

Maternal transfer of methimazole and effects on thyroid hormone availability in embryonic tissues

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Abstract

Methimazole (MMI) is an anti-thyroid drug used in the treatment of chronic hyperthyroidism. There is, however, some debate about its use during pregnancy as MMI is known to cross the mammalian placenta and reach the developing foetus. A similar problem occurs in birds, where MMI is deposited in the egg and taken up by the developing embryo. To investigate whether maternally derived MMI can have detrimental effects on embryonic development, we treated laying hens with MMI (0.03% in drinking water) and measured total and reduced MMI contents in the tissues of hens and embryos at different stages of development. In hens, MMI was selectively increased in the thyroid gland, while its levels in the liver and especially brain remained relatively low. Long-term MMI treatment induced a pronounced goitre with a decrease in thyroxine (T_4) content but an increase in thyroidal 3,5,3'-triiodothyronine (T_3) content. This resulted in normal T_3 levels in tissues except in the brain. In chicken embryos, MMI levels were similar in the liver and brain. They gradually decreased during development but always remained above those in the corresponding maternal tissues. Contrary to the situation in hens, T_4 availability was only moderately affected in embryos. Peripheral T_3 levels were reduced in 14-day-old embryos but normal in 18-day-old embryos, while brain T_3 content was decreased at all embryonic stages tested. We conclude that all embryonic tissues are exposed to relatively high doses of MMI and its oxidised metabolites. The effect of maternal MMI treatment on embryonic thyroid hormone availability is most pronounced for brain T_3 content, which is reduced throughout the embryonic development period.

Key Words

- ▶ methimazole
- ▶ thyroid hormone
- ▶ chicken
- ▶ development
- ▶ brain

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Introduction

Untreated hyperthyroidism in pregnant women increases the risk for a number of adverse outcomes for the mother and foetus, including intrauterine growth restriction,

miscarriage, preterm delivery, pre-eclampsia and congestive heart failure (Luewan *et al.* 2011). Maternal hyperthyroidism can be treated with anti-thyroid drugs such as

propylthiouracil (PTU) and methimazole (MMI). However, these drugs may in turn have a negative impact on foetal development as they can cross the placenta. The present clinical guidelines advise to avoid the use of MMI in early pregnancy because it may increase the risk of specific malformations including choanal and oesophageal atresia and aplasia cutis congenita (Wolf *et al.* 2006, Azizi & Amouzegar 2011, Rodriguez-Garcia *et al.* 2011, Vissenberg *et al.* 2012), but it remains unclear whether these abnormalities are linked to MMI or to the maternal hyperthyroidism itself (Barbero *et al.* 2008).

While it is known that MMI is transferred from the mother to the embryo/foetus, either through the placenta in mammals or through a deposit in the egg in non-mammalian vertebrates, data on the relative MMI concentrations in different embryonic/foetal tissues are scarce. In addition, to what extent maternal MMI reaching the embryo/foetus is present in the reduced or oxidised form has hardly been investigated. While oxidised MMI metabolites are no longer active as anti-thyroid drugs, they have been associated with cytotoxic effects (Kedderis & Rickert 1985, Laurence *et al.* 1998, Heidari *et al.* 2012) and as such they may still disturb normal development.

It also remains unclear to what extent maternal MMI treatment disturbs thyroid hormone (TH)-dependent development in the embryo/foetus. Maternally derived MMI can block the developing thyroid gland as shown repeatedly in rats (Comer & Norton 1985, Ruiz de Ona *et al.* 1988, Calvo *et al.* 1990). However, the early vertebrate brain is highly dependent on adequate 3,5,3'-triiodothyronine (T_3) availability, and it may not yet be able to compensate for a reduced TH supply by an increase in type 2 iodothyronine deiodinase (D2) activity as observed in the adult brain (Ruiz de Ona *et al.* 1988, Van Herck *et al.* 2012). Apart from inhibiting TH production, it has been found more recently that MMI may also have local anti-thyroid effects in tissues by directly suppressing transcriptional activities mediated by T_3 and its receptors (Moriyama *et al.* 2007).

In this study, we have used the chicken embryo to study the effect of maternal MMI treatment on the developing embryo. It has been shown earlier that MMI is taken up from the egg by the embryo and is capable of disturbing development, including brain development (Iqbal *et al.* 1987, Liu & Porter 2004, Kagami & Nishigori 2010). We measured the levels of MMI and its metabolites in different maternal and embryonic tissues and studied the effect of MMI treatment on thyroxine (T_4) and T_3 availability in these tissues.

Materials and methods

Animal treatment and tissue sampling

Experiments were performed on broiler breeder hens (Ross, Belgabroed, Belgium) that were ~30 weeks old. They were fed a standard diet with iodine content within the advised range for euthyroidism (1.9 mg/kg). The animals were divided over six pens, with each containing 20 hens and 3 roosters. Those in four pens were treated with 0.03% 1-methyl-3*H*-imidazole-2-thione (methimazole, Acros Organics, Geel, Belgium) in the drinking water, while those in two pens served as controls. This dose was based on literature data from studies carried out in rats and chickens aimed at inducing hypothyroidism (Iqbal *et al.* 1987, Calvo *et al.* 1990, Rosebrough *et al.* 2006, Sharlin *et al.* 2010). The animals were treated for a total of 16 weeks, and we started collecting eggs after 6 weeks of treatment.

Eggs were incubated in a forced draft incubator at 37.5 °C and 50% relative humidity and automatically turned at a 45° angle every hour. The day on which incubation was started was called day 0 (E0). After 6, 14 and 18 days of incubation (E6, E14 and E18), eggs were opened, and the following tissues were sampled: head and trunk at E6; liver, heart, lung, muscle and brain at E14 and E18. Brain was divided into telencephalon, diencephalon, mesencephalon, cerebellum and hindbrain. Tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C for later use.

At 10, 14 and 16 weeks of treatment, a number of non-incubated eggs were sampled for MMI measurements. Egg yolk and egg white were separated and stored frozen until MMI analysis.

Treatment was stopped for all animals after 16 weeks, and ten control and ten MMI-treated hens were killed at that moment to collect their liver, kidney and different brain regions.

Prior to tissue sampling, blood samples were collected from hens and from E14 and E18 embryos in heparinised tubes. After centrifugation, plasma was stored at -20 °C until analysis.

The Ethical Committee for animal experiments of the KU Leuven approved all the experimental protocols.

Determination of MMI levels

The method for the determination of reduced and total MMI levels was based on derivatisation with 2-chloro-1-methylquinolinium tetrafluoroborate (Bald & Glowacki 2001) and separation and quantification by ion-pairing

reversed-phase liquid chromatography originally developed for human urine (Kusmierek & Bald 2007). Details of the procedure used for chicken tissues, eggs and embryos have been described elsewhere (G Chwatko, V M Darras & E Bald, Personal communication 2013).

Determination of T₄ and T₃ levels

T₄ and T₃ levels in maternal and embryonic tissues were measured following extraction as described previously in detail (Reyns *et al.* 2002, 2005). Typical recoveries of extracted THs ranged from 55 to 75% for T₃ and from 40 to 60% for T₄. The T₃ RIA had a detection limit of 2 fmol and an intra-assay variability of 2.2%. The T₄ RIA had a detection limit of 5 fmol and an intra-assay variability of 2.8%. For the T₃ RIA, cross-reactivity with T₄ was 0.1–0.5%, whereas for the T₄ RIA, cross-reactivity with T₃ was 3.5%. Hormone levels are expressed as picomole per gram tissue.

T₄ and T₃ levels in plasma were measured using the same RIA system without extraction, using a standard in hormone-free serum, and results are expressed as picomole per millilitre plasma.

Thyroid glands were homogenised in 500 µl ice-cold barbital buffer (0.05 M, pH 8.6) using a Glass/Teflon Potter tissue grinder system. This step was followed by further ultrasonic homogenisation (2 × 25 s). Homogenates were then centrifuged and supernatants were transferred into new tubes. Part of the supernatants was directly frozen and stored at –20 °C. For enzymatic degradation of thyroglobulin by addition of 150 µl protease (Sigma P-5147, 6.25 mg protease and 3.13 mg 2-thiouracil in 10 ml Tris-HCl buffer, 0.75 M and pH 8.8) and 15 µl toluol, 150 µl of the supernatants were used. Tubes were stoppered and incubated for 2 days in a shaking water bath at 37 °C.

Finally, tubes were placed in boiling water for 2 min to inactivate the pronase enzyme, snap frozen and stored at –20 °C. Dilutions of untreated homogenates (free hormone levels) and enzymatically digested homogenates (total hormone levels) were analysed by RIA, and free and total hormone contents were calculated per thyroid gland.

RT and quantitative PCR

Total RNA was isolated using TRIzol reagent (Life Technologies) and treated with DNase I (Life Technologies) to remove residual genomic DNA. Total RNA was reverse-transcribed using SuperScript III Reverse Transcriptase and Oligo(dT)_{12–18} primers (Life Technologies). Real-time PCR was performed using a thermal cycler, StepOnePlus Real-Time PCR Systems (Life Technologies), with Fast SYBR Green Mastermix (Life Technologies) and 300 nM of each primer as listed in Table 1. Non-template and water controls were used to detect non-specific amplification. Each experimental and standard sample was analysed in duplicate. Relative expression values were calculated based on the standard curve with the StepOne Software (Life Technologies) using a 1:5 dilution series of pooled cDNA as the standard. The expression data were analysed using the qBase^{PLUS} Software (Biogazelle, Zwijnaarde, Belgium). Two reference genes were used for normalisation: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and cyclophilin for adult hens and *GAPDH* and β-actin for embryos.

Deiodinase enzyme assays

In vitro deiodination activities were determined in microsomes obtained from maternal liver and kidney and in homogenates of the different maternal and embryonic

Table 1 Primer sequences for the quantitative RT-PCR analyses

Name	Accession no. mRNA	Forward sequence (5' → 3')	Reverse sequence (5' → 3')	Amplicon length (bp)
D2	NM_204114	TGT TTC TGA GCC GCT CCA A	ACA CTG GAG TTC GGA GCT TCT C	142
D3	NM_001122648	CAG GAG GAG AAG GTG ATG TAC CA	TCT GGA GCC GGG TTT TGT ACT	100
OATP1C1	NM_001039097	CAT GGG ACG ATA TCA GTA TGA AAG A	CGA GAG TGG AGT TTG GCT TTT CT	97
MCT8	XM_426274	CAA CTC CTT CGG GAT CAT CTA CA	AGC CAA CCC ATG CTG TTT TAA	95
TSH receptor	NM_001033850; NM_001193589; NM_001193588	GGT CCT CTG ATA GCT CTG AAC GAT	CAC TGC TGT GCT TTC AAG AAC TG	119
NIS	XM_429095	CAC CAC GGA GGC TGAT GTT	GCG CAG CCA CCG TGA T	75
TTF1	NM_204616	GCC GTT CTC CTG TTG CAT CT	GAA CAT TTA GCC AGC ATG ATC CA	126
TPO	AM167549	TTC CGA TAA AGG TAA CAA CCA ATG	CAG TGC CAC ATG CAG GAG AT	87
TG	XM_003640868	CAA CGT TTT CCA GGC CAT TG	CCA GTG ATT GGC CAT GTT TAT G	80
β-Actin	NM_205518	ATG GCT CCG GTA TGT GCA A	TGT CTT TCT GGC CCA TAC CAA	120
GAPDH	NM_204305	GAA GCT TAC TGG AAT GGC TTT CC	GAT ATC ATC ATA CTT GGC TGG TTT CTC	97
Cyclophilin A	NM_001166326	GGC TAC AAG GGC TCC TGC TT	CCG TTG TGG CGC GTA AA	77

brain regions as described in detail earlier (Darras *et al.* 1992, Reyns *et al.* 2005). In the D1 activity test, the incubation mixture contained 0.1 mg of protein/ml and 1 μ M rT₃ containing 50 000 c.p.m. of radiolabelled [¹²⁵I]-rT₃, and tubes were incubated at 37 °C for 30 min. D2 activity was assayed in a similar way by incubating 1 mg of protein/ml and 1 nM T₄ containing 50 000 c.p.m. [¹²⁵I]-T₄ at 37 °C for 120 min. A second mixture was prepared with 100 nM T₄ to assess possible interference of D1 activity. Only when iodide production was absent or minimal in the presence of 100 nM T₄, enzyme activity using 1 nM T₄ was considered to be the true D2 activity. In the D3 activity test, the incubation mixture contained 1 mg of protein/ml and 1 nM T₃ containing 150 000 c.p.m. [¹²⁵I]-T₃ as well as 1 μ M rT₃ and 0.1 mM 6*n*-PTU to block possible D1 interference. The mixture was incubated for 120 min at 37 °C. Activities were calculated as picomol (D1) or femtomol (D2 and D3) of hormone deiodinated per milligram protein and per minute.

Statistical analysis

All data comparisons between the control group and the MMI group for the given age and tissue were done by Student's unpaired *t*-test. Comparisons with *P* values <0.05 were considered significantly different.

Results

MMI levels in maternal and embryonic tissues

We measured MMI levels in the hens, eggs and embryos of the MMI-treated groups to evaluate maternal transfer to

the embryos. As not only reduced MMI but also its metabolites may have adverse effects on embryonic development, we used a method allowing discrimination between the total amount of MMI (including oxidised forms) and that of reduced MMI (the anti-thyroid active form). From weeks 10 up to 16 of treatment, the average levels of reduced MMI were 11.0±0.6 μ g/g wet weight for egg yolk (76% of total MMI level) and 12.8±0.7 μ g/g wet weight for egg white (95% of total MMI level; mean ± s.e.m. for a total of 12 samples). MMI levels in maternal and embryonic tissues are given in Table 2. As expected, the highest levels were found in the thyroid gland of adult hens. In these animals, reduced MMI levels were also clearly higher in the peripheral tissues (plasma and liver) than in the brain. A different result was found in embryos where reduced MMI levels were similar in the peripheral tissues and brain. E6 embryos had the highest levels and almost all MMI was present in the reduced form. Total MMI concentrations were comparable at E14 and E18, but the percentage present as reduced MMI was clearly lower at E18 than at E14. However, reduced levels in the embryonic brain remained higher than the levels in the adult brain. Owing to the small size of the embryonic thyroid gland, we were not able to determine MMI levels in this tissue.

Effects on maternal thyroid gland activity

Sixteen weeks of MMI treatment strongly increased the size of the thyroid gland (Table 3). The expression of a number of genes involved in thyroid gland activity was significantly affected, with a decrease in the mRNA levels of thyroid transcription factor 1 (*TTF1* (*NKX2-1*)) and

Table 2 Levels of (MMI*, μ g/g wet tissue weight) in different maternal and embryonic chicken tissues. Embryonic tissues were sampled from eggs collected between weeks 10 and 14 of treatment (eight samples for E6 and three to four samples per tissue for E14 and E18). Samples from hens were collected at the end of the 16-week treatment (ten samples for plasma and three to four samples for tissues). E6, E14 and E18: 6-, 14- and 18-day-old embryos

	Hen		E6		E14		E18	
	Reduced (%) ^a	Total	Reduced (%) ^a	Total	Reduced (%) ^a	Total	Reduced (%) ^a	Total
Plasma	6.6±0.9 ^b (93%)	7.1±1.0						
Thyroid	20.9±2.0 (91%)	22.8±2.6						
Head			7.6±1.2 (91%)	8.3±1.4				
Trunk			7.6±1.2 (97%)	7.9±1.2				
Liver	3.5±1.5 (89%)	3.9±0.7			3.9±0.5 (83%)	4.7±0.2	2.8±0.4 (77%)	3.7±0.5
Telencephalon	2.1±0.1 (92%)	2.2±0.3			4.0±0.6 (90%)	4.4±0.6	2.8±0.4 (68%)	4.1±0.5
Mesencephalon	1.2±0.1 (56%)	2.2±0.3			4.1±0.4 (88%)	4.7±0.6	2.7±0.3 (67%)	4.1±0.5
Cerebellum	1.1±0.1 (59%)	1.9±0.1			3.7±0.6 (66%)	5.6±0.6	2.7±0.4 (69%)	4.0±0.5

^aPercentage of total MMI content present as reduced (anti-thyroid active) MMI.

^bValues represent mean ± s.e.m.

Table 3 Effects of 16 weeks of MMI treatment on maternal thyroid weight (mg) and on total and free thyroid hormone contents (pmol/gland) in adult hens and embryos. Values represent mean \pm s.e.m. for eight to ten samples/group. E14 and E18: 14- and 18-day-old embryos

	Control	MMI	Fold change
Hens			
Weight	190 \pm 18	3299 \pm 454	$\times 17^{\dagger}$
Total T ₄	419 763 \pm 76 320	31 984 \pm 5956	$\times 0.08^{\dagger}$
Free T ₄	18 136 \pm 3178	2048 \pm 450	$\times 0.11^{\dagger}$
Total T ₃	952 \pm 99	2175 \pm 349	$\times 2.3^*$
Free T ₃	15.6 \pm 1.9	115 \pm 32	$\times 7.4^*$
E14			
Total T ₄	164.2 \pm 15.0	18.7 \pm 5.0	$\times 0.11^{\dagger}$
Free T ₄	13.81 \pm 1.40	0.48 \pm 0.12	$\times 0.03^{\dagger}$
Total T ₃	7.67 \pm 0.64	5.59 \pm 0.85	$\times 0.73$
Free T ₃	0.217 \pm 0.55	0.086 \pm 0.021	$\times 0.40^*$
E18			
Total T ₄	3661 \pm 351	1005 \pm 104	$\times 0.27^{\dagger}$
Free T ₄	57.1 \pm 3.6	20.6 \pm 2.6	$\times 0.36^{\dagger}$
Total T ₃	21.36 \pm 1.73	13.30 \pm 2.59	$\times 0.62^*$
Free T ₃	0.299 \pm 0.043	0.581 \pm 0.095	$\times 1.94^*$

* $P < 0.05$ and $^{\dagger}P < 0.001$ compared with the controls (unpaired *t*-test).

thyroglobulin (*TG*) and a significant increase in the mRNA levels of sodium/iodide symporter (*NIS* (*SLC5A5*)), thyroid peroxidase (*TPO*) and thyrotropin receptor (*TSHR*) (Fig. 1).

The amount of T₄ per weight unit of the thyroid gland was more than 100-fold reduced following MMI treatment, and despite the substantial increase in the size of the thyroid gland, both total and free T₄ contents were about tenfold decreased. The amount of T₃ per weight unit was far less affected, and in combination with the higher weight of the thyroid gland, this resulted in a twofold increase in total T₃ content and a sevenfold increase in free T₃ content (Table 3).

TH levels in maternal and embryonic tissues

Blood samples were collected from hens every 2 weeks throughout the treatment period. Circulating T₄ levels in the MMI-treated hens showed a steady decrease until 6 weeks of treatment when they stabilised around 50% of the control levels. Plasma T₃ content was mildly decreased at 10 and 14 weeks of treatment, but remained unchanged at all other time points (data not shown).

T₄ and T₃ levels in maternal tissues are shown in Fig. 2. T₄ levels were strongly decreased in the plasma, liver, kidney and different brain regions of the MMI-treated hens, except in the diencephalon. While T₃ levels in

peripheral plasma, liver and kidney were similar for the control and MMI-treated hens, T₃ content was strongly decreased in the telencephalon and mesencephalon and mildly but not significantly decreased in the diencephalon and cerebellum.

The number of eggs produced by the MMI-treated hens decreased and stabilised around week 8 at 20–25% of the number produced by the control hens. However, the overall quality of the eggs did not change; egg weight, crude energy content, crude protein content and crude lipid content were similar in the MMI-treated and control groups. By contrast, the amount of T₄ and T₃ deposited in the egg yolk was strongly decreased throughout the egg collection period (Fig. 3). T₄ levels in E6 embryos were similar in the trunk and head and were surprisingly not decreased by MMI treatment. At this stage, T₃ levels were clearly higher in the head than in the trunk and were significantly lower in the head of MMI embryos (Fig. 3).

Maternal MMI treatment decreased circulating T₄ levels in E14 and E18 embryos. However, T₄ levels in liver, heart, lung, muscle and different brain regions were not altered (Figs 4 and 5). The situation was different for T₃. While plasma T₃ content was not significantly decreased, levels were, in general, clearly lower in both peripheral tissues and brain in MMI embryos at E14. At E18, T₃ levels were still decreased in the brain but no longer in the peripheral tissues (Figs 4 and 5).

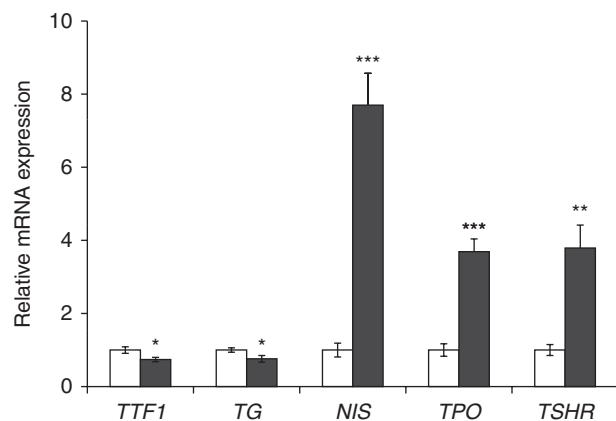
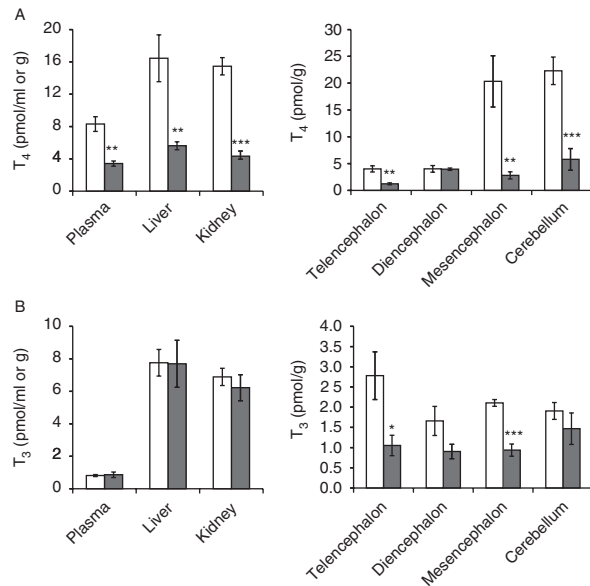


Figure 1

Relative mRNA expression of *TTF1*, *TG*, *NIS*, *TPO* and *TSHR* in the thyroid glands of the control (white bars) and MMI-treated (dark bars) hens following 16 weeks of treatment. Expression levels were normalised using *GAPDH* and cyclophilin as the reference genes. For each gene, levels are expressed relative to the average level in the control group. Values represent mean \pm s.e.m. for five animals per group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (unpaired *t*-test compared with the controls).

**Figure 2**

Levels of T₄ (A) and T₃ (B) (expressed in picomole per millilitre or gram) in the plasma, liver, kidney and brain of the control (white bars) and MMI-treated (dark bars) hens following 16 weeks of treatment. Values represent mean \pm s.e.m. for five animals per group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (unpaired *t*-test compared with the controls).

Expression of TH transporters and deiodinases in maternal and embryonic brain

As MMI treatment clearly affected TH levels in the brain, we measured the mRNA expression of the deiodinases D2 and D3 and the TH transporters *OATP1C1* (*SLCO1C1*) and *MCT8* (*SLC16A2*). Expression was measured in the telencephalon, diencephalon, mesencephalon, cerebellum and hindbrain of the control and MMI-treated hens, but no significant changes were observed. The expression of these four genes was also measured in the telencephalon, diencephalon and mesencephalon of E6 and E14 embryos. No significant changes were found at E6. At E14, the expression of D2 and MCT8 was decreased by respectively 34 and 27% ($P < 0.05$) but only in the diencephalon.

Deiodinase activity in maternal and embryonic tissues

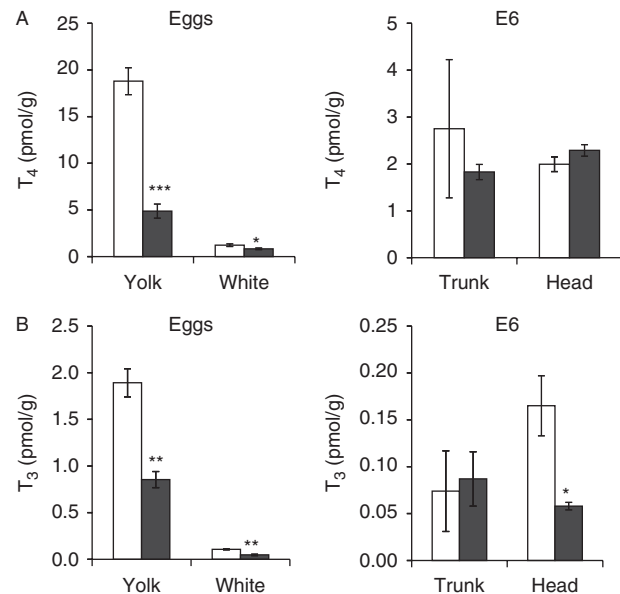
MMI treatment did not significantly affect D1 or D3 activity in maternal liver and kidney, but it strongly increased D2 activity in all regions of the maternal brain (Table 4). Moderate but significant increases were also observed in the diencephalon and mesencephalon of E14 embryos. No increases were found in the embryonic brain at E6, where D2 activity was even significantly lower in embryos from MMI eggs compared with the controls (Table 4).

The only significant change in D3 activity was found in the adult brain where activity was decreased in the telencephalon of the MMI-treated hens (0.597 ± 0.036 fmol T₃ deiodinated/mg protein per min vs 0.766 ± 0.068 in the controls, $P < 0.05$).

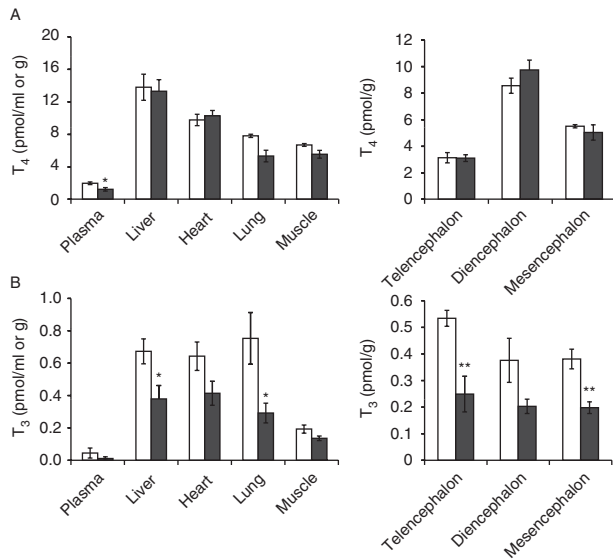
In order to determine whether the presence of MMI as such is able to decrease the efficiency of T₄-to-T₃ conversion, we also tested D2 activity in the brain homogenates of a number of control embryos in the presence or absence of 20 μ g MMI/ml, a concentration corresponding to the highest level observed in tissues (maternal thyroid, 20 μ g/g wet weight). The addition of MMI to the homogenates did not alter *in vitro* D2 activity.

Discussion

The anti-thyroid drug MMI can be absorbed from the gastrointestinal tract and it accumulates in the thyroid gland where it inhibits TH synthesis by interfering with the action of thyroid peroxidase (Nagasaka & Hidaka 1976, Vickers *et al.* 2012). It has been shown in humans and rats that MMI is rapidly metabolised and that a major part is excreted via urine (Skellern & Steer 1981, Hengstmann & Hohn 1985, Cooper 2005, Kusmirek & Bald 2007).

**Figure 3**

Levels of T₄ (A) and T₃ (B) (expressed in picomole per gram) in the eggs and 6-day-old embryos (E6) of the control (white bars) and MMI-treated (dark bars) hens. Eggs were collected following 14 weeks of maternal MMI treatment. Values represent mean \pm s.e.m. for four eggs or embryos per group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (unpaired *t*-test compared with the controls).

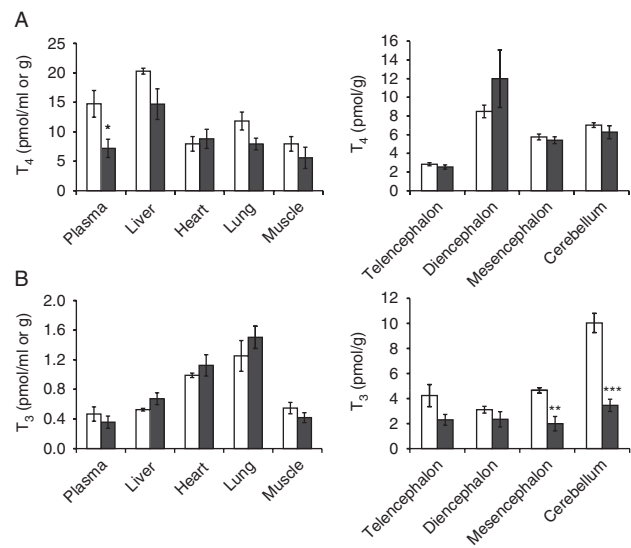
**Figure 4**

Levels of T₄ (A) and T₃ (B) (expressed in picomole per millilitre or gram) in the peripheral tissues and brain of 14-day-old embryos of the control (white bars) and MMI-treated (dark bars) hens. Eggs were collected between weeks 10 and 16 of maternal MMI treatment. Values represent mean \pm s.e.m. for five embryos per group. * $P < 0.05$ and ** $P < 0.01$ (unpaired t -test compared with the controls).

Nevertheless, MMI treatment sometimes induces severe side effects such as agranulocytosis and hepatotoxicity (Cooper 2005, Yang *et al.* 2012), indicating that a significant amount of MMI and/or its metabolites is present in extra-thyroidal tissues. We measured the levels of reduced MMI and its oxidised metabolites in hens following 16 weeks of treatment. The dose that we used was relatively high compared with the ones used in medical practice. Circulating levels were about fourfold higher than the average levels reported for humans (Mortimer *et al.* 1997), but relative differences in tissue levels might also apply to humans where tissue data are scarce. As in humans and rats (Marchant & Alexander 1972, Jansson *et al.* 1983), MMI specifically accumulated in the thyroid gland. About sixfold lower levels were present in the liver, mainly in the reduced form. Total levels in the brain were about half of those in the liver, suggesting that the adult brain is somehow better protected. Interestingly, we found clear differences in the proportion of MMI present as oxidised metabolites. This was more than 40% in the mesencephalon and cerebellum, while this was around 10% in the telencephalon, similar to that observed in the peripheral tissues. So far, it remains unclear whether this difference is the result of a higher local metabolism of MMI or a selective uptake

related to the tissue composition. The fact that total MMI levels are similar could be an element in favour of the first hypothesis.

The major problem with MMI as well as other thionamides in humans and more precisely in pregnant women is that these compounds are transferred to the developing foetus via the placenta and may harm normal development (Cooper 2005, Vissenberg *et al.* 2012). In birds, essential as well as potentially harmful substances are deposited in the egg and taken up by the embryo during development. Two important conclusions can be drawn from the MMI measurements in chicken embryos. The first one is that although both total and reduced MMI levels in embryonic tissues are gradually decreasing from early towards later stages of development, they always remain above the levels found in the corresponding adult tissues. The second one is that the levels in the embryonic brain are as high as those in the embryonic liver, suggesting that the protective mechanisms possibly present in the adult brain are not yet functional before hatching. Most of the MMI is present in the reduced form, although there seems to be a gradual increase in the percentage of oxidised metabolites, especially in the brain. However, although these metabolites no longer function as anti-thyroid drugs, they can be cytotoxic and

**Figure 5**

Levels of T₄ (A) and T₃ (B) (expressed in picomole per millilitre or gram) in the peripheral tissues and brain of 18-day-old embryos of the control (white bars) and MMI-treated (dark bars) hens. Eggs were collected between weeks 10 and 16 of maternal MMI treatment. Values represent mean \pm s.e.m. for five embryos per group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (unpaired t -test compared with the controls).

Table 4 Effects of maternal MMI treatment on type 2 deiodinase activity (fmol T₄ deiodinated/mg protein per min) in maternal and embryonic chicken brain. Values represent mean ± s.e.m. for four pools/group (E6) or eight individual samples/group (E14 and hens)

	Control	MMI
Hens		
Telencephalon	0.499 ± 0.026	0.763 ± 0.057 [†]
Diencephalon	0.180 ± 0.017	0.324 ± 0.016 [†]
Mesencephalon	0.194 ± 0.018	0.279 ± 0.010 [‡]
Cerebellum	0.311 ± 0.027	0.503 ± 0.017 [†]
E6		
Telencephalon	0.638 ± 0.045	0.676 ± 0.053
Diencephalon	0.914 ± 0.068	0.797 ± 0.040
Mesencephalon	0.693 ± 0.032	0.575 ± 0.020*
E14		
Telencephalon	4.094 ± 0.077	4.343 ± 0.177
Diencephalon	4.231 ± 0.090	4.600 ± 0.143*
Mesencephalon	3.477 ± 0.075	3.843 ± 0.115*
Cerebellum	3.089 ± 0.128	3.163 ± 0.167

* $P < 0.05$, [‡] $P < 0.01$ and [†] $P < 0.001$ compared with the controls (unpaired *t*-test).

therefore may still contribute to the adverse effects of MMI on development (Kedderis & Rickert 1985, Genter 1998).

When investigating maternal thyroid status, we found that the MMI-treated hens were able to keep up normal plasma T₃ levels despite a 50% decrease in circulating T₄ levels. The same was true for the TH content in the liver and kidney. One possible mechanism would be an increase in the peripheral conversion of T₄ into T₃ by outer-ring deiodination and/or a decrease in T₃ degradation by inner-ring deiodination. The results of the *in vitro* D1 and D3 activity tests in the liver and kidney suggest that this is not the case. By contrast, we observed dramatic changes at the level of the thyroid gland. The most obvious one was the huge goitre, which can most probably be explained by a decrease in the negative feedback of T₄ at the level of the pituitary thyrotropes (Huang *et al.* 2001, Bianco & Kim 2006). The increased growth of the thyroid gland was accompanied by a highly increased relative expression of the thyrotropin receptor, the sodium/iodide symporter and thyroid peroxidase, while the expression of thyroglobulin and TTF1 was slightly decreased. This is somewhat different from observations in human thyroid cells and rat FRTL-5 cells cultured *in vitro* where short-term treatment with MMI increased the expression of thyroid peroxidase as well as thyroglobulin (Leer *et al.* 1991). However, recent studies on human thyroid slices have shown that the effect of MMI on the expression of thyroid-specific genes may vary according to treatment dose and duration (Vickers *et al.* 2012).

In accordance with the inhibitory effect of MMI on iodine integration into tyrosine, both total and free T₄ contents were strongly decreased. By contrast, long-term MMI treatment resulted in a twofold increase in total T₃ content and a sevenfold increase in free T₃ content. This way the MMI-treated hens could maintain normal T₃ levels by shifting from a predominance of T₃ produced by peripheral deiodination of T₄ to a predominance of T₃ produced within the thyroid gland. A similar mechanism has not been found in pregnant rats where MMI treatment reduced T₄ as well as T₃ levels in plasma, liver and heart, although the relative decrease was smaller for T₃ (Calvo *et al.* 1990). This could be a species-related difference, but it is also possible that the treatment period of 7 days in the rat experiment was too short to induce substantial changes in the ratio of T₃:T₄ content and release of the thyroid gland.

The adaptive mechanism in adult hens seems to be sufficient to maintain T₃ levels in peripheral tissues such as liver and kidney, but not in the brain, which is known to depend heavily on T₄ uptake and local T₄-to-T₃ conversion by D2 (Silva & Matthews 1984, Grijota-Martinez *et al.* 2011). Despite a clear increase in D2 activity, most brain regions did not succeed in compensating for the reduced supply of T₄ and showed low T₃ levels. This finding illustrates once more that the thyroid status of a specific tissue cannot be simply deduced from the TH levels present in circulation.

A tissue-specific control also seems to be active at the level of the ovary, since both T₄ and T₃ concentrations in the eggs were severely reduced despite the normal plasma T₃ levels. This control occurred independently of the overall protein and lipid contents of the eggs, which were not changed by MMI treatment. The fact that the total number of eggs was reduced confirmed earlier data on the inhibitory effect of hypothyroidism on female reproduction in a number of species including fish, amphibians and humans (Van der Geyten *et al.* 2001, Krassas *et al.* 2010, Carr & Patino 2011).

Maternal MMI treatment clearly has an effect on tissue TH availability in developing embryos but, surprisingly, predominantly on T₃. Again, this is not reflected in the circulation since both E14 and E18 embryos from MMI eggs showed significantly reduced plasma T₄ levels, while plasma T₃ content was not significantly different. At E6, T₄ levels in the body of the control and MMI embryos were similar. As at that stage the embryonic thyroid gland is developing but not yet functional, this suggests that the embryo can compensate for the reduced T₄ content in the egg yolk by a more efficient T₄ uptake. The embryonic thyroid gland normally starts contributing to TH

availability in the embryo during the second week of incubation and the effects observed at E14 and E18 should therefore be interpreted as the combined result of the reduced TH content in the yolk and the inhibition of embryonic thyroid gland activity by MMI taken up by the embryo.

As in adult hens, the brain of E14 and E18 embryos from MMI eggs had clearly lower T_3 levels but without a decrease in T_4 content, a combination that is difficult to explain. The possibility of a direct inhibitory effect of MMI on D2 activity in brain homogenates was tested, but it proved to be negative. *In vitro* D2 activity tests on the brain of E14 embryos showed that the amount of active enzyme was higher in the diencephalon and mesencephalon of MMI embryos compared with the controls, indicative of a compensatory response. This response was, however, not present in all brain regions, and it was less pronounced than the strong response in the brain of adult hens. Together with the fact that there was no compensatory increase in D2 activity in the brain at E6, this confirms earlier observations that the typical compensatory response of deiodinases to altered TH availability is not yet mature in young embryos/foetuses (Ruiz de Ona *et al.* 1988, Gereben *et al.* 2008, Sharlin *et al.* 2010, Van Herck *et al.* 2012). The single changes found in *D2* and *MCT8* mRNA in different brain regions of E14 embryos are also not in line with a compensatory response at the level of gene expression and rather point to a delay in the normal ontogenetic expression pattern of these genes in hypothyroid MMI embryos (Geysens *et al.* 2012, Van Herck *et al.* 2012). The fact that hypothyroidism as such did not change the mRNA expression of the deiodinases in the embryonic as well as adult brain is in agreement with literature data from embryonic and post-hatch quail exposed to perchlorate (Chen *et al.* 2008, 2009).

One more interesting observation is that T_3 availability was reduced not only in the brain but also in the peripheral tissues of E14 embryos, while this was no longer the case at E18. During the second half of embryonic development, the relative contribution of the embryonic thyroid gland to total TH availability increases over time and is therefore higher at E18 where the hypothalamo-pituitary–thyroid axis is almost completely mature. As in adult hens, the embryonic thyroid gland may have increased its relative release of T_3 and this is in line with the fact that at E18 free T_3 content was increased in the thyroid gland of MMI embryos. The slower maturation of the thyroid axis in rodents compared with chickens may also help to explain why in rat foetuses from MMI-treated dams both T_4 and T_3 contents are decreased in the

peripheral tissues and brain, similar to the situation in the dams themselves (Calvo *et al.* 1990).

Taken together, our data show that long-term MMI treatment of hens severely reduces T_4 production in the thyroid gland but induces a relative increase in thyroidal T_3 content. As a result, peripheral tissues can maintain normal T_3 levels, but the brain is clearly hypothyroid. As in mammals, the treatment leads to the maternal transfer of MMI and its oxidised metabolites to the developing embryo. Levels in embryonic tissues are relatively high, especially at the early stages, and MMI and/or its oxidised metabolites may therefore have cytotoxic effects on all developing tissues including the brain. Long-term MMI treatment also reduces TH availability in the egg yolk, resulting in lower T_3 availability in the early embryonic brain. At later stages, the maternally derived MMI also disturbs embryonic thyroid gland function. Combined with the lower TH content of the yolk, this results in a reduced T_3 content in the peripheral tissues and brain of 14-day-old embryos and in the brain of 18-day-old embryos. We can conclude that the maternal transfer of MMI and its metabolites to the embryo can be harmful for embryonic development, in particular, for the brain, by a combination of anti-thyroidal and possibly local cytotoxic effects, an observation that may also be of relevance to the human situation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S L J V and S G were responsible for setting up of the experiment, collection of all samples, analysis of gene expression and statistical analysis of all data. E B and G C performed the MMI measurements. E D was responsible for the daily supervision of the MMI treatment of the hens and the collection and quality control of the eggs. E D and R G A assisted in the analysis of embryonic brain samples. V M D supervised the thyroid hormone extractions and deiodination tests and prepared the manuscript.

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