Maternal hypothyroxinemia influences glucose transporter expression in fetal brain and placenta

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Abstract

The influence of maternal hypothyroxinemia on the expression of the glucose transporters, GLUT1 and GLUT3, in rat fetal brain and placenta was investigated. Fetal growth was retarded in hypothyroxinemic pregnancies, but only before the onset of fetal thyroid hormone synthesis. Placental weights were normal, but placental total protein concentration was reduced at 19 days gestation (dg). Immunoblotting revealed a decreased abundance of GLUT1 in placental microsomes at 16 dg, whereas GLUT3 was increased. Fetal serum glucose levels were reduced at 16 dg. In fetal brain, the concentration of microsomal protein was deficient at 16 dg and the abundance of parenchymal forms of GLUT1 was further depressed, whereas GLUT3 was unaffected. Northern hybridization analysis

demonstrated normal GLUT1 mRNA levels in placenta and fetal brain.

In conclusion, maternal hypothyroxinemia results in fetal growth retardation and impaired brain development before the onset of fetal thyroid function. Glucose uptake in fetal brain parenchyma may be compromised directly, due to deficient GLUT1 expression in this tissue, and indirectly, as a result of reduced placental GLUT1 expression. Though corrected by the onset of fetal thyroid hormone synthesis, these deficits are present during the critical period of neuroblast proliferation and may contribute to long term changes in brain development and function seen in this model and in the progeny of hypothyroxinemic women.

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Introduction

Observations in humans and animal models are consistent with the hypothesis that maternal thyroid hormone regulates early fetal brain development (Porterfield & Hendrich 1991, Morreale de Escobar *et al.* 1997, Pickard *et al.* 1997, Sinha *et al.* 1997). For example, children born to hypothyroxinemic mothers in certain iodine deficiency endemias – though themselves clinically euthyroid – display an increased incidence of neurologic disorders, ranging from subclinical deficits in cognitive and motor function to overt neurologic cretinism (Connolly & Pharoah 1989). These disorders can be prevented by iodine supplementation, but only if administered well before the onset of fetal thyroid function, and cognitive and motor function in affected children has been shown to correlate with maternal thyroxine (T₄) levels during pregnancy (Connolly & Pharoah 1989). Associations between maternal hypothyroxinemia and impaired motor and intellectual development have also been reported in iodine-sufficient environments (Man *et al.* 1991).

Studies in the rat have shown that maternal thyroidec-tomy impairs fetal somatic and brain growth (Morreale de

Escobar *et al.* 1985, Bonet & Herrera 1988, Porterfield & Hendrich 1991, Pickard *et al.* 1993). Such deficits are corrected following the onset of fetal thyroid function in a hypothyroxinemic rat dam model, but sensitive markers of brain cell maturation – such as ornithine decarboxylase and neurotransmitter metabolic enzymes – remain abnormal in postnatal life (Pickard *et al.* 1993, Evans *et al.* 1999). Biochemical dysfunction is generally confined to early developing brain regions and is associated with learning and motor deficits in adult progeny (Pickard *et al.* 1997).

Maternal T₄ is transferred to the developing conceptus during pregnancy in humans and rats (Porterfield & Hendrich 1991, Morreale de Escobar *et al.* 1997, Pickard *et al.* 1997, Sinha *et al.* 1997). Although the amount transferred constitutes only a small fraction of the maternal circulating pool, T₄ and metabolites (in particular 3,5,3'-triiodothyronine; T₃) accumulate within the developing brain before the onset of fetal thyroid activity, coincident with nuclear T₃ receptor expression. Fetal brain total T₃ levels are low (ca. 100 pM) at this time (Ferreiro *et al.* 1988, Ruiz de Ona *et al.* 1988), but receptor occupancy approximates 25% since free T₃ concentrations are high in the nucleus relative to the cytosol (Ferreiro *et al.* 1988).

Maternal thyroid hormone may therefore directly influence early fetal brain development, however because it also accumulates within maternal tissues and the placenta (Morreale de Escobar *et al.* 1985), indirect mechanisms of regulation may be important. Placental maldevelopment has been reported in some (Bonet & Herrera 1988, Porterfield & Hendrich 1991) – but not all (Morreale de Escobar *et al.* 1985) – hypothyroid rat dam models, whereas gross placental growth is normal in hypothyroxinemic dams (Pickard *et al.* 1993). More subtle disturbances in placental function cannot be ruled out however.

Thyroid hormone regulates glucose transport in astrocytes in culture (Roeder *et al.* 1988) and postnatal rat brain *in vivo* (Moore *et al.* 1973). Such findings are likely to be of relevance to maternal thyroid hormone action since the fetus is highly dependent upon glucose, both as an energy source and as a precursor for biosynthetic reactions intimately connected with tissue growth (Jones & Rolph 1985). In the rat, the fetal glucose supply is derived from the maternal circulation (Jones & Rolph 1985), with placental transfer of glucose and uptake into fetal tissues being mediated by facilitative glucose transporters (GLUT) (Burant *et al.* 1991, Mueckler 1994). GLUT isoforms exhibit distinct developmental- and tissue-specific patterns of expression, GLUT1 and GLUT3 together serving as the major glucose transporters of rat placenta and brain (Burant *et al.* 1991, Mueckler 1994).

We have therefore examined the influence of maternal hypothyroxinemia in the rat on the ontogeny of GLUT1 and GLUT3 proteins in fetal brain and placenta. We demonstrate that maternal hypothyroxinemia results in reduced expression of GLUT1 protein in fetal brain and, to a lesser extent, placenta before the onset of fetal thyroid hormone synthesis. In contrast, the expression of GLUT3 protein is normal in fetal brain but shows a tendency towards increased levels in placenta. Nevertheless, fetal serum glucose levels are reduced at this stage of pregnancy. The effects on GLUT1 protein expression occur in the absence of changes in transcript levels in fetal brain and placenta, indicating the involvement of translational or post-translational mechanisms of regulation.

Materials and Methods

Materials

Polyclonal antibodies, raised against the C-terminal 13 amino acids of rat GLUT1 and GLUT3, were from Charles River (Southbridge, MA, USA); the GLUT3 antibody was supplied affinity-purified. Oligonucleotides were synthesized by Genosys Biotechnologies (Cambridge, Cambs, UK). Plasmid pSGT containing human GLUT1 cDNA was from the ATCC (Rockville, MD, USA) and pBluescript II SK⁺ from Stratagene Ltd (Cambridge, Cambs, UK). Restriction enzymes were

from Promega (Southampton, Hants, UK) and PCR reagents from PE Applied Biosystems (Warrington, Cheshire, UK). The MAXIscript kit was supplied by AMS Biotechnology (Europe) Ltd (Witney, Oxon, UK). Megaprime DNA and 5'-end labeling kits, Hybond-N and -ECL membranes, protein A (>30 mCi/mg) and Rainbow protein molecular weight markers were from Amersham Life Science Ltd (Little Chalfont, Bucks, UK). RNA and DNA ladders were from Life Technologies Ltd (Paisley, Renfrewshire, UK). dCTP (3000 Ci/mmol), UTP (800 Ci/mmol) and ATP (4500 Ci/mmol) were from ICN Biomedicals Ltd (Thame, Oxon, UK). NICK columns and Microcon-30 microconcentrators were from Pharmacia Biotech (St Albans, Herts, UK) and Amicon Ltd (Stonehouse, Gloucs, UK) respectively. Total T4 and T3 RIA kits were from NETRIA (London, UK), glucose (Trinder) reagent from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) and Coomassie Protein Plus reagent from Pierce & Warriner (UK) Ltd (Chester, Cheshire, UK).

Animal model

Sprague-Dawley rats were maintained in local animal house facilities at 22 °C on a cycle of 14 h light and 10 h darkness. Female rats were partially thyroidectomized (parathyroid-spared) by surgery and, after a recovery period of at least 2 weeks, time-mated with normal males when serum total T4 levels were ≤ 30 nM (TX dams). Pregnant normal (untreated) females constituted the control group (N dams). Animals had free access to water and food (standard small animal laboratory diet); the drinking water of TX dams was supplemented with 0.1% calcium lactate. Animals were stunned then killed by cervical dislocation at 16, 19 or 21 days gestation (dg). Maternal blood was collected by cardiac puncture. Fetuses and placentae were separated on ice, weighed and whole brains quickly dissected. These were cleansed of adherent blood and meninges before weighing. In some experiments, fetal blood was collected from the neck stump after decapitation and pooled from each pregnancy. Other fetal and placental tissues from each litter were also pooled (three to four tissues/pool) before storage at -20 °C.

Isolation of crude microsomes and Western blot analysis

Placentae were chopped (1 mm³ pieces) and washed three times with 9 vol ice-cold TES-PMSF (50 mM Tris-HCl, pH 7.4; 2 mM EDTA; 0.25 M sucrose; 1 mM phenylmethylsulphonyl fluoride). Brains and washed placentae were homogenized with 4 vol TES-PMSF (4 °C) and centrifuged (5000 g; 5 min; 4 °C). The supernatant was further centrifuged (104 000 g; 60 min; 4 °C) to collect the crude microsomal fraction. This pellet was washed once by centrifugation, resuspended in 0.7 vol TES-PMSF and stored at -20 °C.

Electrophoresis was performed using a standard method (Laemmli 1970). Microsomal fractions were diluted in TES-PMSF to the desired protein concentration, then diluted 1:1 with $2 \times$ reducing buffer and incubated at 37°C for 15 min. Samples, including an adult brain control (13 μg microsomal protein), and molecular weight markers were electrophoresed (SDS-PAGE; 10% gel) then transferred to Hybond-ECL membranes (Towbin *et al.* 1979). Blots were blocked with 4% BSA in TBS-T (0.1% Tween-20 in Tris-buffered saline, pH 7.6) for 1 h at 25°C , washed in TBS-T and incubated with either an anti-GLUT1 or an anti-GLUT3 antibody (each diluted 1:2000 in 0.5% BSA in TBS-T) for 1 h at 25°C . Blots were washed in TBS-T and incubated with protein A at 10 ng/ml in antibody diluent. After further washing in TBS-T, blots were exposed to pre-flashed film at -70°C . Radioactive bands (and background control areas) were excised from the filters and counted by γ -spectrometry. Counts were corrected for background and decay, then further corrected for filter-to-filter variation by comparison of the counts for the adult brain control present on each blot.

RNA isolation and Northern blot analysis

Total RNA was isolated (Chomczynski & Sacchi 1987) and aliquots (20 μg) and size markers were electrophoresed (1.2% agarose-formaldehyde gel), blotted on to Hybond-N membranes and UV-fixed. Blots were then hybridized with GLUT1 cDNA or antisense cRNA probes.

The cDNA probe comprised a 545 bp fragment (nucleotides +846 to +1390 of human GLUT1) generated by PCR from the plasmid pSGT. The plasmid was linearized with SmaI and amplified with AmpliTaq DNA polymerase for 25 cycles. Sense and antisense primers were 5'-GCAGCTGTCTGGCAT-3' and 5'-CGATCTCAT CGAAGG-3' respectively; MgCl_2 was 2.5 mM and annealing temperature 41°C . The probe was partially purified (Microcon-30) and radiolabeled using dCTP and the Megaprime DNA labeling system; non-incorporated nucleotide was removed using a NICK column. For generation of the riboprobe, pSGT was digested with XhoII and EcoRI and a 1244 bp fragment (nucleotides +279 to +1476 of human GLUT1 plus a portion of the 3'-untranslated region) subcloned into the BamHI/EcoRI sites of pBluescript II SK⁺. Radiolabeled probe was produced by *in vitro* transcription of XhoI-linearized plasmid using T3 RNA polymerase and UTP. Radiolabeled riboprobe was purified by precipitation in ethanol-ammonium acetate.

For hybridization with the cDNA probe, blots were prehybridized for 8 h at 42°C with $6 \times$ SSPE; $5 \times$ Denhardt's; 0.5% SDS; 50 $\mu\text{g}/\text{ml}$ salmon testis DNA; 50% formamide, then hybridized for 16 h at 42°C with radiolabeled probe at ca. 10^7 c.p.m./ml in $6 \times$ SSPE; 10%

dextran sulphate; $2 \times$ Denhardt's; 0.1% SDS; 20 $\mu\text{g}/\text{ml}$ salmon testis DNA; 50% formamide. For riboprobes, blots were prehybridized for 8 h at 60°C in $5 \times$ SSPE; $2 \times$ Denhardt's; 0.1% SDS; 100 $\mu\text{g}/\text{ml}$ salmon testis DNA and 100 $\mu\text{g}/\text{ml}$ baker's yeast tRNA; 50% formamide, then hybridized for 16 h at 60°C with fresh solution containing radiolabeled probe at ca. 2×10^6 c.p.m./ml.

Hybridized blots were subjected to a final high stringency wash for 20 min in $0.1 \times$ SSC/0.1% SDS at 55°C (cDNA probe) or 75°C (riboprobe), then exposed to preflashed film with an intensifying screen at -70°C . All blots were stripped and rehybridized with an oligonucleotide probe to nucleotides +292 to +321 of rat 18S rRNA, as loading control (Varghese *et al.* 1994). Hybridization was performed at 68°C in $6 \times$ SSPE; $5 \times$ Denhardt's and 50 $\mu\text{g}/\text{ml}$ salmon testis DNA. The probe was 5'-end-labeled with ATP, purified (NICK column), then added to hybridization solution at 1 nM (specific activity 10^6 d.p.m./pmol). The final high stringency wash was in $6 \times$ SSPE/0.1% SDS at 78°C for 2 min.

Autoradiographs were digitized using a Kodak DC-40 digital camera. Densitometric analysis was by NIH Image version 6.1 using an optical density step tablet for calibration.

Determination of thyroid hormone

Total T4 and total T3 levels were determined in maternal serum by RIA using commercial kits.

Determination of glucose

Glucose was determined in maternal and fetal serum by the method of Trinder (1969).

Protein determination

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

Statistical analysis

Data from N and TX dam pregnancies were compared by two-way ANOVA for age and treatment effects; Fisher's protected least significant differences (PLSD) were determined for the *post-hoc* analysis. Homogeneity of variance for all groups was checked using Bartlett's test for samples of unequal sizes and, where necessary, a square root or log transformation was applied before analysis. All results were consistent with a Gaussian distribution and are expressed as mean $\pm$ s.e.m.

Results

Animal model

Premating serum total T4 levels were similar in TX dams irrespective of whether these were studied at 16, 19 or

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

					Tissue weight		
	Dam	Maternal T4 (nM)	Maternal T3 (nM)	Litter size	Fetal body (mg)	Fetal brain (mg)	Placenta (mg)
Days gestation							
16	N	44.0 ± 2.6	1.51 ± 0.24	17.0 ± 0.6	493 ± 9	52.6 ± 1.1	344 ± 13
	TX	13.9 ± 1.6 ^b	0.94 ± 0.22 ^a	11.9 ± 0.9 ^b	445 ± 9 ^b	48.7 ± 1.5 ^a	339 ± 17
19	N	29.9 ± 1.7	1.33 ± 0.17	16.4 ± 1.0	2190 ± 38	127.5 ± 1.9	497 ± 13
	TX	16.2 ± 1.2 ^b	0.87 ± 0.16 ^a	11.5 ± 0.8 ^b	2137 ± 47	128.4 ± 2.3	521 ± 25
21	N	18.3 ± 1.3	1.60 ± 0.31	17.6 ± 1.0	5015 ± 78	186.8 ± 3.4	541 ± 25
	TX	11.6 ± 1.1 ^b	0.91 ± 0.23 ^a	12.1 ± 1.0 ^b	4925 ± 97	185.0 ± 2.2	570 ± 14
Two-way ANOVA							
Treatment	<i>P</i>	<0.001	<0.001	<0.001	<0.005	NS	NS
Age-treatment interaction	<i>P</i>	<0.001	NS	NS	NS	NS	NS

NS, No significant difference by two-way ANOVA. ^a*P*<0.05; ^b*P*<0.001; TX vs N dam pregnancy at the appropriate age (Fisher's PLSD test) *n* ≥ 13 different dams.

21 dg (values were: 16.6 ± 1.8 nM, 16.3 ± 1.6 nM and 17.7 ± 1.3 nM respectively; *n* ≥ 14); each being reduced (by ca. 70%; *P*<0.001) when compared with non-pregnant euthyroid controls (values were: 57.0 ± 1.9 nM). A similar reduction was found in pregnant animals at 16 dg (Table 1). Differences between N and TX dams were less marked at subsequent stages of pregnancy, since circulating total T4 levels declined in N dams, as expected (Morreale de Escobar *et al.* 1985), but remained relatively stable in TX dams (Table 1). Maternal serum total T3 levels remained invariant over the study period, and were reduced by approximately 40% in TX dams (Table 1).

Litter size was consistently reduced by 30% in TX dams at 16–21 dg (Table 1). Fetal body weight was reduced by 10% in TX dams at 16 dg but was normal at subsequent stages of pregnancy (Table 1); a similar pattern was

observed for fetal brain weight (Table 1). Placental weight was normal at all stages of pregnancy studied (Table 1).

Although the concentration of total protein in fetal brain from TX dams was normal between 16 and 21 dg, the proportion of protein recovered in the crude microsomal fraction appeared deficient (Table 2). *Post-hoc* analysis confirmed a significant reduction at 16 dg (68% control level). In TX dam placenta, the concentration of total protein was reduced at 19 dg to 80% of the control level (Table 2).

GLUT1 protein expression

Irrespective of maternal thyroid status, GLUT1 species of 44 and 51 kDa were detected in fetal brain microsomes at 16–21 dg, whereas only the 44 kDa form was observed in

Table 2 Total and microsomal protein in fetal brain and placenta from euthyroid (N) and partially thyroidectomized (TX) rat dam pregnancies

		Fetal brain protein		Placental protein	
		total	microsomal	total	microsomal
Dam		(mg/g tissue)	(% total)	(mg/g tissue)	(% total)
Days gestation					
16	N	51.3 ± 1.8	13.8 ± 1.0	67.7 ± 3.0	14.0 ± 1.9
	TX	53.0 ± 3.6	9.4 ± 1.1 ^a	66.6 ± 4.8	12.9 ± 0.9
19	N	54.5 ± 2.5	13.7 ± 1.4	79.3 ± 6.3	10.3 ± 0.6
	TX	56.1 ± 2.2	12.2 ± 1.3	62.9 ± 5.6 ^b	10.9 ± 1.1
21	N	53.6 ± 3.2	19.3 ± 1.9	59.5 ± 4.7	13.0 ± 1.3
	TX	56.9 ± 2.1	15.1 ± 1.9	69.7 ± 2.5	11.5 ± 0.7
Two-way ANOVA					
Treatment	<i>P</i>	NS	<0.01	NS	NS
Age-treatment interaction	<i>P</i>	NS	NS	<0.05	NS

NS, No significant difference by two-way ANOVA. ^a*P*<0.05; ^b*P*<0.02; TX vs N dam pregnancy at the appropriate age (Fisher's PLSD test) *n* ≥ six different dams.

Figure 1 Effect of maternal hypothyroxinemia on GLUT1 protein expression in fetal brain. A, Western blot of fetal brain microsomes (50 µg protein) from normal (N) and partially thyroidectomized (TX) rat dams immunodetected with a GLUT1-specific antibody and protein A. Blots also included an adult brain microsomal standard (S; 13 µg protein). Only a portion of the autoradiogram is shown and the migration of molecular weight markers is indicated on the right hand side. Note the presence in fetal brain of discrete 44 and 51 kDa species. B, Radioactive bands were excised from filters and counted. Counts were background-subtracted and corrected for filter-to-filter variation using the adult brain standard. Each point is the mean corrected c.p.m. $\pm$ S.E.M. of seven different N (○) or TX (●) dams. Significant treatment effects were found for both 51 kDa ($P < 0.05$) and 44 kDa ($P < 0.02$) forms. * $P < 0.01$; ** $P < 0.005$; TX vs N dam at the appropriate age (Fisher's PLSD test).

adult brain microsomes (Fig. 1). These forms arise by differential glycosylation of a 37 kDa precursor (Wang & Brennan 1988) and are thought to be derived from the brain parenchyma (Walker *et al.* 1988, Wang & Brennan 1988, Dermietzel *et al.* 1992, Vannucci 1994).

In normal fetal brain, the microsomal abundance of the two GLUT1 forms declined between 16 and 19 dg and then remained stable (Fig. 1). Maternal thyroidectomy resulted in deficient expression of both 44 kDa (58% control level) and 51 kDa (67% control level) GLUT1 at 16 dg, although levels of both were normal at 19 and 21 dg (Fig. 1). When age- and treatment-related effects on fetal

brain microsomal protein were taken into account – by expressing results as a tissue concentration – more pronounced deficits in 44 and 51 kDa GLUT1 (40 and 47% control levels respectively) were seen at 16 dg (data not shown).

The GLUT1 antibody detected a discrete 44 kDa species in placental microsomes as well as 58 and 64 kDa species (Fig. 2). It has previously been noted that rat placental GLUT1 exhibits heterogeneous molecular mass due to variable glycosylation (Boileau *et al.* 1995, Shin *et al.* 1997). In normal placenta, levels of total GLUT1 protein increased over the period studied, but the constituent 44 kDa and hyperglycosylated forms exhibited distinct ontogenic profiles (Fig. 2). The abundance of the 44 kDa form remained stable between 16 and 19 dg, then doubled by 21 dg, whereas the hyperglycosylated species increased steadily (3.4-fold) over the study period (Fig. 2). Similar ontogenic profiles were observed in placenta from TX dams, with significant overall treatment effects found for the hyperglycosylated and total transporter forms, though *post-hoc* analysis confirmed only a significant difference for the abundance of total transporter (72% control level) at 16 dg (Fig. 2).

When placental GLUT1 levels were expressed as a function of tissue wet weight, similar ontogenic profiles were obtained but statistically significant treatment-related differences were observed for total ($P = 0.004$), 44 kDa ($P = 0.030$) and hyperglycosylated ($P = 0.008$) forms. *Post-hoc* analysis showed significant ($P < 0.05$) reductions in TX dam placental concentrations of total transporters at 16 dg (8.2 ± 1.1 vs $11.8 \pm 0.8 \times 10^6$ c.p.m./g for controls) and 19 dg (12.8 ± 1.2 vs $19.3 \pm 2.6 \times 10^6$ c.p.m./g for controls), and of hyperglycosylated forms at 19 dg (5.2 ± 0.8 vs $8.5 \pm 1.5 \times 10^6$ c.p.m./g for controls).

GLUT3 protein expression

The GLUT3 antibody recognized two protein species in fetal and adult brain microsomes (Fig. 3). The major 48 kDa species corresponds to authentic GLUT3 protein, while the minor 44 kDa band is thought to arise from cross-reaction with actin (Shepherd *et al.* 1992). The abundance of 48 kDa GLUT3 protein increased steadily in normal fetal brain between 16 and 19 dg, and 19 and 21 dg ($P < 0.05$; Fisher's PLSD test), whereas no increase was noted after 19 dg in TX dams (Fig. 3). A similar difference was apparent when brain GLUT3 levels were expressed as a tissue concentration (data not shown), but treatment and age-treatment interaction effects failed to achieve statistical significance irrespective of the method of expressing results.

In placenta, the GLUT3 antibody recognized slower migrating species (55–87 kDa) in addition to the 48 kDa form noted in brain (Fig. 4). In contrast to GLUT1, the expression of the 48 kDa and hyperglycosylated GLUT3 forms appeared co-ordinately regulated in normal

pregnancy; the abundance of all forms progressively increased between 16 and 21 dg (Fig. 4). Furthermore, the hyperglycosylated forms were expressed at higher levels than the 48 kDa form. Similar findings have been reported by other investigators (Boileau *et al.* 1995, Shin *et al.* 1997). Maternal hypothyroxinemia was without overall effect on the expression of total and hyperglycosylated placental GLUT3 (Fig. 4 and data not shown), though

age-treatment interaction approached statistical significance for 48 kDa GLUT3; *post-hoc* analysis confirmed that this species was significantly elevated (144% control level) in TX dam placenta at 16 dg (Fig. 4). When results were expressed as a tissue concentration, significant age-treatment interaction ($P=0.046$) was noted for the 48 kDa form although no significant differences were found at any individual age by *post-hoc* analysis (data not shown).

GLUT1 mRNA expression

In further experiments, we examined whether maternal hypothyroxinemia modulated GLUT1 transcript levels. This treatment had no effect on the yield of total RNA from fetal brain and placenta at any age (data not shown), allowing a control 18S rRNA probe to be used to correct for differences in sample loading.

Hybridization of Northern blots containing total RNA from placenta, fetal brain and adult brain revealed the presence of a 2.7 kb transcript in all tissues and a minor 1.6 kb transcript in placenta alone (data not shown). This latter transcript was also evident on blots containing poly(A)⁺ placental RNA and was attenuated coincident with the 2.7 kb signal when hybridized blots were washed at increasing stringency (data not shown), indicating that it may represent a splice variant or closely related transcript. Levels of the 2.7 kb transcript were markedly higher in placenta than in adult brain, but barely detectable in fetal brain. Consequently, a riboprobe was utilized to assess the effects of maternal thyroidectomy on fetal brain GLUT1 mRNA expression.

Expression of the 2.7 kb GLUT1 transcript was developmentally regulated in fetal brain in both N and TX dam pregnancies (Fig. 5), with levels decreasing between 16 and 19 dg ($P<0.01$, for both groups; *post-hoc* analysis). Maternal hypothyroxinemia was without any significant effect on fetal brain GLUT1 expression at any age (Fig. 5). Hybridization analysis of Northern blots of placental total RNA with the GLUT1 cDNA probe revealed no

Figure 2 Effect of maternal hypothyroxinemia on GLUT1 protein expression in placenta. A, Western blot of placental microsomes (5 µg protein) from normal (N) and partially thyroidectomized (TX) rat dams immunodetected with a GLUT1-specific antibody and protein A. Blots also included an adult brain microsomal standard (S; 13 µg protein). Only a portion of the autoradiogram is shown and the migration of molecular weight markers is indicated on the right hand side. Note the presence in placenta of 44, 58 and 64 kDa species. B, Radioactive bands corresponding to 44 kDa GLUT1 and hyperglycosylated forms (58 and 64 kDa GLUT1 together) were excised from filters and counted. Counts were background-subtracted and corrected for filter-to-filter variation using the adult brain standard. Each point is the mean corrected c.p.m. ± S.E.M. of at least six different N (○) or TX (●) dams. Significant treatment effects were found for total transporters ($P<0.02$) and hyperglycosylated forms ($P<0.01$). * $P<0.05$; TX vs N dam at the appropriate age (Fisher's PLSD test).

Figure 3 Effect of maternal hypothyroxinemia on GLUT3 protein expression in fetal brain. A, Western blot of fetal brain microsomes (50 µg protein) from normal (N) and partially thyroidectomized (TX) rat dams immunodetected with a GLUT3-specific antibody and protein A. Blots also included an adult brain microsomal standard (S; 13 µg protein). Only a portion of the autoradiogram is shown and the migration of molecular weight markers is indicated on the right hand side. Note the presence in fetal brain of discrete 44 kDa (cross-reacting actin) and 48 kDa (authentic GLUT3) species. B, Radioactive bands corresponding to 48 kDa GLUT3 were excised from filters and counted. Counts were background-subtracted and corrected for filter-to-filter variation using the adult brain standard. Each point is the mean corrected c.p.m. $\pm$ S.E.M. of seven different N (○) or TX (●) dams. No significant treatment or age-treatment interaction effects were found.

significant age- or treatment-related effects for the 2.7 kb transcript (Fig. 5). The placental 1.6 kb band was of low abundance and appeared invariant, and was therefore not quantified.

Maternal and fetal serum glucose levels

Finally, in a further batch of animals, we examined the influence of maternal hypothyroxinemia on fetal serum glucose levels. Glucose was also determined in maternal serum samples from these pregnancies as well as those used in the studies of GLUT protein and mRNA expression. Maternal glucose levels ($n=12$ dams) were found to be normal in TX dams at 16 dg (6.69 ± 0.31 vs 7.12 ± 0.31 mM), 19 dg (6.75 ± 0.34 vs 6.74 ± 0.18 mM) and 21 dg (6.10 ± 0.38 vs 5.65 ± 0.25 mM). In contrast, fetal serum glucose levels showed significant age-treatment interaction ($P=0.036$; $n=8$ different

Figure 4 Effect of maternal hypothyroxinemia on GLUT3 protein expression in placenta. A, Western blot of placental microsomes (25 µg protein) from normal (N) and partially thyroidectomized (TX) rat dams immunodetected with a GLUT3-specific antibody and protein A. Blots also included an adult brain microsomal standard (S; 13 µg protein). Only a portion of the autoradiogram is shown and the migration of molecular weight markers is indicated on the right hand side. Note the presence in placenta of multiple species; estimated sizes are indicated on the left hand side of the autoradiogram. The 44 kDa species is thought to arise from cross-reaction of the antibody with actin. B, Radioactive bands corresponding to 48 kDa GLUT3 and the collection of slower migrating species (hyperglycosylated forms) were excised from filters and counted. Counts were background-subtracted and corrected for filter-to-filter variation using the adult brain standard. Each point is the mean corrected c.p.m. $\pm$ S.E.M. of at least six different N (○) or TX (●) dams. No significant treatment or age-treatment interaction (though $P=0.053$ for 48 kDa GLUT3) effects were found. * $P<0.05$; TX vs N dam at the appropriate age (Fisher's PLSD test).

pregnancies); *post-hoc* analysis confirmed a significant ($P<0.05$) reduction in serum levels in TX dam pregnancies at 16 dg (1.66 ± 0.13 vs 2.18 ± 0.21 mM), but not at 19 dg (1.54 ± 0.11 vs 1.31 ± 0.15 mM) or 21 dg (4.60 ± 0.40 vs 4.91 ± 0.18 mM).

Figure 5 Effect of maternal hypothyroxinemia on GLUT1 mRNA expression in fetal brain and placenta. Northern blots of fetal brain and placental total RNA were hybridized sequentially with GLUT1 and 18S rRNA probes. Ontogenic profiles for A, fetal brain and B, placenta are shown, where each point is the mean GLUT1/18S signal ratio $\pm$ S.E.M. of at least four different normal (○) or partially thyroidectomized (●) dams. No significant treatment or age-treatment interaction effects were found.

Discussion

Female rats were partially thyroidectomized, then allowed at least 2 weeks recovery before mating. Maternal serum total T4 levels were reduced by 60% before the onset of fetal thyroid hormone synthesis, but total T3 levels were less severely affected (40% reduction); consequently these dams are termed 'hypothyroxinemic'. Though not measured here, maternal serum TSH levels are markedly elevated in this model (Evans *et al.* 1999). Because these TX dams are free from factors such as iodine deficiency and anti-thyroid drugs which directly impinge upon fetal thyroid function, the influence of maternal thyroid dysfunction *per se* on fetoplacental development can be investigated. Thyroid hormone deficits in the fetus will therefore be greatest prior to the onset of fetal thyroid activity at 17–18 dg (Morreale de Escobar *et al.* 1985).

Maternal hypothyroxinemia reduced litter size and impaired fetal growth before the onset of fetal thyroid function, but placental weight remained normal, confirming previous observations in this model (Pickard *et al.* 1993, Evans *et al.* 1999). In fetal brain, the total protein concentration was normal, as previously noted (Pickard *et al.* 1993, Evans *et al.* 1999), but the microsomal protein concentration was deficient at 16 dg. This latter fraction is rather crude, and it is not yet known whether plasma membrane, intracellular membrane and/or mitochondrial constituents are affected. In placenta, deficits in the total

protein concentration were seen at 19 dg, as noted earlier (Pickard *et al.* 1993). Maternal hypothyroxinemia also modified GLUT expression in placenta and fetal brain, independent of changes in tissue protein concentration. Thus, the microsomal abundance of GLUT1 protein was reduced in fetal brain and, to a lesser extent, placenta at 16 dg, whereas the 48 kDa GLUT3 species was increased in placenta at this time. These changes were seen in the absence of changes in maternal serum glucose levels, as expected from studies of severely thyroid-deficient rat dams (Porterfield & Hendrich 1991).

GLUT1 is more widely expressed in rat placenta than GLUT3. It is the only glucose transporter in the junctional zone (site of trophoblast proliferation) and is also expressed in the labyrinth, within the limiting membranes of the syncytial layers (barriers to the free diffusion of glucose across the placenta) (Zhou & Bondy 1993, Boileau *et al.* 1995, Shin *et al.* 1997). Here it acts in concert with GLUT3 to mediate glucose uptake from maternal blood into syncytiotrophoblast layer I, but alone facilitates glucose efflux from syncytiotrophoblast layer II into the fetal circulation (Shin *et al.* 1997). Thus, even though the increase in GLUT3 expression in TX dam placenta at 16 dg may serve to maintain, or even enhance, the intrasyncytial glucose concentration, the fetal glucose supply may be diminished if the GLUT1 deficit is present in syncytiotrophoblast layer II. Indeed, fetal serum glucose levels were reduced at this stage of gestation. Placental GLUT1 deficits may also result in aberrant glucose uptake in the junctional zone, although cytotrophoblast proliferation appears normal in this model (Pickard *et al.* 1993).

The 44 and 51 kDa GLUT1 species detected in fetal brain microsomes are thought to constitute parenchymal transporters (Walker *et al.* 1988, Wang & Brennan 1988, Vannucci 1994). Both forms are markedly deficient at 16 dg in TX dam pregnancies, so that glucose uptake into neuronal and astrocytic cell precursors, choroid plexus epithelial cells or ependymal cells (Bondy *et al.* 1992, Dermietzel *et al.* 1992) may be reduced. Expression of the glucose transporter of differentiating neurons, GLUT3, is largely unaffected in contrast, though fetal hypoglycemia may impact upon cellular glucose uptake throughout the brain at 16 dg. Glucose restriction in early brain development may explain the transient deficit in brain cell number seen in this model (Pickard *et al.* 1993).

In postnatal rats, hypothyroidism diminishes, and T4 administration enhances, blood-brain glucose uptake (Moore *et al.* 1973). Maternal iodine deficiency is also associated with reduced blood-brain glucose uptake in weanling progeny, which can be prevented by feeding an iodine-replete diet from parturition (Sunitha *et al.* 1997). Although blood-brain glucose uptake largely reflects the activity of microvascular GLUT1, our results indicate that parenchymal GLUT1 is regulated in a similar manner during early fetal development. Indeed, thyroid hormone

stimulates glucose transport in astrocytes in culture (Roeder *et al.* 1988).

Maternal hypothyroxinemia had no effect on GLUT1 mRNA levels in fetal brain and placenta, suggesting that changes in GLUT1 protein expression are mediated via translational or post-translational control mechanisms. Although thyroid status influences tissue GLUT1 mRNA in other *in vivo* models, close correspondence between GLUT1 protein and mRNA levels is not always apparent (Weinstein & Haber 1992, Castelló *et al.* 1994). Thyroid hormone may modulate factors which bind to translational control elements in GLUT1 mRNA (Boado *et al.* 1996), the incorporation of GLUT1 protein into membranes or its degradation. Further work is required to explore these possibilities, however maternal hypothyroidism results in deficits in translational efficiency in fetal brain and placenta (Hendrich & Porterfield 1996), and thyroid hormone has been shown to stimulate translation in neuronal cell cultures (Pickard *et al.* 1987).

Maternal hypothyroidism in the first half of rat gestation impairs the biosynthesis of metabolic stores which support fetal growth in late gestation (Bonet & Herrera 1988). Furthermore, the administration of growth hormone, which does not cross the placenta, in late gestation to hypothyroid dams, can improve fetal metabolic deficits, though not consistently (Hendrich *et al.* 1997). These results suggest that maternal hypothyroidism induces maternal metabolic dysfunction. This factor is unlikely to impact in the present study since maternal hypothyroxinemia was induced well before, and not after, mating and was less severe. Indeed, body weights, tissue protein concentrations and serum glucose levels are depressed close to term in fetuses of hypothyroid dams (Morreale de Escobar *et al.* 1985, Bonet & Herrera 1988, Porterfield & Hendrich 1991, Hendrich *et al.* 1997) but were only transiently affected in the present study. It is difficult to envisage how fetal development could normalize if maternal metabolic compromise were responsible, since the latter is likely to worsen as pregnancy progresses and the feto-placental burden increases. Rather, the observation that catch-up occurs soon after the onset of fetal thyroid function favours a more direct influence for the maternal thyroid hormone supply on early fetal and placental development.

Placental weights are depressed in certain hypothyroid rat dam models (Bonet & Herrera 1988, Hendrich & Porterfield 1996) but normal in hypothyroxinemic dams (present study; Pickard *et al.* 1993). Deficits in the total protein concentration are seen however in placenta, but only at 19 dg when the fetal thyroid is already functional and placental GLUT expression has normalized. The reasons for this are unclear, but gross placental dysfunction is unlikely since fetal body weights were normal at this age and remained so closer to term.

In conclusion, maternal hypothyroxinemia in the rat results in fetal growth retardation and impaired brain

development before the onset of fetal thyroid hormone synthesis. Glucose uptake into brain parenchyma may be compromised both directly, due to deficient GLUT1 expression in this tissue, and indirectly, as a consequence of fetal hypoglycemia arising from reduced placental GLUT1 expression. Although reversed following the onset of fetal thyroid function, these effects occur during the critical period of blast cell proliferation and may therefore underlie the long term changes in brain development and function noted in this model (Pickard *et al.* 1993, 1997, Evans *et al.* 1999). Maternal hypothyroxinemia in human pregnancy is also associated with cognitive and motor dysfunction in offspring in the absence of change in fetal and placental weights at term (Man *et al.* 1991). Findings in the hypothyroxinemic rat dam model indicate that this brain dysfunction may arise from impaired brain development before active fetal thyroid hormone secretion, when both the fetus and the placenta are dependent upon the maternal circulation for their thyroid hormone supply.

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