

Maternal nutrient deprivation induces sex-specific changes in thyroid hormone receptor and deiodinase expression in the fetal guinea pig brain

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Thyroid hormone deprivation during fetal life has been implicated in neurodevelopmental morbidity. In humans, poor growth *in utero* is also associated with fetal hypothyroxinaemia. In guinea pigs, a short period (48 h) of maternal nutrient deprivation at gestational day (gd) 50 results in fetuses with hypothyroxinaemia and increased brain/body weight ratios. Thyroid hormone action is mediated by nuclear thyroid hormone receptors (TRs) and is dependent upon the prereceptor regulation of supply of triiodothyronine (T₃) by deiodinase enzymes. Examination of fetal guinea pig brains using *in situ* hybridization demonstrated widespread expression of mRNAs encoding TR α 1, α 2 and β 1, with regional colocalization of deiodinase type 2 (D2) mRNA in the developing forebrain, limbic structures, brainstem and cerebellum at gd52. With maternal nutrient deprivation, TR α 1 and β 1 mRNA expression was significantly increased in the male, but decreased in the female fetal hippocampus and cerebellum and other areas showing high TR expression under euthyroid conditions. Maternal nutrient deprivation resulted in elevated D2 mRNA expression in males and females. Deiodinase type 3 (D3) mRNA expression was confined to the shell of the nucleus accumbens, the posterior amygdalohippocampal area, brainstem and cerebellum, and did not change with maternal nutrient deprivation. In conclusion, maternal nutrient deprivation resulted in sex-specific changes in TR mRNA expression and a generalized increase in D2 mRNAs within the fetal brain. These changes may represent a protective mechanism to maintain appropriate thyroid hormone action in the face of fetal hypothyroxinaemia in order to optimize brain development.

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Thyroid hormones play an important role in the development of the mammalian central nervous system (CNS), and evidence suggests that this influence extends back into fetal life (Calvo *et al.* 1990, 2002). Studies in humans have shown that mild maternal hypothyroidism in early pregnancy is associated with adverse neuropsychological outcomes in children (Haddow *et al.* 1999; Pop *et al.* 2003), reflecting the sensitivity of the fetal CNS to changes in thyroid status.

Intrauterine growth restriction (IUGR), with fetal brain weight maintained relative to body weight, is often secondary to malplacental syndromes. IUGR babies contribute significantly to perinatal mortality

and childhood morbidity, including neurodevelopmental delay (Gaffney *et al.* 1994; Kok *et al.* 1998). Cordocentesis studies show that circulating thyroxine (T₄) and triiodothyronine (T₃) concentrations are significantly reduced in human fetuses with IUGR (Thorpe-Beeston *et al.* 1991; Kilby *et al.* 1998). In this connection, hypothyroxinaemia may be a protective mechanism for maintaining viability in IUGR by reducing metabolic demands, and hence oxygen consumption, at the expense of disrupting neurodevelopment (Thorpe-Beeston & Nicolaidis, 1996). Often in severe, early onset intrauterine growth restriction there is vascular redistribution of blood flow, sparing of fetal brain growth and utilization

of glycogen stores within the fetal liver. However, CNS abnormalities have been reported, and in animal models of IUGR (Mallard *et al.* 1998) these abnormalities are similar to those described in thyroid hormone deficiency (Balazs, 1971). This observation has led to the hypothesis that fetal hypothyroxinaemia contributes to the pathogenesis of the neurodevelopmental impairment in IUGR.

Guinea pigs, like humans, show extensive neuro-endocrine maturation and rapid brain growth in late fetal life (Dobbing & Sands, 1979; Matthews, 1998). Maternal nutrient deprivation (MND) in guinea pigs results in fetuses with increased brain/body weight ratios and modified fetal endocrine function, including a significant reduction in circulating T_4 concentrations (Lingas *et al.* 1999). In human maternal undernutrition, similar constraints on fetal growth have been reported (Lumey, 1992) and this has been linked to increased cardiovascular, metabolic and endocrine morbidity in adult life (Barker *et al.* 1989; Barker, 1999). These similarities therefore make the guinea pig a suitable animal model (and human analogue) with which to study the effects of MND on the role of thyroid hormone in fetal brain development.

Thyroid hormone effects are mediated by the expression of thyroid hormone receptors (TRs) and are dependent on the local delivery of the active ligand, T_3 , determined in part by enzymes that metabolize thyroid hormones, especially the iodothyronine deiodinases (Bianco *et al.* 2002). The TR isoforms $\alpha 1$, $\beta 1$ and $\beta 2$ are nuclear transcription factors which bind T_3 to regulate the transcription of thyroid hormone responsive genes (Yen, 2001). Such genes include neurogranin and myelin basic protein, expression of which is reduced in the developing CNS in hypothyroxinaemia (Ibarrola & Rodriguez-Pena, 1997; Dowling & Zoeller, 2000). In addition, the non-ligand binding TR $\alpha 2$ isoform modulates the function of other TRs (Koenig *et al.* 1989).

The major circulating thyroid hormone is the prohormone, T_4 , which is converted to T_3 by deiodinase type 1 (D1) or type 2 (D2) (Visser *et al.* 1983). Deiodinase type 3 (D3) is responsible for the inactivation of T_4 and T_3 to inactive metabolites (Kaplan & Yaskoski, 1980). Deiodinases are prereceptor regulators of local thyroid hormone action, and their actions may potentially compensate for changes in concentrations of circulating thyroid hormone in disease states (Visser *et al.* 1983; Visser, 1996). Evidence from *in vivo* and *in vitro* studies has suggested that D2 and D3 both participate in the homeostatic regulation of local T_3 concentrations in the mammalian CNS with findings of D2 up-regulation and D3 down-regulation associated with hypothyroidism (Cavaliere *et al.* 1986; Ruiz de Ona *et al.* 1991; Guadano-Ferraz *et al.* 1999; Tu *et al.* 1999).

This study was designed to identify thyroid hormone responsive regions in the fetal guinea pig brain and to demonstrate the localization of deiodinase subtypes. This

involved partial sequencing of the guinea pig genes for the TR isoforms and the deiodinases. We hypothesized that maternal nutrient deprivation, which is known to induce hypothyroxinaemia and IUGR (Lingas *et al.* 1999), will result in modification of TR expression and compensatory changes in deiodinase expression in the fetal brain.

Methods

Animals and treatments

Animal breeding and treatments were performed at the University of Toronto according to protocols approved by the Animal Care Committee and in accordance with the Canadian Council for Animal Care as previously described (Lingas *et al.* 1999). Pregnant guinea pigs were deprived of all food for 48 h on gestational day (gd) 50 ($n = 7$) or allowed to feed normally (control, $n = 5$) (term = 70 days). Water was available *ad libitum*. On gd52 (after 48 h treatment), pregnant guinea pigs were killed by decapitation. Fetuses were quickly removed and the brain dissected and stored at -80°C until processing.

As published previously (Lingas *et al.* 1999), plasma T_4 concentrations were similar in control and deprived mothers but fetal T_4 concentrations were significantly lower in the nutritionally deprived group (control 226.7 ± 20.3 ng ml $^{-1}$; deprived 112.6 ± 13.4 ng ml $^{-1}$; $P < 0.001$). We were unable to determine plasma T_3 concentrations due to very limited plasma volume. The fetal weights were significantly different (control 44.18 ± 1.22 g; deprived 40.13 ± 0.63 g; $P < 0.01$) and the brain/body weight ratio was significantly higher in the deprived group (control 0.0438 ± 0.001 ; deprived 0.0488 ± 0.0006 ; $P < 0.001$) while the weights of the thyroid gland were not different. Normal litter size is two to three fetuses, and where possible both male and female fetuses were taken from each litter for subsequent brain analysis (control: male $n = 5$, female $n = 8$; deprived: male $n = 8$, female $n = 8$).

Gene sequencing and probe design

Partial sequencing of each of the mRNAs encoding the guinea pig TR isoforms and deiodinase subtypes was undertaken using polymerase chain reaction (PCR)-based techniques previously described (McCabe *et al.* 1999). Briefly, guinea pig tissue (liver, brain striatum or diaphragmatic skeletal muscle; 100 mg) was homogenized, total RNA prepared using a one-step guanidinium phenol-chloroform extraction method (Tri-reagent, Sigma-Aldrich UK) and reverse transcribed using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA), to yield cDNAs for use as templates. Degenerate primers were designed

Table 1. Probe sequences used for each thyroid hormone receptor (TR) isoform and deiodinase subtype for *in situ* hybridization and the close sequence homology it has to other species; the periods over which slides of the forebrain and brainstem/cerebellum were exposed to the radiographic film are also indicated

Gene	Bases of coding sequence*	<i>In situ</i> hybridization probe (45 bases)	Percentage comparative homology to				Exposure times required	
			Human	Rat	Mouse	Pig	Fore-brain	Brainstem/Cerebellum
TR α 1	1136–1180	TCATGTGGAGGAAGCGGCTGG CGTGGCAGGCCCGATCATGCGGA	100	100	100	100	3w	6w
TR α 2	1112–1156	CTGCCGCTGCCCCCTGTACAG AATCGAACTCTGCACTTCTCT	100	100	100	100	4d	7d
TR β 1	493–537	TCTCTTCTGTTTGGAGGTCTG AGCACTAGAGATGCTCTGATCAT	90	—	88	92	4w	5w
TR β 2	156–200	CCAGGGTAACTACAGGTATAAG GCTGATCACTGCCAGGCCTGT	100	93	100	—	6w	—
D1	215–259	CAGATGGTGCCTTCTCTCTCTG AGAGGCAAACCACCGAGCAGTCT	80	—	88	—	—	—
D2	125–169	GGTCAGCATGCGCCGCACTCTCC GCGAGTGGACTTGGAGCGGCT	100	97	100	—	4w	6w
D3	29–73	GCGGGAACAGCACGAGGCAC GAGGCGGTCTGGGCGCAGAGCCTCA	97	100	100	—	8w	6w

*From ORF of human gene.

from human sequences, aimed at amplifying regions which included unique sequences of each TR isoform and deiodinase subtype. (Primer Express programs from DNA Star (Madison, USA) and Applied Biosystems (USA) Mac software.)

The products from the PCR performed using Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany) were separated by electrophoresis using 2% Tris-acetate-EDTA (Eppendorf AG, Hamburg, Germany) agarose gels. Fragment sizes were gauged in relation to a 1 kb pair DNA mass ladder (Pharmacia, Uppsala, Sweden). PCR products of the expected sizes for each gene were purified after gel excision and eluted using QIAQuick gel purification kits (Qiagen, Hilden, Germany). The PCR products (50–100 ng) were used in each sequencing reaction, which consisted of the Big Dye cycle sequencing kit (3 μ l) containing AmpliTaq (Perkin Elmer Applied Biosystems, Foster City, CA, USA) and 3.2 pmol of the respective forward or reverse primer with annealing temperatures set as for the PCR reactions for each gene. PCR fragments encompassing over 80% of the cDNA were sequenced in both directions on an ABI 377 sequencer and electropherograms analysed using DNASTar software.

The sequenced guinea pig gene fragments showed considerable homology with the human: TR α 1 (100%), TR α 2 (99%), TR β 1 (90%) and D1 (86%). The resulting partial sequences were used to design a 45 base oligonucleotide probe specific for the guinea pig mRNA for each gene (Sigma-Genosys, Mississauga, Ontario, Canada). Attempts at sequencing guinea pig D2 and D3 were unsuccessful while TR β 2 was not sequenced. Therefore

for these three genes, consensus sequences derived from several different vertebrate species (rat, mouse, chicken, human, killifish, *Xenopus*; sequences from NCBI PubMed database) were used to design the antisense probes.

A BLAST search (NCBI PubMed) was performed for each oligonucleotide probe sequence to ensure there was no significant resemblance to other sequenced genes. The probe sequences chosen were also highly homologous to other known mammalian sequences for each gene (Table 1). A sense probe with a similar G and C content was used as control. Preliminary experiments on guinea pig tissues known to express specific TRs and deiodinases revealed a positive expression for each probe, while no hybridization was seen with the control probe. (TR α 1, TR α 2, TR β 1, TR β 2 in the CNS; D1 in kidney and liver; D2 in the anterior pituitary gland; D3 in placenta; not illustrated.)

In situ hybridization (ISH)

The methods used have been described in detail previously (Matthews & Challis, 1995). Coronal cryosections (12 μ m) of fetal brain (ranging from the bregma to the posterior hippocampus, and the lambda to the posterior lobe of the cerebellum) were mounted onto poly L-lysine coated slides and postfixed with 4% paraformaldehyde. The probes were labelled using terminal deoxynucleotidyl transferase (Gibco BRL, Burlington or Pharmacia LKB, Ontario, Canada) and ³⁵S-labelled deoxyadenosine 5'-(α -thio)triphosphate (³⁵S-dATP; 1300 Ci mmol⁻¹; Du Pont, Canada) to a

specific activity of $0.7\text{--}1.0 \times 10^9$ c.p.m. μg^{-1} . Labelled probe in hybridization buffer was applied to slides at a concentration of $250\text{--}500$ c.p.m. μl^{-1} . Slides were incubated overnight in a moist chamber (42°C). After several washes with SSC, sections were dehydrated in ethanol and dried. Slides were exposed to autoradiographic film (Biomax, Kodak) together with sections incubated with the sense probe and ^{14}C -standards (American Radiochemical, MO, USA). The latter were used to ensure that analysis was undertaken in the linear range of the autoradiographic film. Exposure times are listed in Table 1.

Data analysis

Sections from the forebrain and brainstem/cerebellum (BS/CB) were hybridized separately. However, for each gene in either the forebrain or BS/CB, sections were processed simultaneously to allow direct comparison between the control and deprived groups. The relative optical density (ROD) of the signal on autoradiographic film was quantified, after subtraction of background values, using a computerized image analysis system (Imaging Research, St Catharines, Ontario, Canada) (Matthews & Challis, 1995; Lingas *et al.* 1999).

In the forebrain, measurements were undertaken at the level of the anterior hippocampus (Figs 1 and 2) for the following areas: the cingulate cortex (Cg), cerebral cortex (Cx; all layers measured together, excluding piriform cortex), hippocampus (CA1/2, CA3 and CA4 subfields measured separately), upper blade of the dentate gyrus (DG). The lateral ventricles/shell of the accumbens nuclei (LV/ACBsh) and caudate putamen were measured from more cranial sections. The LV/ACBsh were measured together as they were not distinguishable on film. Measurements of the amygdala (Am) were taken where there was detectable expression. D3 mRNA was only measured in the LV/ACBsh, the capsule of the amygdalohippocampal nucleus (AHiPM) and posteromedial cortical amygdaloid nucleus (PMCo).

In the brainstem and cerebellum, measurements were taken at the inferior colliculus (IC), pontine nuclei (PN), the brainstem as a whole (BS; average of readings at three different levels: caudal to the IC, caudal to the pons, lower medulla at the level of the dorsomotor nucleus of the vagus (DM)) and the cerebellum as a whole (CB; average of readings at the same three levels). In addition, TR α 1 and TR α 2 signals were quantified in the lateral areas of the brainstem (LatBS), which includes the cochlea nucleus (CN). D2 mRNA levels were also measured in the vestibular nuclei (VN), CN (average of ventral and dorsal areas) and cerebellar flocculus (FL). D3 mRNA was measured in the brainstem and cerebellum, each as a whole, at the level caudal to the pons.

Statistical methods

Group data were statistically analysed using a three-way ANOVA (treatment (MND) \times sex \times region) followed by Duncan's *post hoc* analysis. As this revealed differences between the sexes for some genes (detailed in results), we subsequently analysed the data using (1) a two-way ANOVA (sex \times region) to detect differences in localization within the control group and (2) a two-way ANOVA (treatment \times region) to detect the effects of MND within each sex separately. The ANOVA was followed by Bonferroni's method of all pair wise *post hoc* multiple comparisons. Group data are presented as means \pm s.e.m. and statistical significance was set at $P < 0.05$.

Results

Localization of TR and deiodinase mRNA in fetal guinea pig brain

Forebrain. There were marked regional differences in the expression of mRNA transcripts encoding all the TR isoforms ($P < 0.001$) and D2 ($P < 0.001$). TR α 1, TR α 2 and TR β 1 mRNAs were widely expressed throughout the cerebral (Cx) and cingulate (Cg) cortices, LV/ACBsh and caudate putamen (CP) (Figs 1 and 3). Overall TR β 2 mRNA expression was more limited, with expression confined to the Cx, Cg and hippocampus. Expression of each TR isoform was highest in the hippocampus. The TR α isoforms were most abundantly expressed in the CA1–3 subfields with moderate expression in CA4 and DG, while the TR β isoforms were predominantly expressed in CA1/2 with very low expression in the other subfields and DG.

The expression of D2 transcripts was localized to the primary regions of TR expression, though detailed cellular colocalization studies were not undertaken. Highest levels of D2 mRNA were detected in the cingulate cortex (Cg) with more moderate expression in the hippocampus (Figs 2 and 4). D3 expression was confined to the shell of the nucleus accumbens (ACBsh), capsule of the amygdalohippocampal nucleus (AHiPM) and posteromedial cortical amygdaloid nucleus (PMCo) and to the lateral ventricle (LV). No expression was observed in the cortex or hippocampus. There was no D1 mRNA expression in any region of the forebrain.

Posterior midbrain, brainstem and cerebellum. There were marked regional differences in the expression of mRNA encoding the TR α 1, α 2, β 1 isoforms ($P < 0.001$), D2 ($P < 0.001$) and D3 ($P < 0.01$). These three TR isoforms were expressed in the inferior colliculus (IC), pontine nuclei (PN), dorsomotor nuclei of the vagus (DM), within various regions of the brainstem and diffusely within the cerebellum, with slightly higher expression in the Purkinje cell layers (Figs 1 and 5).

Within the brainstem, the highest levels of TR α 1 and α 2 transcripts were identified in the lateral regions (Lat BS; which include the cochlea and vestibular nuclei) while TR β 1 mRNA was highest in the pontine nuclei (Figs 1 and 5). There was strong expression of D2 mRNA in the inferior colliculus (IC), pontine nuclei (PN), the ventral and dorsal cochlea nuclei (CN), vestibular nuclei (VN) and the cerebellum (especially the flocculus). D3 mRNA expression was more diffuse throughout the brainstem

and cerebellum, with no particular nuclei distinguishable (Figs 2 and 6). There was no TR β 2 or D1 mRNA expression in the BS/CB.

The effect of maternal nutrient deprivation (MND) on TR and deiodinase mRNA levels

Thyroid hormone receptors in the forebrain. There was a trend for increased TR α 1 mRNA expression in males

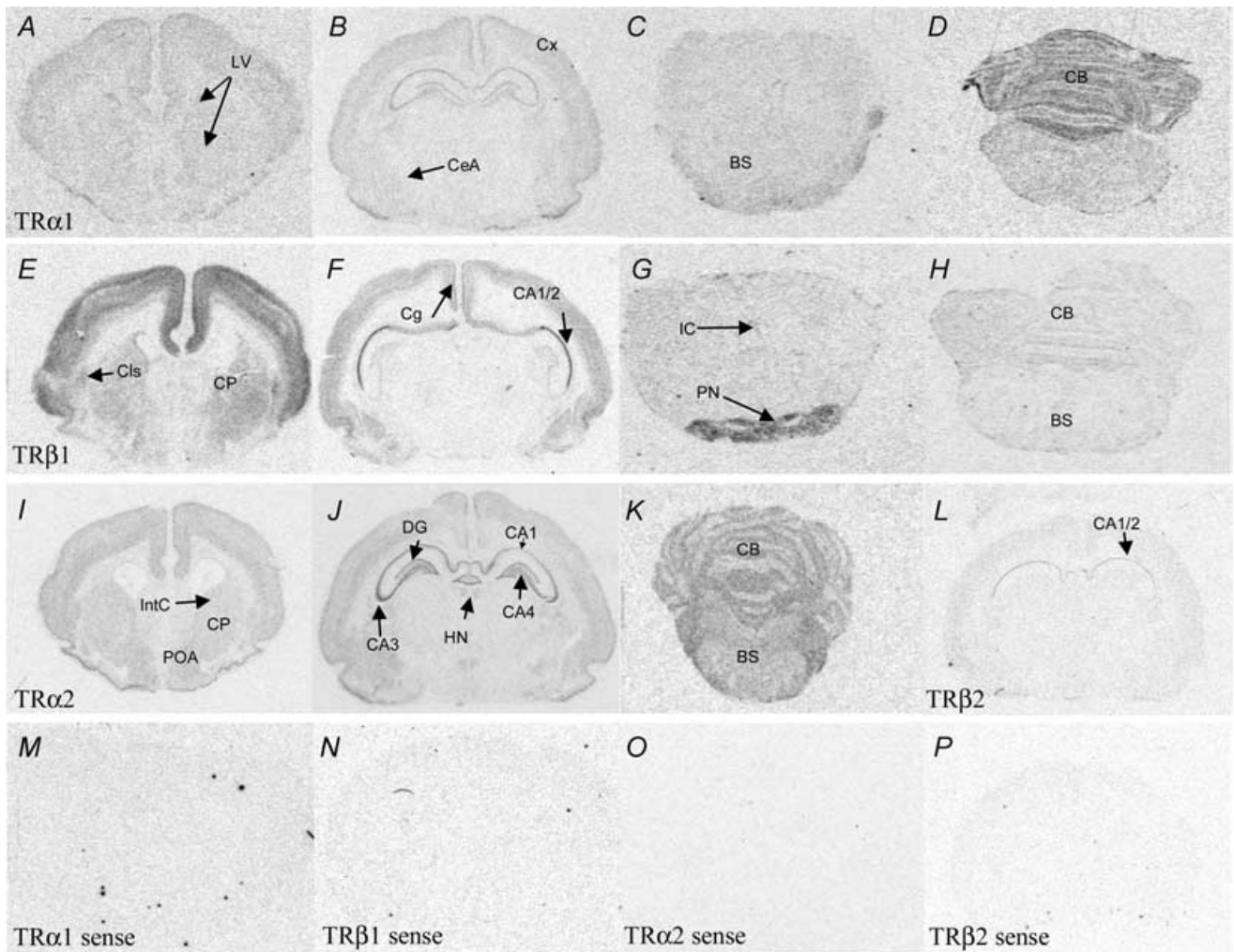


Figure 1. Representative coronal sections illustrating relative expression patterns of mRNA encoding for TR α 1 (A–D), TR β 1 (E–H), TR α 2 (I–K) and TR β 2 (L) in the brain, following a rostral caudal gradient from the forebrain to the brainstem (note: not all areas illustrated)

TR α 1 mRNA expression was detected in the lining of the lateral ventricles (LV), cortex (Cx) hippocampal formation (CA1/2 and DG; see F) and central nucleus of the amygdala (CeA). Very low levels were detected in the brainstem (BS), while relatively high levels were observed in the cerebellum (CB). TR β 1 mRNA was highly expressed in the cerebral cortex, claustrum (Cls) and caudate putamen (CP). Specific expression was detected in the cingulate cortex (Cg) and the CA1/2 subfield of the hippocampus. High expression was observed in the pontine nuclei (PN) with low expression in the rest of the brainstem and cerebellum (BS/CB). TR α 2 mRNA was expressed throughout the frontal cortex, including the internal capsule (IntC), caudate putamen (CP) and preoptic areas (POA). High levels were found in the hippocampal formation (CA1–4), dentate gyrus (DG) and habenular nuclei (HN). Low levels were detected in the brainstem (BS) with higher expression in the cerebellum (CB). TR β 2 mRNA was expressed at low levels in the cerebral cortex and the CA1/2 subfield of the hippocampus. As a negative control, sense probes for all thyroid hormone receptors were made. Sections hybridized with sense probes for TR α 1 (M), TR β 1 mRNA (N), TR α 2 (O) and TR β 2 mRNA (P); no signal was detected.

and reduced expression in females when MND fetuses were compared with controls (three-way ANOVA: effect of sex $P < 0.01$; Fig. 3A). However, subsequent *post hoc* analysis revealed that the differences were not regionally specific in either males or females. Overall, MND did not significantly modify TR α 2 mRNA levels compared with controls in either male or female fetuses (Fig. 3B). TR β 1 mRNA expression in the control fetuses was higher in females than in males (effect of sex $P < 0.01$; effect of sex \times region $P < 0.001$) with *post hoc* analysis revealing significantly higher expression in the CA1/2 subfield of the hippocampus of female control fetuses compared with male control fetuses ($P < 0.001$; Fig. 3C). MND significantly modified TR β 1 mRNA levels (three-way ANOVA: effect of sex \times treatment $P < 0.001$; Fig. 3C) with different responses observed between male and female fetuses. In male fetuses (two-way ANOVA: effect of treatment not significant), *post hoc* analysis revealed a significant increase in TR β 1 mRNA in the CA1/2 hippocampal subfield ($P < 0.01$) with MND. In female fetuses (two-way ANOVA: effect of treatment $P < 0.001$; effect of treatment \times region $P < 0.001$) there was a significant reduction in TR β 1 mRNA expression in the CA1/2 hippocampal subfield ($P < 0.001$) with MND compared with control. Maternal nutrient deprivation had no effect on TR β 2 mRNA expression in either male or female fetuses (Fig. 3D).

Deiodinases in the forebrain. D2 mRNA expression was higher in female controls than in male controls ($P < 0.001$), with significantly greater D2 mRNA expression in the dentate gyrus (DG; $P < 0.05$) and corpus callosum ($P < 0.001$) of female controls compared to male control fetuses (Fig. 4A). MND caused a significant widespread increase in D2 mRNA expression in both male and female fetuses (effect of treatment $P < 0.001$; Fig. 4A). In male fetuses, there was a significant increase of D2 transcripts particularly in the cingulate cortex (Cg; $P < 0.01$), CA1/2 ($P < 0.05$), CA3 ($P < 0.05$), CA4 ($P < 0.05$) subfields of the hippocampus and dentate gyrus (DG; $P < 0.05$) with MND compared with control. In female fetuses, significant increases were detected in the region of the lateral ventricle and shell of the nucleus accumbens (LV/ACBsh; $P < 0.05$) following MND. MND had no effect on D3 mRNA expression in either males or females (Fig. 4B).

Thyroid hormone receptors in the posterior midbrain, brainstem and cerebellum. Basal TR α 1 mRNA expression in the control fetuses was higher in females than in males ($P < 0.001$) with significantly higher TR α 1 mRNA expression in the pontine nuclei ($P < 0.05$), lateral brainstem ($P < 0.05$) and cerebellum ($P < 0.05$) in female control fetuses compared with male control fetuses (Fig. 5A). There was increased TR α 1 mRNA expression

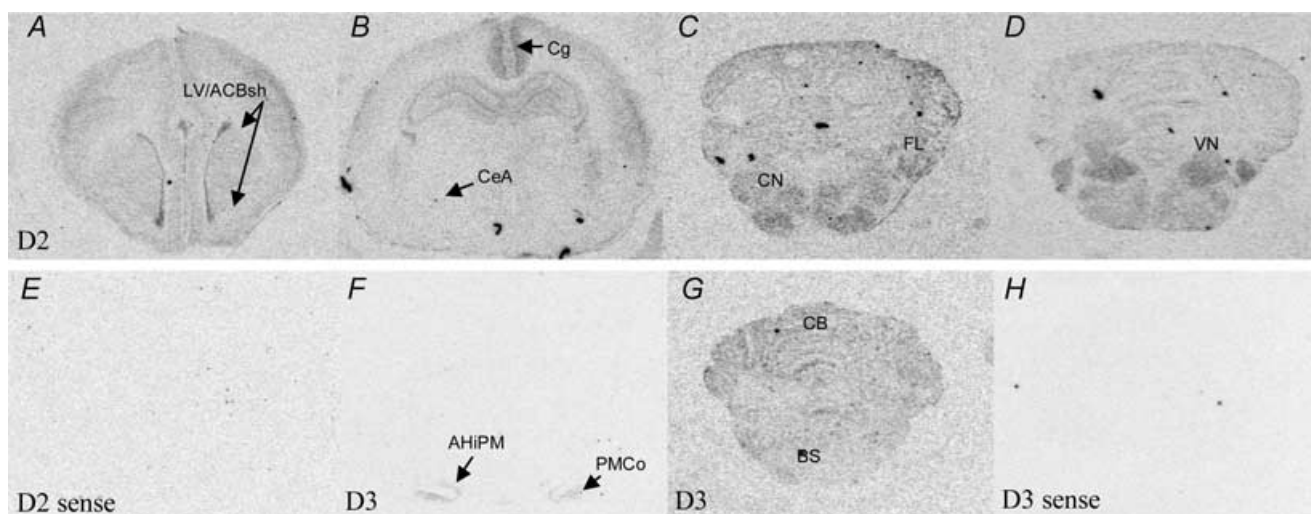


Figure 2. Representative coronal images of brain sections illustrating relative expression patterns of mRNA encoding deiodinase type 2 (D2) following a rostral-caudal gradient from the forebrain to the brainstem (note: not all areas illustrated)

D2 mRNA (A–D) expression was detected in the lining of the lateral ventricles (LV) and shell of the nucleus accumbens (ACBsh), cerebral cortex, cingulate cortex (Cg) and hippocampal formation, with low levels in the central nucleus of amygdala (CeA). D2 mRNA was expressed throughout the brainstem and cerebellum with particularly high levels being detected in the cochlear nuclei (CN), cerebellar flocculus (FL), and vestibular nucleus (VN). Deiodinase type 3 mRNA (D3, F and G) was found specifically in the capsule of the amygdalohippocampal nucleus (AHIPM) and posteromedial cortical amygdaloid nucleus (PMCo) in the forebrain but was more diffusely expressed in the brainstem (BS) and cerebellum (CB). As a negative control, sense probes for the deiodinase subtypes were made. Sections hybridized with sense probes for D2 (E) and D3 mRNA (H); no signal was detected.

in male fetuses and reduced expression in the females with MND compared with control fetuses (effect of sex \times treatment $P < 0.00001$; Fig. 5A). *Post hoc* analysis showed a significant increase in TR α 1 mRNA expression in the lateral brainstem (Lat BS; $P < 0.01$) and cerebellum (CB; $P < 0.01$) in male fetuses, while in female fetuses there was a significant reduction in TR α 1 mRNA expression in the pontine nuclei (PN; $P < 0.01$), the brainstem (BS; $P < 0.05$) and cerebellum (CB; $P < 0.05$). Overall, there was a general trend of increased TR α 2 mRNA in male fetuses but reduced expression in the females with MND compared with control fetuses (effect of sex \times treatment $P < 0.05$; Fig. 5B) but when male and female fetuses were considered separately, *post hoc* analysis failed to reveal any statistically significant differences in all the regions measured.

When both sexes were considered together, MND had a significant effect overall on TR β 1 mRNA levels ($P < 0.05$; Fig. 5C). However, when the sexes were analysed separately, there was no significant differences seen for the effect of MND but *post hoc* analysis did show a significant reduction in the expression of TR β 1 mRNA in the pontine nuclei ($P < 0.05$) with MND compared with control in female fetuses only.

Deiodinases in the posterior midbrain, brainstem and cerebellum. Maternal nutrient deprivation resulted in a widespread significant increase in D2 mRNA expression in both male and female fetuses ($P < 0.0001$; Fig. 6A) with a more marked increase in female fetuses compared with male fetuses (three-way ANOVA: effect of sex \times treatment $P < 0.01$). In male fetuses, even though the overall effect of MND was significant ($P < 0.05$), no statistically significant differences were found in specific regions. In contrast, in female fetuses, the overall effect of MND was highly significant ($P < 0.001$) with significant increases in D2 transcripts seen in the inferior colliculus (IC; $P < 0.001$), pontine nuclei (PN; $P < 0.01$), vestibular nuclei (VN; $P < 0.01$), cochlea nuclei (CN; $P < 0.01$) and flocculus (FL; $P < 0.001$) in fetuses from nutrient deprived mothers compared with control. Of note, MND had no effect on D2 mRNA expression within the cerebellum in male or female fetuses. Though there was a trend towards a reduction in D3 mRNA levels with MND this failed to reach statistical significance (three-way ANOVA: effect of treatment $P = 0.06$; two-way ANOVA: not significant for both sexes; Fig. 6B).

Discussion

This is the first study to have simultaneously determined the developmental profile of both the TR isoforms and deiodinases in the fetal brain of any species. This study has localized and defined the relative levels of mRNAs

encoding specific TR isoforms and deiodinase subtypes in fetal guinea pig brain during the phase of rapid brain growth (gd50–52). We also describe for the first time sex-specific expression of TRs and deiodinases in the brain during development, as well as profound effects of maternal nutrient deprivation (MND) on their expression.

The widespread yet regionally specific expression patterns of the TR isoforms and deiodinase subtypes in the fetal guinea pig brain indicate they may play important and specific roles in neurodevelopment. The distribution of TR isoforms at gd50 in the guinea pig is consistent with that described in the neonatal rat brain (Mellstrom *et al.* 1991), which is in line with the notion that maximal brain growth in the guinea pig occurs at gd50, whereas it does not occur until post-natal days 5–8 in the rat (Dobbing & Sands, 1979). As a generalization, the thyroid hormone-responsive areas of the brain (i.e. those expressing TRs) also expressed D2. The D2 enzyme contributes significantly to the local supply of the active ligand, T₃, in the CNS (Crantz *et al.* 1982). These areas also correspond to brain structures implicated in human neurobehavioural deficits (learning and memory (hippocampus), attention (caudate), visuospatial and auditory abilities and cognition (cerebral cortex), locomotor coordination (cerebellum)) observed in children exposed to hypothyroxinaemia early during the course of development (Rovet, 1999).

D2 is the most widely expressed of the three deiodinase subtypes in the fetal guinea pig brain, and this is consistent with reports of widespread expression of D2 transcripts in the neonatal rat brain (Guadano-Ferraz *et al.* 1997). However, we did not identify high levels of D2 mRNA in the lining of the third ventricle and the median eminence as reported previously in the neonatal (Guadano-Ferraz *et al.* 1997) and adult (Tu *et al.* 1997) rat. It is possible that D2 expression in these areas occurs after gd52 in the guinea pig. In this study, we localized D2 and D3 expression within the lateral ventricles, and these enzymes may have a role in the regulation of thyroid hormone supply to the CNS from the circulation via the choroid-plexus (Chanoine *et al.* 1992; Verhoelst *et al.* 2004). The highly localized pattern of D3 mRNA expression to specific limbic structures in the fetal guinea pig brain has also been reported in the neonatal rat (Escamez *et al.* 1999). This pattern of expression contrasts with more extensive D3 mRNA distribution described in the adult rat brain (Tu *et al.* 1999), perhaps further indicating a targeted protective role for D3 in the developing brain.

The absence of D1 activity in both human fetal and adult brains indicates that this subtype is unlikely to play a significant role in the human CNS (Chan *et al.* 2002), and the present findings suggest this may also be the case

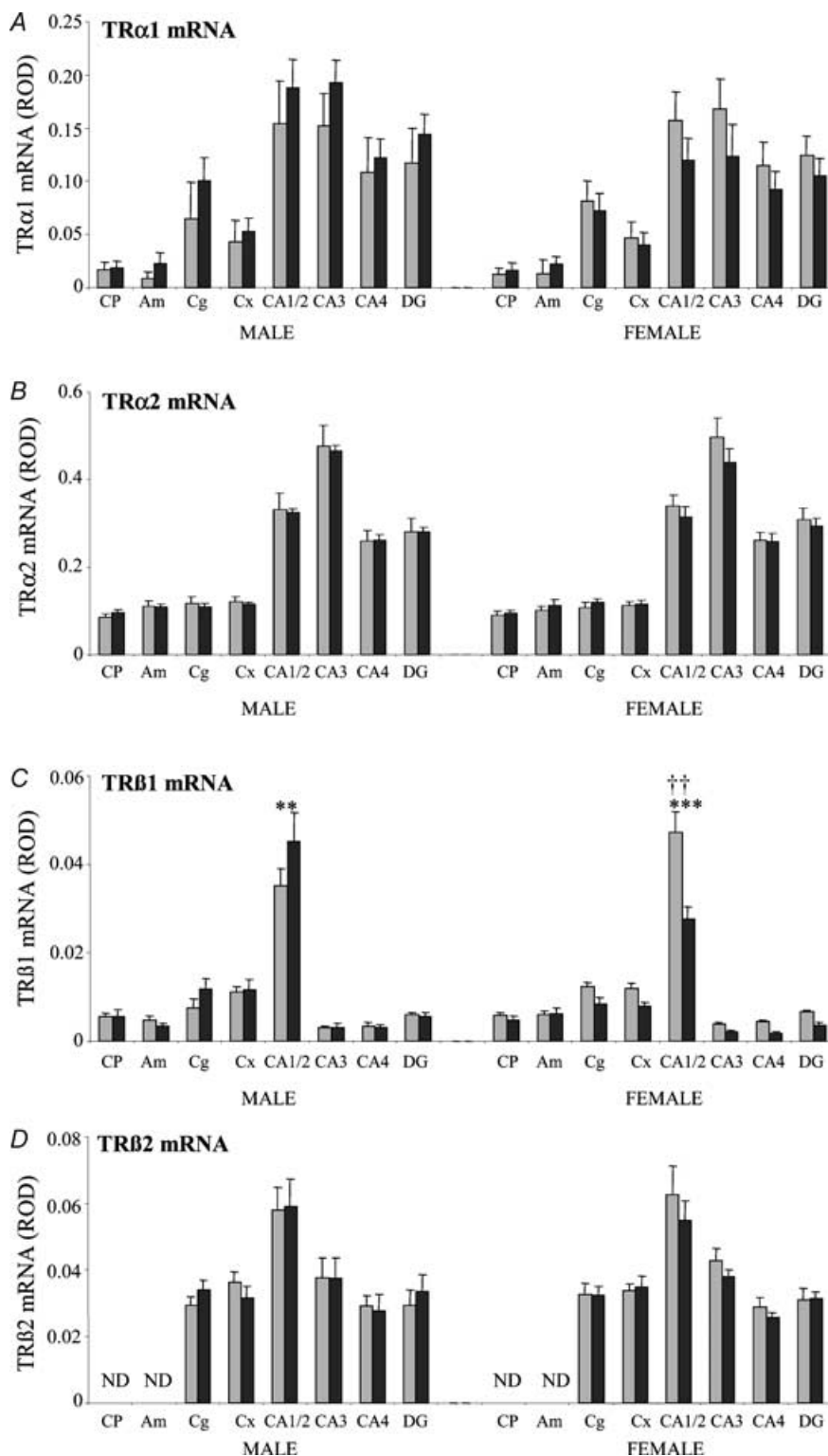


Figure 3. Densitometric analysis of expression of mRNAs encoding the TR isoforms (A: TR α 1; B: TR α 2; C: TR β 1; D: TR β 2) in the forebrain and limbic structures in control animals and after 48 h of nutrient deprivation in male (control (grey) $n = 5$, deprived (black) $n = 8$) and female fetuses (control $n = 8$, deprived $n = 8$)

Results are expressed as mean \pm s.e.m. relative optical density (ROD). Statistical significance is indicated as: ** $P < 0.01$, *** $P < 0.001$. $\dagger\dagger$ indicates a significant difference between males and females in the control group. CP, caudate putamen; Am, amygdala; Cg, cingulate cortex; Cx, cerebral cortex; CA1/2, CA3, CA4 (hippocampal subfields); DG, upper blade of the dentate gyrus. ND, expression not detected.

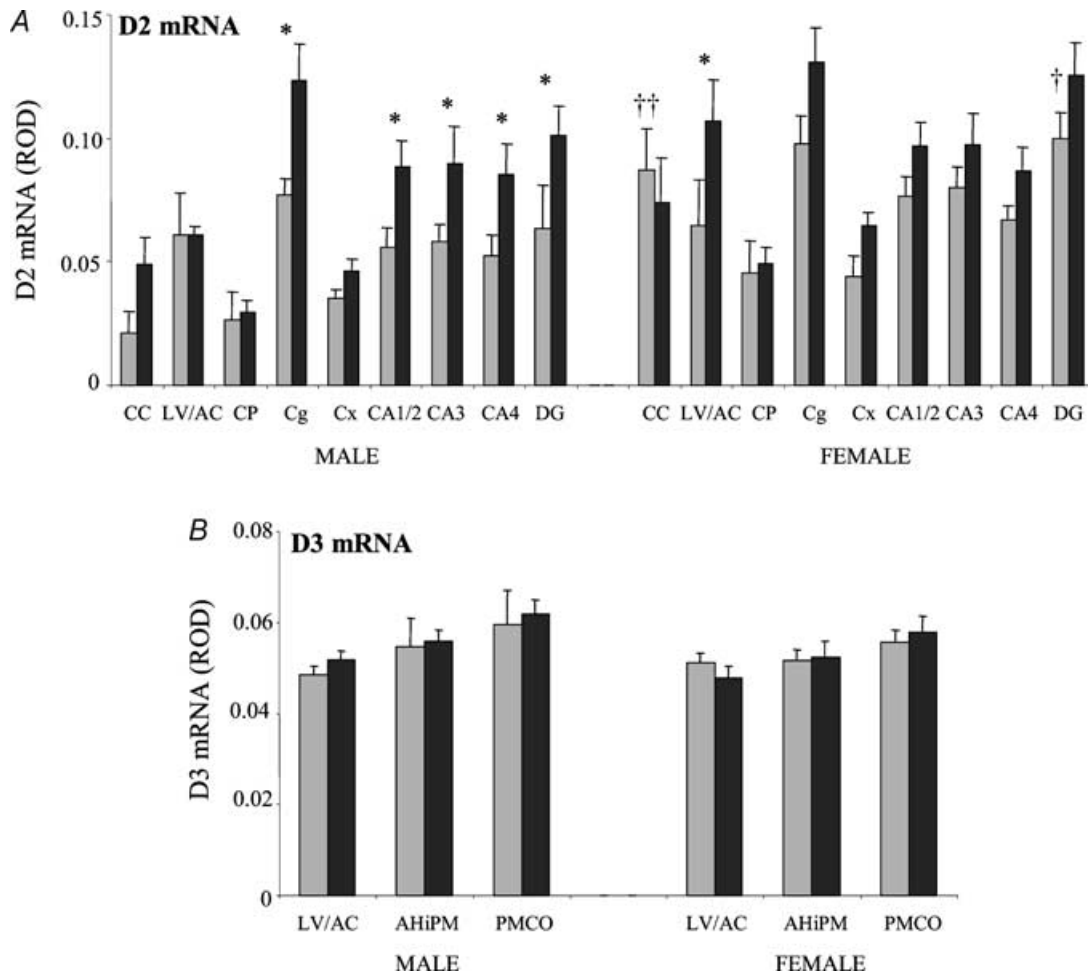


Figure 4. Densitometric analysis of expression of mRNAs encoding the iodothyronine deiodinase type 2 (D2; A) and type 3 (D3; B) in the forebrain and limbic structures in control animals and after 48 h of nutrient deprivation in male (control (grey) $n = 5$, deprived (black) $n = 8$) and female fetuses (control $n = 8$, deprived $n = 8$)

Results are expressed as mean \pm s.e.m. relative optical density (ROD). Statistical significance is indicated as: * $P < 0.05$. $\dagger\dagger$ indicates a significant difference between males and females in the control group. CC, corpus callosum; LV/AC, lateral ventricle and shell of the nucleus accumbens; CP, caudate putamen; Cg, cingulate cortex; Cx, cerebral cortex; CA1/2, CA3, CA4 are the hippocampal subfields; DG, upper blade of the dentate gyrus; AHiPM, capsule of the amygdalohippocampal nucleus; PMCO, posteromedial cortical amygdaloid nucleus.

in the guinea pig. In contrast, D1 enzyme activity has been described in the adult rat brain (Visser *et al.* 1982).

Maternal nutrient deprivation increased mRNA levels for the major transactivating TR isoforms in male fetal brain, whereas expression decreased in females. The areas that expressed the highest amounts of TR α 1 mRNA (cerebellum) and TR β 1 mRNA (CA1/2; pontine nuclei) under euthyroid (control) conditions were also the areas which were most affected during periods of nutrient-induced hypothyroxinaemia. Conversely, the expression of the nonligand binding isoform TR α 2 and the weakly expressed TR β 2 were largely unaffected.

The mechanisms by which MND alters TR isoform expression are at present unclear but our results suggest

that fetal hypothyroxinaemia may play a role. T₃ treatment of several CNS cell types *in vitro* results in an increase in TR β 1 mRNA expression (Lebel *et al.* 1993; Chan *et al.* 2003b), with little effect on mRNAs encoding the TR α isoforms. This is likely to be mediated via direct T₃ regulation at the two thyroid hormone response elements described in the promoter regions of the TR β (*c-erbA β*) gene (Sakurai *et al.* 1992). This would explain the decrease in TR β 1 mRNA in response to MND seen in female fetuses. On the other hand, androgens may alter the effect of MND on TR expression in male fetuses resulting in increased TR β 1 mRNA expression. Sexual differentiation of the fetal guinea pig brain occurs at approximately gd50, a time when male androgen levels are known to be elevated (Resko & Roselli, 1997). In this connection,

MND also has sex-specific effects on the expression of other steroid hormone receptors within the developing brain, including mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) (Lingas *et al.* 1999).

In patients with non-thyroidal illness, an increase in TR expression seen in non-CNS cell types may compensate for decreased circulating thyroid hormone levels (Williams *et al.* 1989). Although no similar studies have been carried

out in the brain, it is possible that an up-regulation of TRs may partially compensate for the reduced availability of thyroid hormone, akin to that reported for other steroid hormone receptors (e.g. MR) in the fetal brain (McCabe *et al.* 2001). However, it is now recognized that during CNS development, TR α 1 and β 1 play important roles in the basal repression of gene transcription, which helps to regulate the timing of developmental events (Anderson

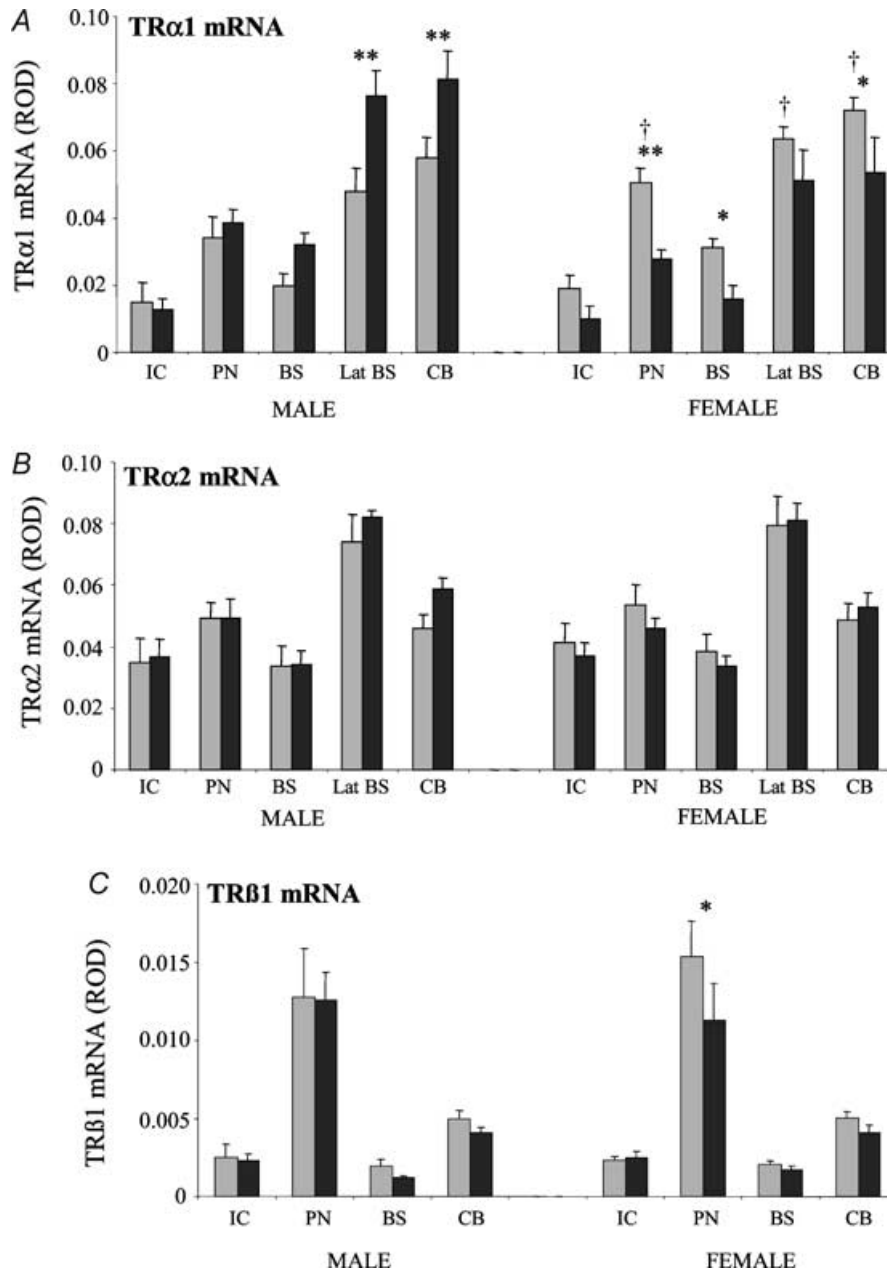


Figure 5. Densitometric analysis of expression of mRNAs encoding the TR isoforms (A: TR α 1; B: TR α 2; C: TR β 1) in the brainstem and cerebellum in control animals and after 48 h of nutrient deprivation in male (control (grey) $n = 5$, deprived (black) $n = 7$) and female fetuses (control $n = 8$, deprived $n = 7$). Results are expressed as mean \pm s.e.m. relative optical density (ROD). Statistical significance is indicated as: * $P < 0.05$, ** $P < 0.01$. † indicates a significant difference between males and females of the control group. IC, inferior colliculus; PN, pontine nuclei; BS, brainstem; Lat BS, lateral regions of the brainstem; CB, cerebellum.

et al. 2003), as well as the conventionally understood trans-activating role of inducing thyroid hormone responsive gene transcription. So up-regulation of TR expression may not necessarily afford protection to brain development. Since thus far no clinical study of hypothyroxinaemia has described sex differences in neurodevelopmental outcomes in children, the divergent responses in TR expression to MND in male fetuses compared with female fetuses may represent sex-specific methods of compensation for hypothyroxinaemia or merely result from different developmental profiles at the time of MND. The latter possibility would agree with our findings of significant sex-specific differences in TR expression in specific brain regions under euthyroid conditions.

Fetuses of nutritionally deprived mothers demonstrated increases in D2 mRNA expression throughout the brain. Based on studies in adult rat cerebral cortex which

have demonstrated that hypothyroidism has a greater up-regulatory effect on D2 activity than D2 mRNA compared with euthyroid controls (Burmeister *et al.* 1997), it is likely that in fetal guinea pig brains increased D2 mRNA would reflect increased D2 activity. This would maintain local thyroid hormone concentrations in the face of hypothyroxinaemia, supporting the possibility that D2 plays a crucial part in the homeostatic regulation of local T₃ supply. The stimulatory effect that a reduction in T₃ has on D2 transcription is likely to be indirect since a negative TRE has not been identified in the D2 promoter (Bianco *et al.* 2002). One possible mechanism is through the effect of cortisol, the primary glucocorticoid in guinea pigs, which is dramatically up-regulated in fetuses of nutritionally deprived mothers (Lingas *et al.* 1999). Dexamethasone administration to chick embryos has resulted in increased D2 activity in the brain (Reyns *et al.* 2005) and synthetic glucocorticoids have been shown

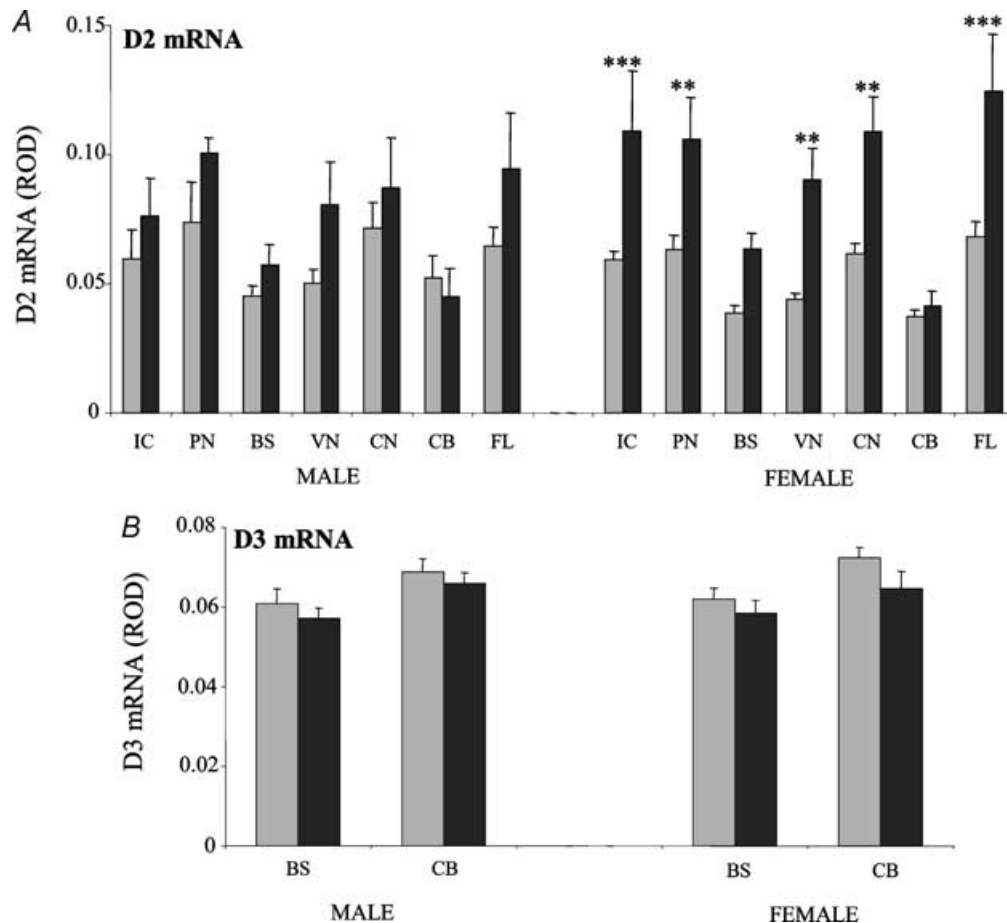


Figure 6. Densitometric analysis of expression of mRNAs encoding the iodothyronine deiodinase type 2 (D2; A) and type 3 (D3; B) in the brainstem and cerebellum in control animals and after 48 h of nutrient deprivation in male (control (grey) $n = 5$, deprived (black) $n = 7$) and female fetuses (control $n = 8$, deprived $n = 7$)

Results are expressed as mean \pm S.E.M. relative optical density (ROD). Statistical significance are indicated as: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. IC, inferior colliculus; PN, pontine nuclei; BS, brainstem; VN, vestibular nucleus; CN, cochlea nucleus; CB, cerebellum; FL, flocculus.

to induce the transcription of D2 in a rat pituitary tumour cell line, an effect reduced in the presence of T₃ (Kim *et al.* 1998).

In general, greater increases in D2 transcripts were seen in the forebrain of male fetuses with MND, while in female fetuses greater increases in D2 transcripts were detected in the brainstem. This may reflect a sex difference in compensatory mechanisms aimed at modulating the influence of hypothyroxinaemia in the brain and suggests that sex hormones may also have a role in the regulation of D2 expression. Androgens have been shown to suppress phenolic (outer-ring) mono-deiodination activities (probably by D1 and D2) in the pineal gland of Syrian hamsters (Rubio *et al.* 1996) while oestrogen, but not progesterone and androgens, alters D2 activity in pituitaries of adult rats (Donda *et al.* 1990; Lisboa *et al.* 2001). These data suggests that the effects of sex hormones on D2 expression may be different across species, between different tissue types and CNS areas, and during various stages of development. It is notable that the cerebellum, a well-established thyroid hormone responsive organ, did not exhibit an increase in D2 expression except in the flocculus following MND. The lack of a compensatory response in the cerebellum makes it particularly vulnerable to hypothyroxinaemia and this may be associated with findings that fine motor skills are significantly poorer in children with congenital hypothyroidism than in euthyroid controls (Rovet, 1999).

We have previously noted a lack of correlation between changes in D3 transcript levels and D3 activity in fetal cerebral cortex (Chan *et al.* 2002) and placenta (Chan *et al.* 2003a) across gestation in normal human development, which is highly suggestive of post-transcriptional regulation but the mechanisms by which this occur are yet to be established. Thus in the present study the lack of change seen in D3 mRNA expression in response to hypothyroxinaemia following MND does not exclude the possibility of a compensatory down-regulation in D3 activity.

Fetuses of nutritionally deprived mothers also represent a model of poor fetal growth. Unlike the findings in this study, we have previously reported that the cerebral cortex and cerebellum of human fetuses with severe IUGR demonstrate a reduction in the expression of all the TR isoform proteins (determined by semiquantitative scoring of immunohistochemical staining) (Kilby *et al.* 2000). These data were derived from biopsies of cerebral cortex rather than completely intact tissues. Also, male and female fetuses were not analysed separately and were from a wide range of gestational ages. Our current study in guinea pigs is a more comprehensive assessment of TR expression in the CNS (whole brains) associated with, albeit less severe, growth restriction *in utero* in fetuses of the same gestational age.

Modification of the fetal environment by MND has permanent effects on the hypothalamo–pituitary–adrenal (HPA) axis development in guinea pigs (Lingas *et al.* 1999). Moreover, the development of GR systems in limbic structures and subsequent disruptions in HPA activity have been linked to thyroid hormone effects on the ascending serotonergic fibres innervating the limbic system (Meaney *et al.* 1987; Liu *et al.* 1997). We had previously found that GR mRNA was down-regulated specifically in the paraventricular nucleus (PVN) and CA1/2 region of the hippocampus in both male and female fetuses following MND (Lingas *et al.* 1999). Interestingly, TR β 1 and D2 mRNA expression were also affected in the CA1/2 region of the hippocampus. However, unlike the case for GR, the effect was strikingly sexually dimorphic for TR β 1 mRNA. Alterations in TR and deiodinase expression in the brainstem of IUGR fetuses and fetuses of nutritionally deprived mothers may also be implicated in the pathogenesis of an abnormal HPA axis.

In conclusion, we have shown that MND dramatically alters transcription of the two major TR isoforms, α 1 and β 1, in the fetal guinea pig brain in a sex- and region-specific manner, especially in the hippocampus, brainstem and cerebellum. MND-induced changes in mRNA expression, coupled with elevated levels of D2 mRNA may represent a fetal-specific, protective mechanism to maintain local T₃ supply and minimize aberrant brain development.

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