. Fetuses and placentae were separated on ice, and fetal brains quickly dissected, freed of blood and meninges, weighed, and 5–7 brains from each litter pooled. Maternal brain was also collected from a control dam for use as an immunoblotting standard. All tissues were stored at –20 °C. Animals were handled within local regulations and the Animals (Scientific Procedures) Act 1986.

Sample preparation and Western blotting

Brains were homogenised with 9 vol TE buffer (2 mM EDTA, 0.5% v/v Triton X-100 and 10 mM phenylmethylsulphonylfluoride in 50 mM Tris-HCl; pH 6.8), diluted to the required protein concentration with TE buffer, then boiled for 3 min under reducing conditions (Laemmli 1970). After SDS-PAGE, samples were electrophoretically transferred (2 h; 0.36 A; 4 °C) to nitrocellulose membranes (Towbin *et al.* 1979).

Blots were blocked with 10% (w/v) dried milk powder in TBS-T (0.1% v/v Tween-20 in Tris-buffered saline; pH 7.6) for 1 h at 25 °C, before incubation with either anti-GFAP, anti-vimentin, anti- α -internexin or anti-NF-L antibodies (diluted in blocking solution at 1:20 000, 1:2000, 1:5000 and 1:2000 respectively) for 1 h at 25 °C. Blots were washed in TBS-T before incubation for 1 h with secondary antibody (diluted 1:1000). After further washing with TBS-T, blots were processed using the manufacturers' ECL protocol, and exposed to pre-flashed film. Blots were stained with India Ink (Hancock & Tsang 1983) in order to confirm equal sample loading.

Images of autoradiograms were captured using a Kodak DC40 digital camera and the band intensities estimated



using NIH Image software calibrated with an optical density (OD) step tablet. Sample bands were corrected for filter-to-filter variation using standard signals by application of the correction factor $[x/S_{ave}] \cdot S_{filt}$, where x is the OD of the sample band; S_{ave} is the average OD of standards on all filters and S_{filt} is the OD of the standard on the same filter as the sample band (x).

Protein determination

Protein was determined by a dye-binding method (Bradford 1976).

Determination of maternal thyroid hormone levels

Maternal serum total T_4 and total T_3 levels were determined by radioimmunoassays according to manufacturers' protocols.

Statistical analysis and linear regression

Data from N and TX dam pregnancies were compared by two-way ANOVA using Fisher's protected least significant difference (PLSD) test for *post-hoc* analysis. Homogeneity of variance for all groups was verified using Bartlett's test for samples of unequal sizes and, where appropriate, a square root or log transformation was applied before statistical analysis. All values are expressed as means \pm s.e.m. The relationship between cytoskeletal protein level and fetal brain weight was evaluated by linear regression analysis.

Results

Animal model

Pre-mating serum total T_4 levels in TX dams were reduced to 20–25% of controls (Table 1). During late

Figure 1 The expression of vimentin and GFAP in fetal brain from normal (N) and partially thyroidectomized (TX) rat dams. (A) Representative portions of Western blots probed with vimentin (upper panels) and GFAP (lower panels) antibodies. Vimentin blots contained samples and a 16 dg normal fetal brain standard (S), all at 2.5 μ g protein loading. GFAP blots contained samples (10 μ g protein) and an adult brain standard (S; 0.25 μ g protein). Migration of molecular weight markers is indicated on the left and authentic GFAP on the right. The GFAP antibody cross-reacts with a minor 60 kDa protein species, which was not studied further. (B) Ontogenic profiles of vimentin (upper panel) and GFAP (lower panel). Each point is the mean \pm s.e.m. of at least six different N (open symbols) or TX (closed symbols) dams. Significant treatment ($P < 0.05$) and age-treatment ($P < 0.05$) effects were found for GFAP; * $P < 0.01$, TX compared with N dam (Fisher's PLSD). (C) Regression of GFAP levels on fetal brain weight; the linear relationships found for N (open symbols) ($r^2 = 0.64$; $P = 0.0006$) and for TX (closed symbols) ($r^2 = 0.61$; $P = 0.0026$) dams were not significantly different in slope or elevation.

pregnancy, differences in maternal T_4 levels between N and TX dams were less marked (Table 1), largely due to the expected decline in circulating T_4 in N dams (Morreale de Escobar *et al.* 1985). Maternal serum total T_3 was also reduced in TX dams, to between 40–50% of control levels (Table 1). TX dams carried fewer fetuses than N dams, and fetal body weights were reduced at 19 and 21 dg to 88% and 73% of control levels respectively. Fetal brain weight was also reduced (90% of controls) in TX dams at 21 dg, but fetal brain protein concentration was normal at all ages (Table 1). Placental weight was also normal at all ages (data not shown).

Vimentin expression

The vimentin antibody recognised a single 57 kDa band in fetal brain (Fig. 1), as expected (Fliegner & Liem 1991). The abundance of vimentin remained constant between 16 and 19 dg, and decreased ($P < 0.01$) markedly thereafter (Fig. 1). Maternal thyroidectomy was without effect on vimentin levels in fetal brain.

GFAP expression

The GFAP antibody recognised a 50 kDa protein species, as expected, and a less abundant slower migrating species (ca. 60 kDa) in fetal and adult brain (Fig. 1). The 50 kDa band was specific for nervous tissue, whereas the 60 kDa band was detected in fetal liver, lung and heart (data not shown) and was therefore not studied further. GFAP was undetectable at 16 dg, but present at similar abundance in both groups at 19 dg. Levels increased fivefold between 19 and 21 dg in N dam progeny, but only threefold in TX dam progeny. Consequently, GFAP was markedly deficient in TX fetal brain at 21 dg (44% of control level). Since fetal brain weight was also reduced at this age in TX dam progeny, we compared the relationship between GFAP abundance and brain weight between 19 and 21 dg in both groups. Regression of GFAP on fetal brain weight resulted in superimposable regression lines (Fig. 1), indicating appropriate GFAP expression for fetal brain weight in TX dam progeny.

α -Internexin expression

The α -internexin antibody recognised the expected 66 kDa species in fetal and adult brain (Fliegner & Liem 1991) (Fig. 2). In N fetal brain, abundance declined ($P < 0.01$) between 16 and 19 dg and then remained stable. Maternal thyroidectomy was associated with reduced levels at 16 dg (71% of control value), but increased levels of expression at 21 dg (147% of control value). α -Internexin abundance exhibited an inverse linear relationship with fetal brain weight in N dams throughout

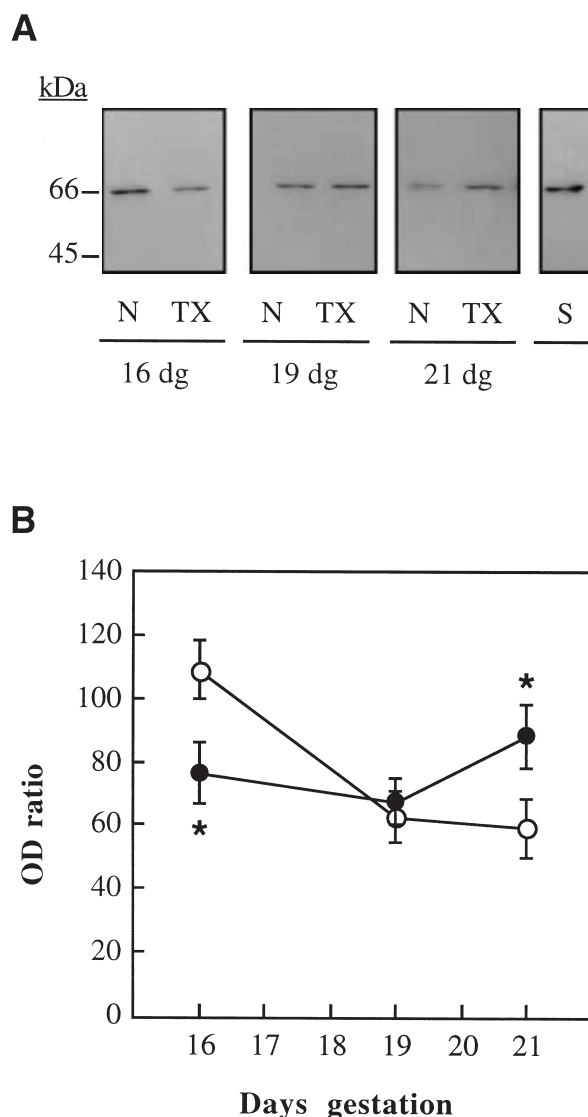


Figure 2 The expression of α -internexin in fetal brain from normal (N) and partially thyroidectomized (TX) rat dams. (A) Representative portions of Western blots containing samples and an adult brain standard (S) all at 2.5 μ g protein loading. Migration of molecular weight markers is indicated on the left. (B) Ontogenic profile of α -internexin. Each point is the mean \pm S.E.M. of at least seven different N (open symbols) or TX (closed symbols) dam pregnancies. A significant age–treatment interaction occurred ($P < 0.01$). * $P < 0.05$, TX compared with N dam (Fisher's PLSD).

the study period ($P = 0.0008$, $r^2 = 0.4$), but was unrelated to fetal brain weight in TX dams (data not shown).

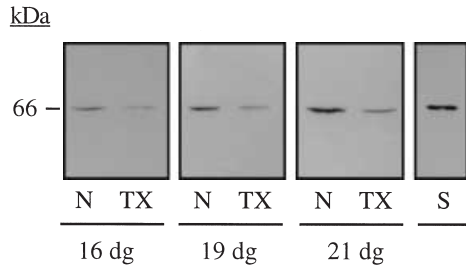
NF-L expression

The NF-L antibody recognised a 68 kDa species in fetal and adult brain (Fig. 3) as expected (Fliegner & Liem 1991), the abundance of which increased ($P < 0.05$)

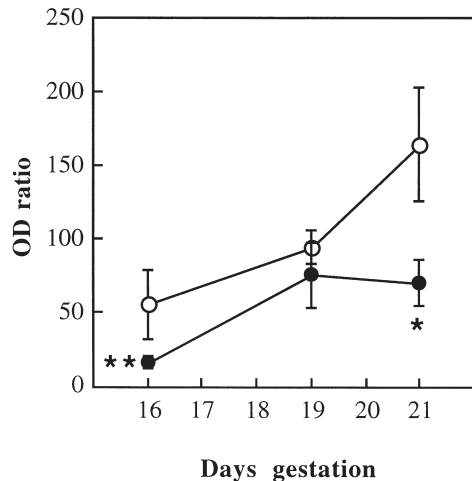
between 16 and 19 dg in both N and TX dam progeny. Maternal thyroidectomy resulted in deficient expression of

NF-L at 16 dg (39% of control level) and 21 dg (43% of control level). Regression of NF-L levels on fetal brain between 16 and 21 dg, revealed linear relationships in N and TX dam progeny of identical slope but differing elevation (Fig. 3). Thus, for a given fetal brain weight, NF-L levels were lower in TX than N dam progeny.

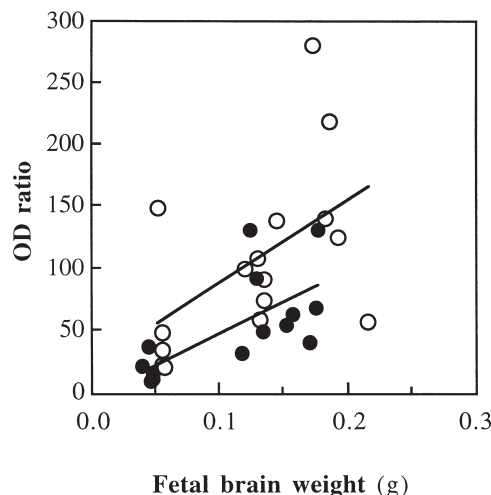
A



B



C



Discussion

Partial thyroidectomy depressed pre-mating maternal serum total T_4 to around one quarter of control levels and was associated with a reduction in litter size. Fetal body and brain weights were depressed close to term, but placental weights were normal, consistent with previous studies of severely hypothyroid dams (Morreale de Escobar *et al.* 1985). In moderate maternal hypothyroxinaemia (pre-mating total $T_4 > 16.2$ nM), however, late fetal growth appears normal (Pickard *et al.* 2000).

Vimentin is the predominant IF protein in several neuronal and astrocytic precursors. Maternal hypothyroidism had no effect on vimentin expression in fetal brain over the study period, indicating normal acquisition of neuronal and astrocytic precursor cells.

GFAP was detectable only after the onset of fetal TH secretion; levels were initially normal at 19 dg, but markedly deficient at 21 dg in TX dam progeny. This indicates that maternal hypothyroidism impairs astrocytic maturation in the fetal brain towards the end of pregnancy. Models of combined materno-fetal hypothyroidism have shown that vimentin expression appears normal, but GFAP levels are reduced in perinatal hippocampus (Rami & Rabie 1988, Martinez-Galan *et al.* 1997).

Although materno-fetal T_4 transfer may contribute as much as one fifth of the fetal extrathyroidal T_4 pool at 21 dg (Morreale de Escobar *et al.* 1990), the onset of fetal secretion rapidly corrects fetal cerebral thyroid hormone deficits in overtly hypothyroid rat dams (Morreale de Escobar *et al.* 1985). Consequently, fetal brain hypothyroidism is unlikely to account for the GFAP deficit in TX dam progeny. Rather, this latter effect appears related to the retarded fetal brain growth, since TX dam fetuses express appropriate levels of GFAP for fetal brain weight.

Figure 3 The expression of NF-L in fetal brain from normal (N) and partially thyroidectomized (TX) rat dams. (A) Representative portions of Western blots containing samples (10 μ g protein) and an adult brain standard (S; 0.5 μ g protein). Migration of molecular weight markers is indicated on the left. (B) Ontogenic profile of NF-L. Each point is the mean \pm S.E.M. of at least 5 different N (open symbols) or TX (closed symbols) dams. A significant ($P < 0.001$) treatment effect was found: $*P < 0.05$; $**P < 0.01$, TX compared with N dam (Fisher's PLSD). (C) Regression of NF-L levels on fetal brain weight. The linear relationships found for N (open symbols) ($r^2 = 0.27$; $P = 0.038$) and TX (closed symbols) ($r^2 = 0.47$; $P = 0.0051$) dams had identical slopes but different ($P < 0.05$) elevations.

α -Internexin is the first neuronal intermediate filament to be expressed in newly differentiating neurons and constitutes the major neuronal IF protein prior to the onset of fetal thyroid hormone secretion. To our knowledge, the influence of thyroid status on α -internexin expression has not previously been investigated, despite its suggested role in the stabilisation of small calibre axons during early brain development (Fliegner *et al.* 1994). Maternal hypothyroidism was associated with a deficit in α -internexin abundance at 16 dg and the absence of the expected ontogenic decline of this protein during late fetal life. Consequently α -internexin levels were markedly elevated in TX fetal brain near term. This may have serious consequences if not corrected soon after birth, since over-expression of α -internexin is associated with motor coordination deficits in transgenic mice (Ching *et al.* 1999). Ornithine decarboxylase and DOPA decarboxylase also have important roles in the acquisition of sensorimotor functions, and it is of some interest that they display comparable ontogenic profiles to α -internexin in normal fetal brain and are similarly disturbed as a consequence of maternal hypothyroxinaemia (Pickard *et al.* 1993, Evans *et al.* 1999).

NF-L is expressed subsequent to α -internexin during early neuronal differentiation (Kaplan *et al.* 1990, Fliegner *et al.* 1994). Maternal hypothyroidism was associated with reduced NF-L expression in fetal brain, both before the onset of fetal thyroid function and near term. Although N and TX dam progeny displayed similar rates of increase of NF-L with fetal brain weight, the elevations of these profiles were distinct. Consequently, NF-L levels in TX dam progeny were approximately half those in their normal counterparts for a given fetal brain weight.

The early deficit in NF-L is consistent with the recent report that thyroid hormone positively regulates NF-L expression in postnatal rat brain (Ghosh *et al.* 1999). However, the early deficit in α -internexin expression may also be influential, as this protein is thought to form the primary scaffold around which neurofilament triplet proteins co-assemble during development (Fliegner *et al.* 1994, Balin & Miller 1995, Ching & Liem 1998).

Together, these findings suggest that maternal hypothyroidism in the rat disrupts early stages of neuronal differentiation in fetal brain, and that such disturbances are not corrected by the onset of fetal thyroid hormone secretion. Astrocytic differentiation – although initially normal – is perturbed near term concomitant with a more general delay in fetal brain growth. These findings confirm the existence of a critical period of maternal thyroid hormone-dependency for fetal brain development (Pickard *et al.* 1997), be it mediated directly (through interaction with fetal brain TH nuclear receptors) or indirectly, at the levels of maternal metabolism and/or placental function (Bonet & Herrera 1988, Hendrich & Porterfield 1992, Leonard *et al.* 1999, Pickard *et al.* 1999, 2000).

Disruption of the co-ordinate maturation of neuronal and glial cells is likely to impinge upon the establishment of vital neural connections (Rakic 1990), possibly contributing to the behavioural deficits in postnatal progeny of hypothyroid rat dams (Attree *et al.* 1992, Sinha *et al.* 1997). The human brain at birth corresponds to the rat brain at 10 postnatal days so that most neuronal differentiation occurs prenatally in man but postnatally in rats (Porterfield & Hendrich 1993). Studies in humans have emphasised the importance of the maternal TH environment prior to the onset of fetal thyroid function (18–20 weeks gestation in humans) with respect to fetal brain development (Connolly & Pharoah 1989, Pop *et al.* 1999). α -Internexin, NF-L and GFAP are expressed in human fetal brain from at least 9 weeks gestation (Voronina & Preobrazhensky 1994, Arnold & Trojanowski 1996). Thus, similar compromise to that seen in the rat may contribute to the cognitive and motor dysfunction seen in the offspring of hypothyroid women (Connolly & Pharoah 1989, Man *et al.* 1991, Haddow *et al.* 1999). However, given the differences in materno-fetal TH economy and placentation between rats and humans, extrapolations between these two species must be approached with sufficient caution.

The evidence presented here, together with previous studies, demonstrates that maternal thyroid status influences several developmental parameters in fetal brain. Experiments in progress in our laboratory aim to elucidate the spatial changes and molecular mechanisms underlying this regulation.

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