

Chronic local inflammation in mice results in decreased TRH and type 3 deiodinase mRNA expression in the hypothalamic paraventricular nucleus independently of diminished food intake

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Abstract

During illness, changes in thyroid hormone metabolism occur, known as nonthyroidal illness and characterised by decreased serum triiodothyronine (T_3) and thyroxine (T_4) without an increase in TSH. A mouse model of chronic illness is local inflammation, induced by a turpentine injection in each hind limb. Although serum T_3 and T_4 are markedly decreased in this model, it is unknown whether turpentine administration affects the central part of the hypothalamus–pituitary–thyroid axis (HPT-axis). We therefore studied thyroid hormone metabolism in hypothalamus and pituitary of mice during chronic inflammation induced by turpentine injection. Using pair-fed controls, we could differentiate between the effects of chronic inflammation *per se* and the effects of restricted food intake as a result of illness. Chronic inflammation increased interleukin (IL)-1 β mRNA expression in the hypothalamus more rapidly than in the pituitary. This hypothalamic cytokine response was associated

with a rapid increase in local D2 mRNA expression. By contrast, no changes were present in pituitary D2 expression. TSH β mRNA expression was altered compared with controls. Comparing chronic inflamed mice with pair-fed controls, both preproTSH releasing hormone (TRH) and D3 mRNA expression in the paraventricular nucleus were significantly lower 48 h after turpentine administration. The timecourse of TSH β mRNA expression was completely different in inflamed mice compared with pair-fed mice. Turpentine administration resulted in significantly decreased TSH β mRNA expression only after 24 h while later in time it was lower in pair-fed controls. In conclusion, central thyroid hormone metabolism is altered during chronic inflammation and this cannot solely be attributed to diminished food intake.

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Introduction

Acute systemic illness induced by bacterial endotoxin (lipopoly saccharide; LPS) administration, results in altered peripheral and central thyroid hormone metabolism, so-called nonthyroidal illness (NTI; Papanicolaou 2000, Koenig 2005). Pituitary D2 and TRH mRNA expression decrease while hypothalamic D2 mRNA expression (Boelen *et al.* 2004) and activity (Fekete *et al.* 2004) increase shortly after LPS administration. The D2 increase is associated with increased hypothalamic interleukin (IL)-1 β mRNA expression (Boelen *et al.* 2004). It is known that chronic inflammation in mice, induced by an s.c. injection of turpentine in each hind limb also results in altered peripheral thyroid hormone metabolism (Chopra *et al.* 1987, Woloski & Jamieson 1987) and we have shown recently that besides decreased serum triiodothyronine (T_3) and thyroxine (T_4) levels liver type 1 deiodinase (D1) did not change and type 3 deiodinase (D3) activity increased in inflammatory cells at the site of inflammation (Boelen *et al.* 2005).

The observed decrease in serum thyroid hormone levels during chronic inflammation might be due in part to

diminished food intake during the first days of illness. It is known that in rats starvation results in decreased serum thyroid hormones and thyroid-stimulating hormone (TSH) concentrations and decreased TSH releasing hormone (TRH) mRNA expression in the hypothalamic paraventricular nucleus (PVN) associated with a modest increase in hypothalamic D2 activity (Diano *et al.* 1998). These alterations are partly regulated by leptin which is downregulated during starvation (Ahima *et al.* 1996, Legradi *et al.* 1997, Coppola *et al.* 2005). The observed modest increase in D2 activity during starvation is in contrast with the rapid, cytokine-related, more than a threefold increase in D2 activity (Fekete *et al.* 2004) and mRNA expression (Boelen *et al.* 2004) observed shortly after LPS administration. Faggioni *et al.* (1998) showed that both LPS and turpentine administration results in markedly increased serum leptin levels despite the fact that these mice decrease their food intake. IL-1 β is essential for leptin induction during inflammation because neither LPS nor turpentine increases leptin levels in IL-1 β ^{0/0} mice while leptin is normally induced in IL-6^{0/0} mice (Faggioni *et al.* 1998). However, data addressing changes in thyroid hormone-related gene expression in pituitary and hypothalamus are sparse.

The aims of the present study were to (1) evaluate pituitary and hypothalamic thyroid hormone metabolism during chronic inflammation and (2) differentiate between the effects of chronic inflammation *per se* and the effects of restricted food intake as a result of illness on pituitary and hypothalamic thyroid hormone metabolism. Thyroid hormone metabolism was evaluated by determining hypothalamic TRH, D2, D3 and TR β 1 mRNA expression and pituitary TSH β and D2 mRNA expression in relation to serum T₄, T₃ and reverse T₃ (rT₃) levels.

Materials and Methods

Animals

Female C57Bl6 mice (Harlan Sprague–Dawley, Horst, The Netherlands) weighing approximately 20 g were used at 6–12 weeks of age. The mice were kept in 12 h light:12 h darkness cycles (light on from 0700 to 1900 h), in a temperature-controlled room (22 °C) and food and water were available *ad libitum*. A week before and during the experiment, the mice were housed in groups according to the experimental set-up. Local chronic inflammation was induced by a s.c. injection of 100 μ l steam-distilled turpentine in each hind limb. Control mice received saline in each hind limb. Due to the diurnal variation of thyroid hormone-related genes, the experiments were performed using the same time schedule starting at 0900 h ($t=0$).

We performed two experiments. In the first experiment, we compared mice with chronic inflammation with control mice who received saline and food *ad libitum*. In the second experiment, pair-fed control mice were used as controls because food deprivation as a result of illness by itself affects thyroid hormone metabolism. At various time points after turpentine injection ($t=0, 8, 24, 48$ and 120 h), four to five mice per group were anaesthetised with isoflurane, blood was taken by cardiac puncture and then mice were killed by cervical dislocation. Serum was stored at -20 °C until analysed. The pituitary and hypothalamus (defined rostrally by the optic chiasm, caudally by the mamillary bodies, laterally by the optic tract and dorsally by the apex of the third ventricle) were isolated and stored immediately in liquid nitrogen. Based on anatomical landmarks (the apex of the third ventricle; Franklin 1997), both paraventricular nuclei, adjoining the third ventricle were obtained by punching the bilateral PVN region with a hollow needle (diameter 1100 μ m). These samples may have included (part of) the dorsomedial nucleus (DMN). The same instrument was used to obtain the arcuate nucleus/median eminence region. The study was approved by the local animal welfare committee.

Serum determinations

Serum T₃, T₄ and rT₃ levels were measured with in-house RIAs (Wiersinga & Chopra 1982). To prevent interassay variation, all samples of one experiment were measured within the same assay.

RNA isolation and RT-PCR

mRNA was isolated from whole hypothalamus, PVN and pituitary using the Magna Pure apparatus and the Magna Pure LC mRNA isolation kit II (tissue; Roche Biochemicals) according to the manufacturer's protocol, and cDNA synthesis was performed with the First Strand cDNA synthesis kit for RT-PCR (AMV; Roche Molecular Biochemicals). In order to check whether the isolated cDNA contains genomic DNA, an RT control reaction was performed after each RNA isolation. Published primer pairs were used to amplify hypoxanthine phosphoribosyl transferase (HPRT, a house-keeping gene; Sweet *et al.* 2001). We designed primer pairs for IL-1 β (oligo-spanning), D2, D3 (oligo-spanning), TR β 1, TSH β and preproTRH (IL-1 β forward: TTGACGGACCC-CAAAAGAT and reverse: GAAGCTGGATGCTCTCAT-CTG; D2 forward: GATGCTCCCAATTCC AGTGT and reverse: AGTGAAAGGTGGTCAGGTGG; D3 forward: CTACGTCATCCAGAGTGGCA and reverse: CTGTTC-ATCATAGCGCTCCA; TR β 1 forward: CACCTGGATC-CTGACGATGT and reverse: ACAGGTGATGCAGCG ATAGT; TSH β forward: TCAACACCACCATCTGTGCT and reverse: TTGCCACACTTGCAGCTTAC; pre-proTRH forward: TCGTGCTAACTGGTATCCCC and reverse: CCCAAATCTCCCCTCTCTTC; Boelen *et al.* 2004). Real-time PCR was performed for quantitation of the above-mentioned mRNAs. cDNA standards for the different mRNAs were prepared from RNA of murine liver, pituitary or hypothalamus. For each mRNA assayed, a standard curve was generated using tenfold serial dilutions of this target standard PCR product and the same primers used to amplify the cDNA. For each gene, the standard protocol was optimised by varying MgCl₂ concentrations. PCR were set up with cDNA, MgCl₂ (25 mM), SybrGreenI (Roche Molecular Biochemicals), forward and reverse primers and H₂O. The reactions were then cycled in the LightCycler (Roche Molecular Biochemicals) as described previously (Boelen *et al.* 2004). The LightCycler software generated a standard curve (measurements taken during the exponential phase of the amplification) which enabled the amount of each mRNA in each test sample to be determined. All results were corrected as to their mRNA content using HPRT mRNA. Relative expression was presented by arbitrary units, the magnitude depends on the amount of tissue and cDNA dilution used and could be different for each tissue. Samples were individually checked for their PCR efficiency (Ramakers *et al.* 2003). The median of the efficiency was calculated for each assay and samples with >0.05 difference of the efficiency median value were not taken into account.

Statistics

Data were normally distributed (Shapiro–Wilk test) and presented as the mean \pm S.E.M. Variation between turpentine-treated mice and (pair) fed control mice, was evaluated by ANOVA with two grouping factors (time and treatment).

P values in the figures represent the significant effect of treatment. In case of time-related changes in the control group, times×treatment (interaction) values are also given. ANOVA was followed by Tukey's test for pair-wise comparisons (symbols in the figures represent the pair-wise *P* values.) In case of unequal variances, data were rank transformed prior to ANOVA. All analyses were carried out in SPSS 11.5.1, (SPSS Inc., Chicago, IL, USA). *P* values <0.05 were considered statistically significant.

Results

Effect of chronic inflammation on serum thyroid hormone levels

Turpentine administration causes a sterile abscess at the site of injection (hind limb), which results in serious discomfort. Serum T_3 , T_4 and rT_3 levels were significantly decreased 24, 48 and 120 h after injection compared with $t=0$. Since chronic illness results in diminished food intake, by itself affecting thyroid hormone metabolism, we also used pair fed mice as controls. Serum T_3 levels were similar in chronic inflamed mice compared with pair fed controls, while serum

T_4 and rT_3 levels were significantly lower during chronic inflammation relative to pair fed controls (ANOVA, $P<0.01$). The T_3/rT_3 ratio was significantly higher in turpentine-treated animals compared with pair-fed control mice (ANOVA, $P<0.001$) (Fig. 1).

Effect of chronic inflammation on hypothalamic and pituitary thyroid hormone metabolism

mRNA expression of IL-1 β and several thyroid hormone metabolism-related genes was measured in the hypothalamus and pituitary of mice with chronic inflammation compared with overall *ad libitum* fed control mice. The results are presented in Fig. 2. Turpentine administration resulted in a significant increase in IL-1 β mRNA expression in the whole hypothalamus as well as an increase in D2 mRNA expression shortly after turpentine administration ($t=8$ h, $P<0.05$). Hypothalamic D3 mRNA expression was not different in mice with chronic inflammation compared with fed control mice (Fig. 2). PreproTRH and TR β 1 mRNA expression in the whole hypothalamus did not change after turpentine administration (data not shown). Pituitary IL-1 β mRNA expression was significantly increased 120 h after turpentine

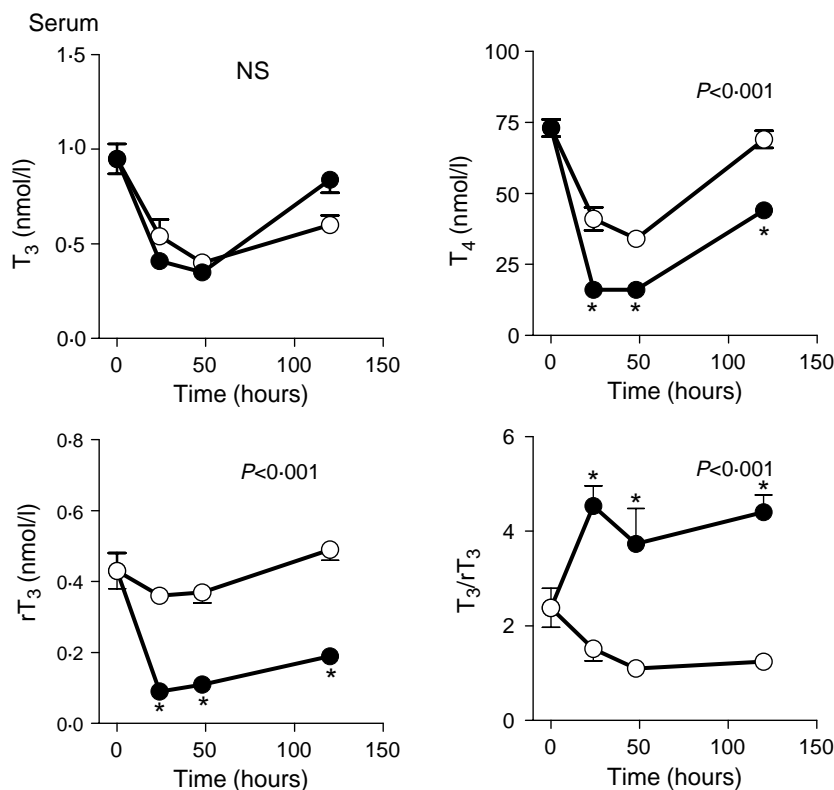


Figure 1 Serum levels of T_3 , T_4 and rT_3 and T_3/rT_3 ratio of mice after turpentine-induced chronic inflammation (●-●) compared with saline-treated, pair fed control mice (○-○). Mean values \pm S.E.M. ($n=5$) are depicted; *P* values indicate treatment difference between groups by ANOVA and statistical difference between groups at a single time point is indicated by symbols; * $P<0.01$. NS, not significant.

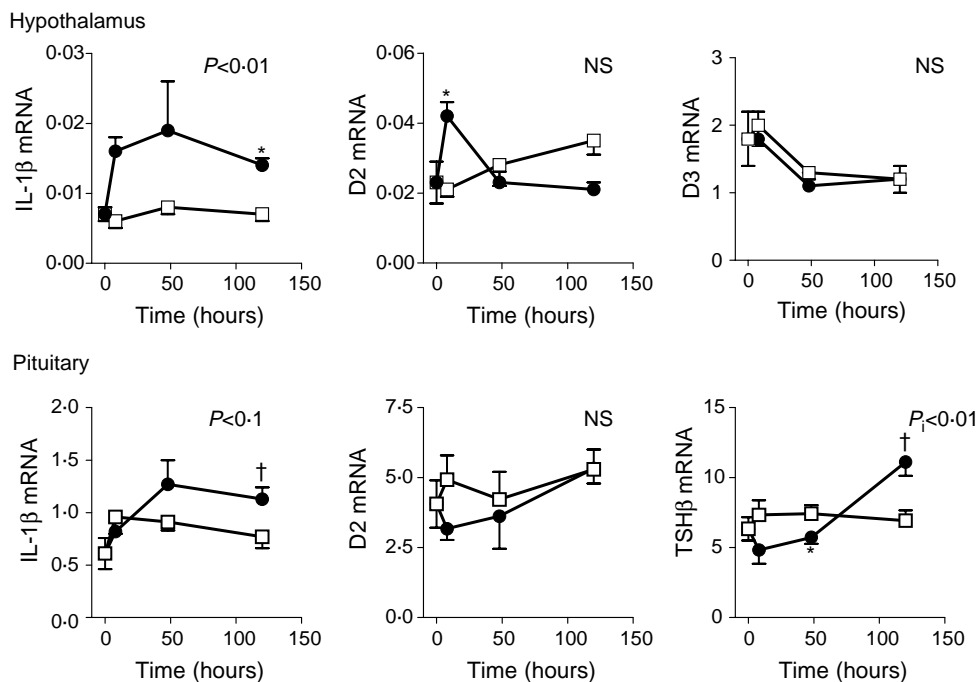


Figure 2 Relative expression of hypothalamic IL-1 β , D2 and D3 mRNA (upper panel) and pituitary IL-1 β , D2 and TSH β mRNA expression (lower panel) of mice after turpentine-induced chronic inflammation (●-●) compared with saline-treated control mice who received food *ad libitum* (□-□). Mean values of expression relative to HPRT \pm s.e.m. ($n=4$) are depicted; P values indicate treatment difference between groups by ANOVA, P_i value indicates significant interaction between time and treatment and statistical difference between groups at a single time point is indicated by symbols: * $P < 0.05$ and † $P < 0.01$. NS, not significant.

administration ($P < 0.01$) compared with fed control mice but was not significantly different during the whole time period ($P < 0.1$). Pituitary TSH β mRNA expression was different in infected mice compared with fed mice (ANOVA, $P_{\text{interaction}} < 0.01$) with significantly decreased TSH β mRNA expression 48 h after turpentine administration and significantly higher TSH β mRNA at 120 h after turpentine administration (Fig. 2).

Effect of chronic inflammation on hypothalamic and pituitary thyroid hormone metabolism compared with food restriction

In order to differentiate between the effects of the inflammatory response by itself and illness-induced decreased food intake on central thyroid hormone metabolism, chronic inflammation was compared with pair fed controls. It is known that TRH mRNA expression of neurons located in the hypothalamic PVN is markedly decreased by restricted food intake and starvation. We therefore measured IL-1 β , preproTRH, D2, D3 and TR β 1 mRNA expression in this nucleus. D2 mRNA expression was also measured in the arcuate nucleus/median eminence region (ARC). ANOVA revealed significantly higher IL-1 β mRNA expression

($P < 0.01$) and lower preproTRH mRNA expression in the PVN ($P < 0.05$) after turpentine administration compared with pair fed controls (Fig. 3). IL-1 β mRNA expression in the PVN represents a biphasic response; in the beginning increased expression due to turpentine administration and after 120 h re-increased expression due to a developing systemic acute phase response. D2 and TR β 1 mRNA expression after turpentine administration and restricted food intake (pair fed controls) did not change compared with basal levels at $t=0$ (data not shown), while D3 mRNA expression in the PVN during chronic inflammation was marginally lower compared with pair fed controls ($P=0.056$) especially at 48 h ($P < 0.05$). D2 mRNA expression in the ARC did not increase significantly during chronic inflammation even though a trend could be found ($P=0.15$; Fig. 3). Pituitary IL-1 β mRNA expression was increased 120 h after turpentine administration compared with pair fed controls (Fig. 4). Pituitary D2 expression was similar in chronically inflamed and pair fed control mice. The timecourse of changes in pituitary TSH β mRNA expression during the observed 120 h was different in infected mice compared with pair fed mice (ANOVA, $P_{\text{interaction}} < 0.01$) with significantly decreased TSH β mRNA expression 24 h after turpentine

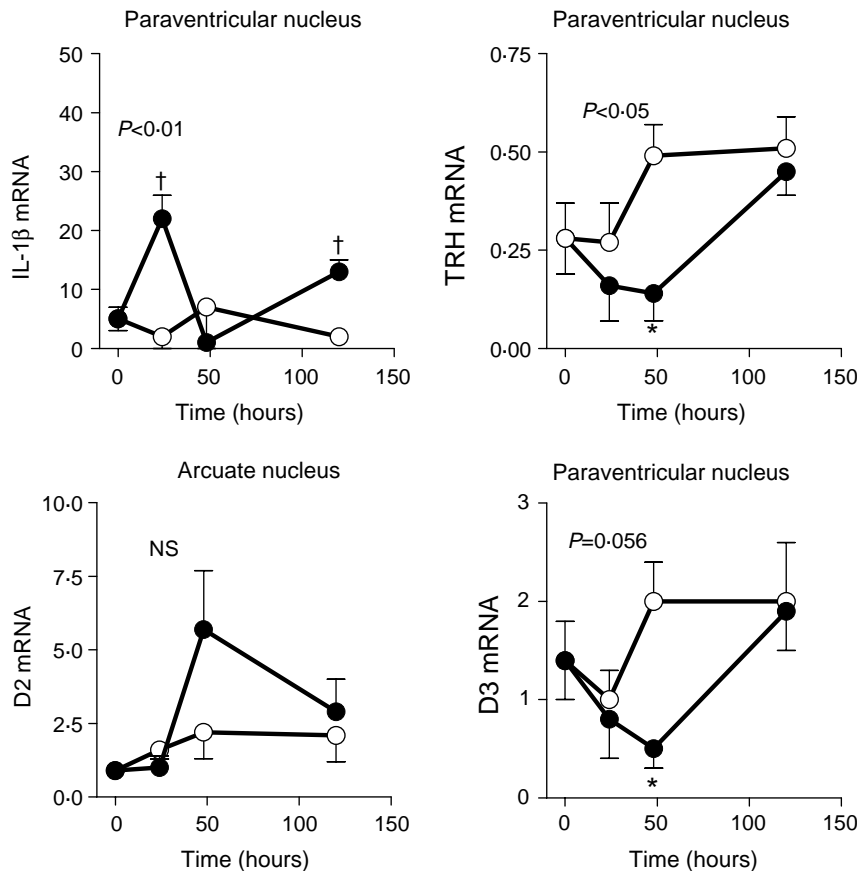


Figure 3 Relative expression of IL-1 β , preproTRH and D3 RNA expression in the PVN and D2 mRNA in the ARC of mice after turpentine-induced chronic inflammation (●-●) compared with saline-treated, pair fed control mice (○-○). Mean values of expression relative to HPRT \pm S.E.M. ($n=5$) are depicted; P values indicate treatment difference between groups by ANOVA and statistical difference between groups at a single time point is indicated by symbols: * $P<0.05$ and † $P<0.01$. NS, not significant.

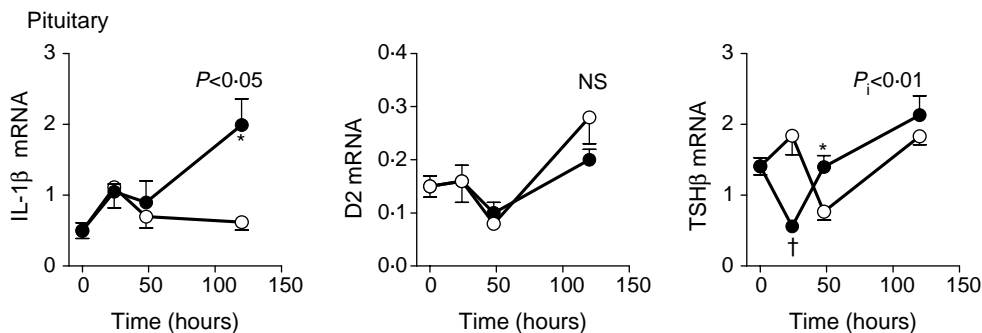


Figure 4 Relative expression of pituitary IL-1 β , D2 and TSH β mRNA expression of mice after turpentine-induced chronic inflammation (●-●) compared with saline-treated, pair fed control mice (○-○). Mean values of expression relative to HPRT \pm S.E.M. ($n=5$) are depicted; P value indicates treatment difference between groups by ANOVA, P_i value indicates significant interaction between time and treatment and statistical difference between groups at a single time point is indicated by symbols: * $P<0.05$ and † $P<0.01$. NS, not significant.

administration and significantly lower TSH β mRNA in pair fed controls at $t=48$ h.

Discussion

It is known that profound alterations occur in the central part of the hypothalamic-pituitary-thyroid (HPT)-axis during illness. We and other researchers have shown that acute, systemic illness induced by bacterial endotoxin (LPS) results in downregulation of pituitary thyroid hormone metabolism and an increase in hypothalamic D2 mRNA expression (Boelen *et al.* 2004) and activity (Fekete *et al.* 2004) in rodents, while TRH expression is downregulated in direct relation with pre-mortem serum TSH in patients with nonthyroidal illness (Fliers *et al.* 1997). Starvation on the other hand also results in altered thyroid hormone metabolism in both hypothalamus and pituitary, with decreased TRH expression in the PVN in association with a modest increase in hypothalamic D2 activity (Diano *et al.* 1998). Both pituitary TSH β and D2 mRNA expression decrease during starvation (Boelen *et al.* 2006). While the time course of acute illness is too short to show influence of restricted food intake on the observed alterations, the effects of restricted food intake as a result of illness during a longer period may influence central thyroid hormone metabolism. We therefore studied pituitary and hypothalamic thyroid hormone metabolism during chronic inflammation in mice differentiating between the effects of restricted food intake and chronic inflammation on pituitary and hypothalamic thyroid hormone metabolism in relation to serum thyroid hormone levels.

A turpentine-induced abscess is a relatively mild model of chronic inflammation with serial activation of specific cytokines resulting in a characteristic acute phase response (Leon 2002). We found a small but significant increase in hypothalamic IL-1 β in association with a rapid increase in hypothalamic D2 mRNA expression. This is in agreement with earlier observations in acute illness (Boelen *et al.* 2004). In the present study, we did not observe illness-induced alterations in TRH mRNA expression in whole hypothalamus. However, in punched tissue samples containing the PVN region, we observed significant decreased preproTRH mRNA expression after turpentine administration compared with pair fed controls, which was associated with a significant decrease in D3 mRNA expression 48 h after turpentine administration. Increased IL-1 β mRNA expression was more pronounced in this specific region compared with the whole hypothalamus indicating the presence of IL-1 β producing cells in this area. Furthermore, D2 mRNA expression in punched tissue samples containing the arcuate nucleus/median eminence region tended to increase 48 h after turpentine administration (also compared with pair fed controls). D2 mRNA expression did not increase in pair fed animals compared with basal levels at time point 0 indicating that restricted food intake *per se* did not influence hypothalamic D2 expression in our experimental model.

Although we cannot rule out the presence of adjacent nuclei (the DMN, which contains also TRH-expressing neurons) in the PVN punches, it might be plausible that only TRH-expressing hypophysiotropic neurons were affected, since pituitary TSH β mRNA expression and serum thyroid hormone levels were also decreased.

Increased D3 expression during hyperthyroidism and undetectable D3 expression during hypothyroidism have been reported in rat CNS, but hypothalamic D3 expression was of moderate magnitude (Tu *et al.* 1999). Alkemade *et al.* (2005) recently showed D3 immunoreactivity in neurons of selective nuclei of the human hypothalamus, perhaps explaining the difference in D3 expression in the present study between whole hypothalamus and punched tissue samples containing PVN more selectively. D3 expression in the human hypothalamus showed a strong overlap with TR expression suggesting that D3 is expressed in T₃-responsive neurons. Our results suggest that during chronic illness, T₃ bioavailability in the PVN and mediobasal hypothalamus is relatively increased by a downregulation of D3 activity in the PVN and an upregulation of D2 activity in the ARC. This may act, in turn, to decrease preproTRH mRNA in PVN neurons, thereby contributing to persistent low serum concentrations of thyroid hormone. This putative adaptation mechanism was not observed during restricted food intake.

Pituitary thyroid hormone metabolism was partly affected during chronic illness: D2 mRNA expression did not change while TSH β mRNA expression decreased slightly in the beginning during chronic inflammation and ends up higher 120 h after turpentine administration compared with fed controls. The use of pair fed controls showed that this decrease was not solely due to diminished food intake. The TSH β mRNA decrease in pair fed controls was observed after 48 h while after 24 h TSH β mRNA was decreased in the inflamed animals despite the same amount of ingested food. This indicates that food-related signals can be overridden by inflammatory mediators. A possible candidate may be leptin since turpentine administration has been shown to result in a significant increase in serum leptin levels despite decreased food intake (Faggioni *et al.* 1998). Furthermore, other hypothalamic or pituitary neuropeptides induced during the inflammatory response and specific cytokines could play a role despite the fact that we did not find a predominant IL-1 β response in the pituitary.

The observed decrease in serum T₃ levels during chronic inflammation could be ascribed to diminished food intake during the first days of illness, in contrast to the decrease in serum T₄ and rT₃. It is known that liver D1 is unaffected by turpentine-induced chronic illness which may explain similar serum T₃ levels, but additional peripheral deiodinases may be involved. It is tempting to speculate about the role of muscle D2 as it has been shown that muscle D2 is probably the major source of circulating T₃ in humans (Maia *et al.* 2005). This seems, however, less likely in rodents, since Wagner *et al.* (2003) showed that D2 mRNA expression can be detected in a variety of mouse tissues but not in skeletal muscle, although

a recent paper showed that the majority of iodide generated by D1 was not derived from T_4 (Schneider *et al.* 2006). Furthermore, a study of Streckfuss *et al.* (2005) showed that serum thyroid hormone levels were not different in mice lacking hepatic selenoenzyme expression compared with controls. The early decrease in pituitary TSH β mRNA expression could explain the more severe decrease in serum T_4 levels during chronic inflammation. The induction of enzymes other than deiodinases during illness could also play a role in the observed differences in serum T_4 and rT_3 levels during chronic inflammation compared with pair fed controls. Sulphation of several iodothyronines by sulphotransferases (SULT) is an important pathway in the metabolism of thyroid hormones by, amongst others, affecting deiodination of T_4 and T_3 by D1. Sulphation of T_4 (T_4S) and rT_3 (rT_3S) facilitates inner ring deiodination, while sulphation of T_4 impairs outer ring deiodination suggesting that altered sulphotransferases activity affects thyroid hormone levels by itself. However, Peeters *et al.* (2005a) reported increased T_4S levels in critically ill patients but attributed this to decreased liver D1 activity and not to increased SULT1 activity. Furthermore, it has been shown in mice that one member of the SULT2 family (SULT2A1) decreased in liver during the acute-phase response (Kim *et al.* 2004). These results in combination with the unaltered liver D1 activity in our mouse model (Boelen *et al.* 2005) did not suggest a prominent role of sulphotransferases in lowering serum T_4 and rT_3 levels during chronic inflammation. The T_3/rT_3 ratio was increased during chronic inflammation due to more severely decreased rT_3 levels compared with pair fed controls, which is in contrast with results obtained in humans. Peeters *et al.* (2005b) have shown that the T_3/rT_3 ratio, a prognostic marker of survival in critically ill patients reflecting alterations in peripheral thyroid hormone metabolism, was lower in nonsurvivors than in survivors. In rodents, however, the kinetics of rT_3 are opposite, since Burgi *et al.* (1986) have already shown in 1986 that serum rT_3 increases during fasting but not so during bacterial infection. We observed unchanged serum rT_3 levels in pair fed controls, while inflammation resulted in decreased rT_3 levels.

In summary, chronic inflammation induces an early increase of hypothalamic D2 mRNA expression potentially resulting in locally increased tissue T_3 concentrations. This, in turn, might inhibit preproTRH mRNA expression in hypophysiotropic neurons in the PVN. Proinflammatory cytokines might be involved in the observed D2 mRNA increase as it has been shown that the D2 promoter region contains multiple nuclear factor-kappa B (NF-kB)-binding sites (Zeold *et al.* 2006). In addition to the early increase in total hypothalamic D2 expression, D3 mRNA expression in the PVN was decreased and D2 mRNA expression seemed to increase in the ARC 48 h after turpentine administration. We are aware of the fact that we did not measure D2 and D3 activity levels in our study, although previous studies have shown that pituitary and hypothalamic D2 mRNA expression during illness correlates well with D2 activity (Boelen *et al.*

2004, Fekete *et al.* 2004). Furthermore, the sample size of our experiments in combination with punching hypothalamic regions could be the reason for not reaching statistical significance but we are confident that the results we obtained reflect thyroid hormone metabolism during chronic inflammation. The major function of D2 and D3 in the brain is to regulate local bioavailability of T_3 (Lechan & Fekete 2005) and both increased D2 expression and decreased D3 expression may be expected to result in locally increased T_3 tissue concentrations. The mechanism involved in hypothalamic D3 mRNA inhibition is unknown at present. D3 mRNA expression may be induced by several growth factors (Huang *et al.* 2005) and cytokine-related pathways (Pallud *et al.* 1999). Locally produced glucocorticoids as a result of inflammation might represent an alternative factor capable of downregulating hypothalamic D3 mRNA expression as it is known that IL-1 β activates the central part of the hypothalamic-pituitary-adrenal (HPA)-axis (Turnbull *et al.* 2003). Finally, diminished food intake as a result of illness in our chronic inflammation model does not explain the illness-induced alterations in hypothalamic D2 and D3 mRNA expression.

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