Journal of Neuroendocrinology, 2000, Vol. 12, 1149–1159



# Chronic Iodine Deprivation Attenuates Stress-Induced and Diurnal Variation in Corticosterone Secretion in Female Wistar Rats

L. A. Nolan,* R. J. Windle,*† S. A. Wood,*† Y. M. Kershaw,*† H. R. Lunness,* S. L. Lightman,* C. D. Ingram† and A. Levy*

*University Research Centre for Neuroendocrinology, University of Bristol, Bristol Royal Infirmary, Bristol, UK.
†Department of Anatomy, School of Medical Sciences, University of Bristol, Bristol, UK.

Key words: hypothyroidism, HPA axis, iodine deficiency.

## Abstract

Many millions of people throughout the world are at risk of developing iodine deficiency-associated disorders. The underlying effects of iodine deficiency on neuroendocrine function are poorly defined. We have studied stress-induced and diurnal variation in corticosterone secretion in female rats rendered chronically hypothyroid by feeding them an iodine-free diet for 6 months. Corticosterone secretory responses in iodine deficient animals were compared to those seen in animals rendered hypothyroid with propylthiouracil and untreated controls. By using a well-validated, automated blood sampling system to collect small samples of blood over the complete daily cycle in unrestrained animals, we have demonstrated for the first time that the normal diurnal rhythm of corticosterone secretion is lost in chronic iodine deficiency and that the corticosterone secretory response to the psychological stress of 10 min exposure to white noise is attenuated. Despite restoration of circulating triiodothyronine and thyrotropin releasing hormone- and thyroid stimulating hormone $\beta$-transcript prevalence in the hypothalamus and pituitary, respectively, 1 month after restoration of normal iodine-containing diet both the diurnal variation in corticosterone levels and the corticosterone secretory response to the noise stress remained reduced in amplitude compared to control animals. Thus, chronic hypothyroidism induced by iodine deficiency significantly attenuates hypothalamo-pituitary-adrenal axis activity, an effect that persists after functional recovery of the thyroid axis.

Iodine is an essential trace element which is critical for the formation of thyroid hormone and hence, for normal growth and development. Hypothyroidism resulting from an inadequate supply of iodine during foetal life can give rise to profound developmental defects of the central nervous system, whereas the major effect of early postnatal hypothyroidism is stunting of growth. The specific effects on neuroendocrine function following untreated, long-term hypothyroidism induced by chronic dietary iodine deficiency remain to be fully elucidated.

As well as producing the expected upregulation of thyrotropin releasing hormone (TRH) and thyroid stimulating hormone (TSH) levels and downregulation of serum triiodothyronine ($T_3$) and thyroxine ($T_4$), rat models of acute hypothyroidism such as surgical thyroidectomy or propylthiouracil (PTU) treatment are known to affect the hypothalamo-pituitary-adrenal (HPA) axis (1–6). Such changes will have effects on metabolism, immune function and the homeostatic response to stressful stimuli. There is general agreement that in the absence of thyroid hormone, the basal plasma corticosterone concentration remains either unchanged or is reduced, and that the stress- or corticotropin releasing hormone (CRH)-induced increase in plasma corticosterone observed in euthyroid animals is attenuated or abolished. In addition, it has been shown that the amplitude of the normal diurnal rise in circulating plasma corticosterone is gradually reduced over a 5-week period following thyroidectomy in female rats (1), and is restored within 2 weeks by the re-introduction of either $T_3$ or $T_4$.

All previous studies of chronic hypothyroidism in rats have been based on work using either surgical thyroidectomy or treatment with PTU. In the present study, we have investigated the effects of a more physiologically relevant model of long-term iodine deficiency on the diurnal pattern of plasma corticosterone levels, the corticosterone secretory response to a 10 min white noise stress and rapid glucocorti-

*Correspondence to:* Dr Lesley Nolan, University Research Centre for Neuroendocrinology, University of Bristol, Bristol Royal Infirmary, Lower Maudlin Street, Bristol BS2 8HW, UK (e-mail: lesley.a.nolan@bris.ac.uk).

coid feedback. This has been compared to the effects of long-term PTU ingestion.

## Materials and methods

### *Animals and diets*

Female Wistar rats, aged 3 weeks at the start of feeding each experimental diet were used. Animals were allowed continuous free access to normal drinking water and were housed in groups of up to four on a 14 : 10 h light : dark cycle (lights on at 05.00 h). All rats were weighed at intervals of 4 weeks throughout the experimental period. Two groups of rats (n = 16) were allowed free access to either normal powdered diet (Rat Standard Diet no. 1, Bantin & Kingman Ltd, Hull, Humberside, UK), or a commercially prepared, powdered, low-iodine containing diet (LID diet number TD 95007; Harlan, Bicester, UK) for a period of 6 months. This period of treatment was chosen following a previous study in our laboratory during which marked physiological effects were produced in iodine-deprived rats particularly in relation to fertility (7). The maximum level of iodine in the normal diet was 3.04 mg/kg compared with 0.05 mg/kg in the low iodine diet. A third group of rats were fed the low-iodine containing diet for 6 months and were then returned to a normal powdered diet for 4 weeks before the study of hormone levels. For comparative purposes, two additional groups of rats were allowed free access for 6 months to a powdered diet containing either 0.5% (wt/wt) PTU (Sigma, Poole, Dorset, UK) or a powdered diet containing 0.5% PTU and 40 μg/kg $T_3$ (Link Pharmaceuticals, Horsham, UK). All animal procedures were carried out in accordance with UK Home Office animal welfare regulations.

### *Jugular vein cannulation*

Cannulation was carried out as described previously (8). Animals were anaesthetized using a combination of Hypnorm (0.32 mg/kg fentanyl citrate and 10 mg/kg fluanisone, i.m., Janssen Pharmaceuticals, Oxford, UK) and diazepam (2.6 mg/kg i.p., Phoenix Pharmaceuticals, Gloucester, UK). The right jugular vein was exposed and a silastic-tipped polythene cannula (Dow Corning, Midland, MI, USA; o.d., 0.96 mm; i.d., 0.58 mm; Portex, Hythe, UK) filled with 10 U/ml heparinized isotonic saline was inserted into the vessel until it lay close to the entrance of the right atrium. The free end of the cannula was exteriorized through a scalp incision and then tunnelled through a protective spring that was anchored to the parietal bones using two stainless steel screws and self-curing dental acrylic. After recovery, animals were moved to individual housing cages, and the end of the spring was attached to a mechanical swivel that rotated through a vertical plane, giving the animals maximum freedom of movement. The cannulae were flushed daily with heparinized saline solution. The perioperative mortality during the surgical and recovery phases of these experiments was slightly higher in PTU-treated rats than in rats in other dietary groups.

### *Experimental paradigm*

Four days after surgery, the cannulae were connected to an automated sampling system via air-tight swivels, as previously described (9). The animals were connected to the system at 18.00 h and sampling initiated at 06.00 h the next morning. Samples (30 μl) were collected every hour for a period of 24 h to determine the basal profiles of corticosterone release. At 06.00 h the following day, the sampling system automatically switched to collection of blood samples at 10 min intervals. After a further 2-h period, a white noise generator was activated and the animals were exposed to 114 dB (12,000–60 000 Hz) for 10 min in order to test for responses to acute psychological stress. Sampling continued at 10 min intervals for a further 3 h after which the animals were left undisturbed without further sampling. All blood samples were collected at a 1 : 4 dilution in heparinized saline i.e. a total sample volume of 150 μl. The plasma fraction was separated by centrifugation and used for the measurement of corticosterone concentration.

To examine possible variation in the glucocorticoid feedback on both basal plasma corticosterone levels and the response to white noise stress, on day 7 after surgery animals were sampled again between 06.00 h and 11.00 h, and were given intravenous injections of 2 mg methylprednisolone (Pharmacia & Upjohn Ltd, Milton Keynes, UK) via the indwelling cannula 40 min prior to a period of noise stress. This high dose of methylprednisolone was chosen to be well in excess of that required for maximum feedback so that differences in body weight on glucocorticoid clearance would have no effect on the results.

Up to 4 days following the exposure to the noise stress, rats were killed by decapitation, between 14.00 h and 15.00 h. The brains and pituitary glands were collected, frozen on dry ice and stored at −80°C for *in situ* hybridization studies. Trunk blood was collected into heparinized tubes for separation of plasma fraction and measurement of $T_3$ and corticosterone binding protein (CBG) concentrations.

### *Recording of behavioural responses*

The behaviour of animals was recorded onto videotape using a system comprising of individual video cameras (WV-BP 100, Panasonic, Osaka, Japan), a sequential camera selection system (Gem Mono Multiplexer, Norbain Security Ltd, USA) and a high performance VCR unit (HS5424, Mitsubishi Electric Company, Osaka, Japan). Observations were monitored from a separate room. Total active time and the number of rearings made by each animal were retrospectively scored for 10 min prior to the application of noise stress, 10 min during noise stress and for 10 min immediately following stress. Results were expressed as mean ± SE.

### *Hormone and CBG assays*

Total plasma corticosterone concentrations were measured directly in plasma by RIA using a citrate buffer at pH 3.0 to denature the binding globulin (4 μl diluted plasma fraction in 100 μl buffer), antiserum kindly supplied by Prof. G. Makara (Institute of Experimental Medicine, Budapest, Hungary) and [$^{125}$I]-corticosterone (ICN Biomedicals, Irvine, CA, USA) with a specific activity of 2–3 mCi/μg (8). The assay had a limit of detection of 5 ± 1 ng/ml, and intra- and interassay coefficients of variation were measured at 12.4% and 16.0%, respectively.

To confirm the comparative degree of hypothyroidism induced by the two experimental models, plasma $T_3$ concentrations were measured using a commercially available kit (Amerlex-M T3 RIA kit, Ortho-Clinical Diagnostics, Amersham International plc, Amersham, Bucks, UK) according to the manufacturer's instructions. The sensitivity of the assay was 0.098 ng/ml. Serum $T_4$ levels were not measured in this study but have previously been shown in our laboratory to be reduced to approximately 10% of control values after 4–6 months of dietary iodine deprivation (7).

As increased levels of free plasma corticosterone may effectively occur in the presence of a normal total corticosterone concentration when combined with reduced plasma CBG concentration, plasma CBG levels were determined by single point assays as previously described (8). Protein content was measured using the Bradford method and the final values were expressed as picomoles of [$^3$H]corticosterone bound per mg protein.

### In situ *hybridization histochemistry*

*In situ* hybridization histochemistry was performed on 12 μm-thick coronal brain sections cut through the paraventricular nucleus (PVN), the hippocampus at Bregma −5.3 mm (10) and axial pituitary sections cut at −18C and thaw mounted onto gelatin-coated slides as previously described (11, 12). Briefly, for oligodeoxynucleotide probes, sections were warmed to room temperature and allowed to dry for 10 min before fixing in 4% formaldehyde in PBS for 10 min. Sections were washed in phosphate-buffered saline and then incubated in 0.25% (v/v) acetic anhydride and 1.4% (v/v) triethanolamine in 0.9% saline for 10 min at room temperature. Sections were transferred through 70% (1 min), 80% (1 min), 95% (2 min) and 100% (1 min) ethanol, 100% chloroform (5 min), 100% (1 min) and 95%(1 min) ethanol before being air dried. For brain sections, hybridization was carried out using $^{35}$S-dATP 3′ end-labelled synthetic 48mer oligonucleotide probes complementary to either TRH ($2 \times 10^5$ counts/slide), CRH ($2 \times 10^5$ counts/slide), or vasopressin (AVP) (25 000 counts/slide) mRNA. For pituitary sections, hybridization was carried out using similarly labelled probes complementary to either TSH$\beta$ ($2 \times 10^5$ counts/slide) or pro-opiomelanocortin (POMC) ($10^5$ counts/slide). All mRNA oligonucleotide probe sequences are as described previously (4, 13). After hybridization, sections were washed for 1 h in four changes of 1 × SSC and for a further hour in two changes of 1 × SSC at room temperature.

Studies of the expression of mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) were undertaken in order to determine whether variations in receptor gene expression could account for any variation in either the circadian rhythm of HPA activity, feedback responses to glucocorticoids or the variable stress response. Full-length sense and antisense rat MR and GR transcripts incorporating $^{35}$S-UTP were generated from the vectors prMREH-GEM-4 and prGR-GEM-3Z, respectively (kindly provided by Prof. J. Seckl, Molecular Medicine Centre, University of Edinburgh, UK) using an Sp6/T7 transcription kit according to the manufacturers instructions (Boehringer Mannheim, Lewes, East Sussex, UK). Tissue sections were fixed in 4%

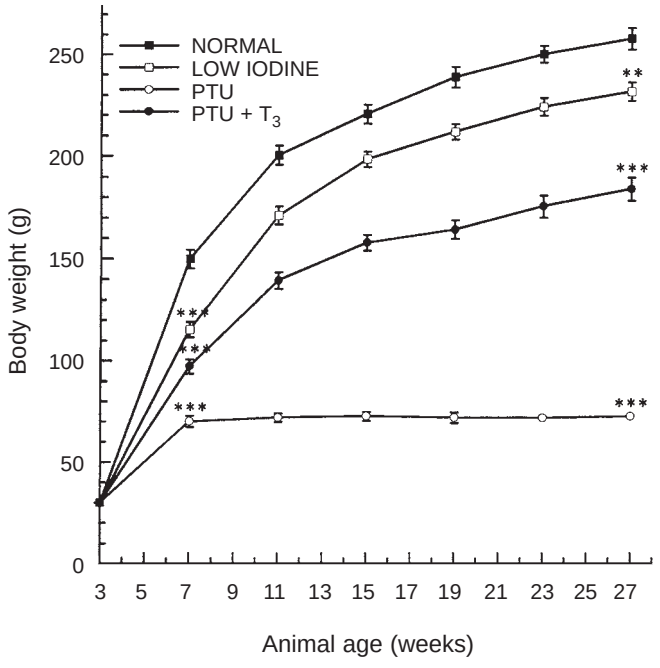


FIG. 1. The effects of hypothyroidism on body weight. Experimental diets were started at age 3 weeks. Means ± SE are shown; n = 16. Statistical marks (**$P < 0.01$; ***$P < 0.001$ compared to controls) are shown for 7 and 27 weeks only.

paraformaldehyde, prehybridized as above and hybridized overnight at 50°C in 45 µl of hybridization buffer containing 50% formamide, 4 × SSC, 1 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 10% dextran sulphate (molecular weight 500 000) and $10^6$ counts of probe/slide. Before adding riboprobe to the hybridization buffer, it was mixed with 2 µl of nucleic acid solution/slide (500 µg/ml sheared, single stranded salmon testis DNA and 250 µg/ml yeast transfer RNA), heated to 65°C for 5 min and quenched on ice. Following hybridization, coverslips were gently lifted off in 2 × SSC at room temperature and the slides washed for 15 min in two changes of 2 × SSC/50% formamide at 50°C. Sections were then rinsed briefly in 2 × SSC at 37°C and incubated in 2 × SSC containing 20 µg/ml RNAase A for 30 min at 37°C. Sections were again rinsed in 2 × SSC, then washed in 3 × 15 min changes of 2 × SSC/50% formamide at 50°C, followed by two room temperature washes in 2 × SSC for 5 min each.

Slides were briefly dipped in water then air-dried. Dry hybridized sections were opposed to autoradiography film (Hyperfilm MP, Amersham International plc) for up to 14 days, and the resulting images were analysed densitometrically using a Macintosh IIci computer equipped with an image capture board (Scion, Walkersville, MD, USA) running the program Image by Wayne Rasband, NIMH (Bethesda, MD, USA). For AVP mRNA expression in the PVN, only the medial half of the nucleus was included in the area analysed in order to exclude the signal from the lateral magnocellular regions. Results were expressed either as the mean deviation from controls (% ± SE) or as arbitrary units representing integrated optical densities (means ± SE).

*Statistical analysis*

GraphPad Instat™ (GraphPad Software, San Diego, CA, USA) was used to perform statistical calculations. Differences between multiple groups were evaluated using one-way ANOVA followed by Tukey-Kramer multiple comparison post tests. $P < 0.05$ was considered statistically significant.

## Results

### *Effect of diet on body weight*

In both experimental models, hypothyroid rats failed to grow at the same rate as normal controls (Fig. 1). Within 4 weeks of changing to a low iodine diet, animals weighed significantly less than those receiving a normal diet, and by the age of 27 weeks remained 10% smaller (low iodine 231 ± 4.5 g *vs* controls 257 ± 5.5 g; means ± SE; n = 16; $P < 0.01$). Weights of iodine-deprived rats restored to a normal control diet for the following four-week period are not shown. In contrast to this mild, early growth retardation, rats rendered hypothyroid by administration of dietary PTU failed to grow at all past the age of 7 weeks and weighed only 72 ± 5.5 g at 27 weeks, an effect which was partially ameliorated by concurrent treatment with $T_3$.

### *$T_3$, CBG and transcriptional effects of hypothyroidism*

As expected, chronic iodine deprivation resulted in a four-fold increase in PVN TRH transcripts (389 ± 101% of control; n = 6; $P < 0.01$; Fig. 2A) and a five-fold increase in anterior pituitary TSH$\beta$ transcripts (526 ± 46% of control; n = 6; $P < 0.001$; Fig. 2B). Within 1 month of restoration of these animals to a normal dietary iodine intake, both TRH and TSH$\beta$ transcript levels were restored to control values. No significant changes were detected in PVN CRH, parvocellular AVP or anterior pituitary POMC transcripts in iodine deprived rats (Fig. 2C–E), although 1 month after restoration of a normal dietary intake CRH transcript levels were significantly higher than controls (180 ± 20% of control; n = 6; $P < 0.01$; Fig. 2C).

PTU-induced hypothyroidism also resulted in increases in PVN TRH and anterior pituitary TSH$\beta$ transcripts similar to the low iodine group, but concurrent $T_3$ replacement (at 40 µg/kg food) alone was not sufficient to prevent these PTU-induced changes. In contrast to low-iodine diet, PTU treatment for 6 months resulted in a very marked reduction in CRH transcripts in the PVN to 12 ± 2.7% of the control value (n = 4; $P < 0.01$; Fig. 2C), together with a downward trend in parvocellular AVP and anterior pituitary POMC transcripts (Fig. 2D,E) similar to that previously demonstrated after just 2 weeks treatment (4). Concurrent $T_3$ replacement prevented the reduction in CRH transcript levels.

Serum $T_3$ levels were slightly but not significantly reduced following chronic iodine deprivation compared to normal controls and significantly reduced in animals treated with PTU (n = 4; $P < 0.001$; Fig. 3A), thus confirming the less profound nature of the hypothyroidism induced by long-term iodine deficiency. However, we were unable to investigate the precise relationship of any phase shifts in $T_3$ *vs* corticosterone diurnal rhythms over a complete 24-h period due to the small sample size obtainable from the frequent sampling system employed. Return of iodine-deprived rats to a normal dietary intake for 1 month also restored serum $T_3$ levels whereas concurrent treatment with PTU and $T_3$ maintained $T_3$ levels below that found in normal control animals.

Plasma CBG concentrations were unaffected by chronic iodine deprivation but were significantly reduced following treatment with PTU (n = 4; $P < 0.001$; Fig. 3B). Concurrent $T_3$ replacement only partially restored the CBG concentration towards that found in normal control animals.

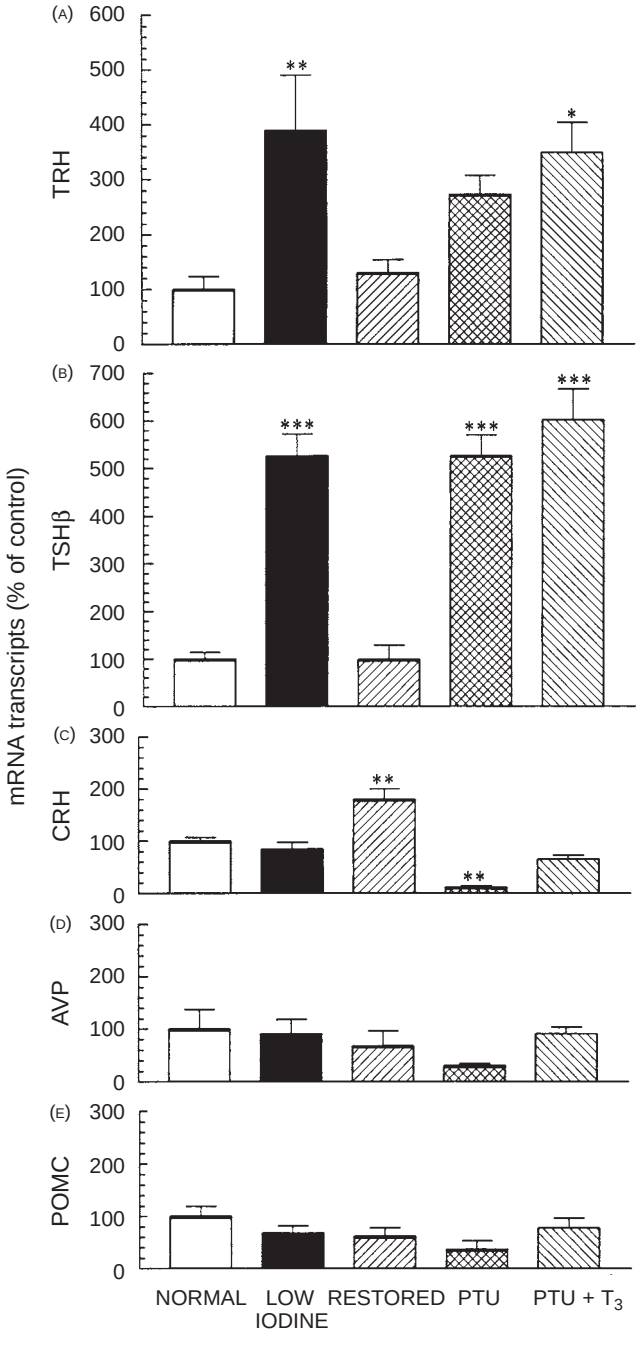


FIG. 2. The effects of a normal powdered diet for 6 months (NORMAL), a low iodine diet for 6 months (LOW IODINE), a low iodine diet for 6 months followed by restoration of a normal diet for 1 month (RESTORED), dietary propylthiouracil (PTU) for 6 months (PTU) and dietary PTU + $T_3$ for 6 months (PTU + $T_3$) on the prevalence of (A) paraventricular nucleus (PVN) thyrotropin releasing hormone (TRH), (B) pituitary thyroid stimulating hormone (TSH) $\beta$, (C) PVN corticotropin releasing hormone (CRH), (D) medial PVN vasopressin (AVP) and (E) pituitary pro-opiomelanocortin (POMC) mRNA transcripts. Mean percentage deviation from controls ± SE; n = 4–6. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to controls.

*Diurnal variation in plasma corticosterone*

The mean plasma corticosterone concentration in rats fed normal powdered diet showed a characteristic, diurnal rhythm with the highest levels occurring between 18.00 h and 22.00 h and a peak value of 159 ± 14 ng/ml at 18.00 h (Fig. 4). Mean corticosterone concentrations varied significantly between the nadir and acrophase showing a 2.5-fold difference in levels (Table 1). The diurnal variation in circulating corticosterone was abolished and the mean nadir levels of corticosterone reduced by both iodine deprivation (Fig. 4A) and by dietary PTU treatment for 6 months (Fig. 4B and Table 1). Normal diurnal corticosterone variation was not re-established by restoration of a normal iodine containing diet for one month or by the addition of $T_3$ to PTU-containing diet.

*HPA and behavioural response to noise stress*

In animals fed a normal diet, a 10-min noise stress beginning at 08.00 h elicited a rapid increase in corticosterone release, which peaked 20 min after the onset of noise when the plasma corticosterone concentration reached 224 ± 29 ng/ml, before rapidly declining (Fig. 5). During a period of maximum inhibition between 60 and 90 min after the onset of the stress, hormone levels fell below baseline before recovering to pre-stress levels. Chronic iodine deprivation resulted in a reduction in the plasma corticosterone response to noise measured 20 min after the onset (124 ± 20 ng/ml; n = 6; $P < 0.05$ compared to controls), which was partially restored after a return to an iodine-replete diet for 1 month (Fig. 5A). There was no effect on the timing of the peak, the subsequent rapid decline to pre-stress levels or the period of inhibition. In contrast, the plasma corticosterone response to noise stress was completely abolished in PTU-treated animals with a partial recovery achieved in animals receiving concurrent PTU and $T_3$ replacement (Fig. 5B). Furthermore, the PTU treated animals showed no post-stress decline in levels during the period of maximal inhibition.

Mean basal plasma corticosterone concentrations for each animal were calculated from the samples obtained during the 2 h period immediately prior to the application of noise stress, and subtracted from each of the six samples collected immediately following the onset of noise. Differences in (a) the plasma corticosterone concentration 20 min after the onset of noise and (b) the total corticosterone response made during the 60-min period immediately following the onset of noise ('Δ area under the curve'), are shown in Table 2. Chronic iodine deficiency and PTU administration significantly reduced the plasma corticosterone response to noise stress (20 min after the onset of noise) compared to control ($P < 0.05$ and $< 0.01$, respectively), and reduced the area under the corticosterone response curve, which reached significance in the PTU-treated animals.

All groups of animals showed increases in both total activity and number of rearings during the period of noise stress (Fig. 6), although this did not reach statistical significance in the PTU- or the PTU + $T_3$-treated groups of rats due to the higher level of spontaneous activity (Fig. 6D,E). With the exception of iodine-deprived rats restored to a normal diet, the period of total activity began to decline during the 10 min following the cessation of noise. There were no statistically significant differences in the behaviour patterns

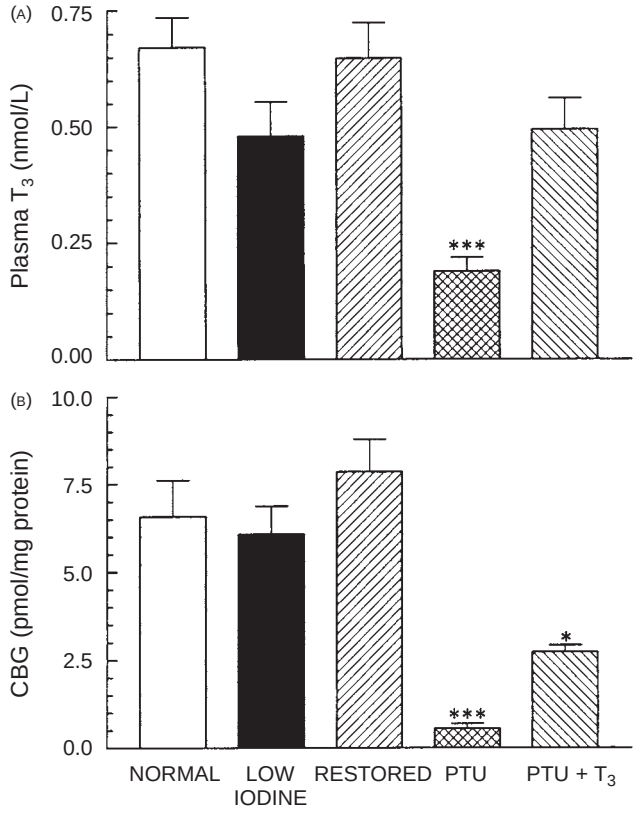


FIG. 3. The effects of a normal powdered diet for 6 months (NORMAL), a low iodine diet for 6 months (LOW IODINE), a low iodine diet for 6 months followed by restoration of a normal diet for one month (RESTORED), dietary propylthiouracil (PTU) for 6 months (PTU) and dietary PTU + $T_3$ for 6 months (PTU + $T_3$) on the plasma concentration of (A) $T_3$ and (B) corticosterone binding protein (CBG) protein. (Means ± SE; n = 4–6). *$P < 0.05$; ***$P < 0.001$ compared to controls.

observed between any of the dietary groups in any individual time period.

### *Suppression of secretory corticosterone responses by prednisolone*

In view of the possibility that the change of diurnal rhythmicity or dynamic response to stress may be due to altered glucocorticoid negative feedback, the effect of exogenous prednisolone was tested on basal and stress-induced corticosterone secretion. An intravenous bolus of prednisolone 40 min prior to the application of noise stress blocked the increase in plasma corticosterone concentration seen in the absence of prednisolone in all rats (Fig. 7). In the normally fed rats, the mean baseline plasma corticosterone concentration also decreased from 46 ± 6.9 ng/ml prior to the administration of prednisolone to 5.5 ± 2.2 ng/ml for the period between 1 and 3 h after the onset of noise stress (n = 5; $P < 0.001$; Fig. 7). Similar significant reductions in mean plasma corticosterone concentrations following the period of noise stress also occurred in all dietary groups of animals although mean plasma corticosterone levels measured in PTU-treated rats following the period of noise stress remained significantly higher than those measured in normally fed animals (Fig. 7C).

### *Effect of hypothyroidism on MR and GR mRNA expression*

The relative expression of GR and MR mRNA transcripts showed the expected pattern of distribution within the PVN and subfields of the hippocampus. Whilst MR mRNA expression was found through all regions of the hippocampus, it was not detected in the PVN, and GR mRNA was low in the CA3 subfield but detected in all other hippocampal areas and in the PVN. No significant variation in this pattern was detected in any of the treatment groups (Fig. 8). The anterior pituitary expressed both GR and MR mRNA transcripts and whilst the treatments did not affect MR expression, GR mRNA was significantly increased in PTU-treated animals (Fig. 8A), an effect partially reversed by concurrent treatment with $T_3$.

## Discussion

This study has demonstrated for the first time that chronic iodine deficiency is associated with loss of the normal diurnal rhythm of corticosterone secretion and an attenuated corticosterone response to acute psychological stress. This contrasts with the results of a study reported 25 years ago in which no changes in the diurnal rhythm of corticosterone were detected in iodine-deficient Sprague-Dawley rats (14). The reasons for this difference are unknown but may relate to their method of sample collection in ether-anaesthetized animals. Thyroidectomy has been shown to result in a progressive loss of diurnal rhythm of corticosterone which can be restored by either $T_3$ or $T_4$ (1, 15), and the current study has shown that both iodine deprivation and PTU have a similar effect. However, while the loss of rhythm in PTU-treated animals may be explained by loss of CRH synthesis, this does not appear to underlie the effect in iodine-deprived animals, whose abnormalities in HPA function occur in the presence of normal or only marginally reduced levels of PVN CRH and AVP transcripts, anterior pituitary POMC transcripts, hippocampal and pituitary GR and MR transcripts and serum CBG concentrations. Because this study has not attempted to address potential changes in the levels of functional hypothalamic, hippocampal and pituitary peptides, it remains possible that the presence or absence of measurable

TABLE 1. The Effects of Iodine Deprivation and Propylthiouracil (PTU) on Mean Nadir and Acrophase Plasma Corticosterone.

| | Circulating corticosterone (ng/ml) | |
|---|---|---|
| Group (n ≥ 5) | Nadir | Acrophase |
| Normal diet | 57 ± 15 | 145 ± 17 |
| Low iodine diet | 29 ± 6 | 44 ± 13*** |
| Iodine restored | 41 ± 11 | 51 ± 17*** |
| PTU | 33 ± 7 | 28 ± 18*** |
| PTU + $T_3$ | 39 ± 13 | 33 ± 18*** |

Means ± SEM are shown. ***$P < 0.01$ compared with controls.

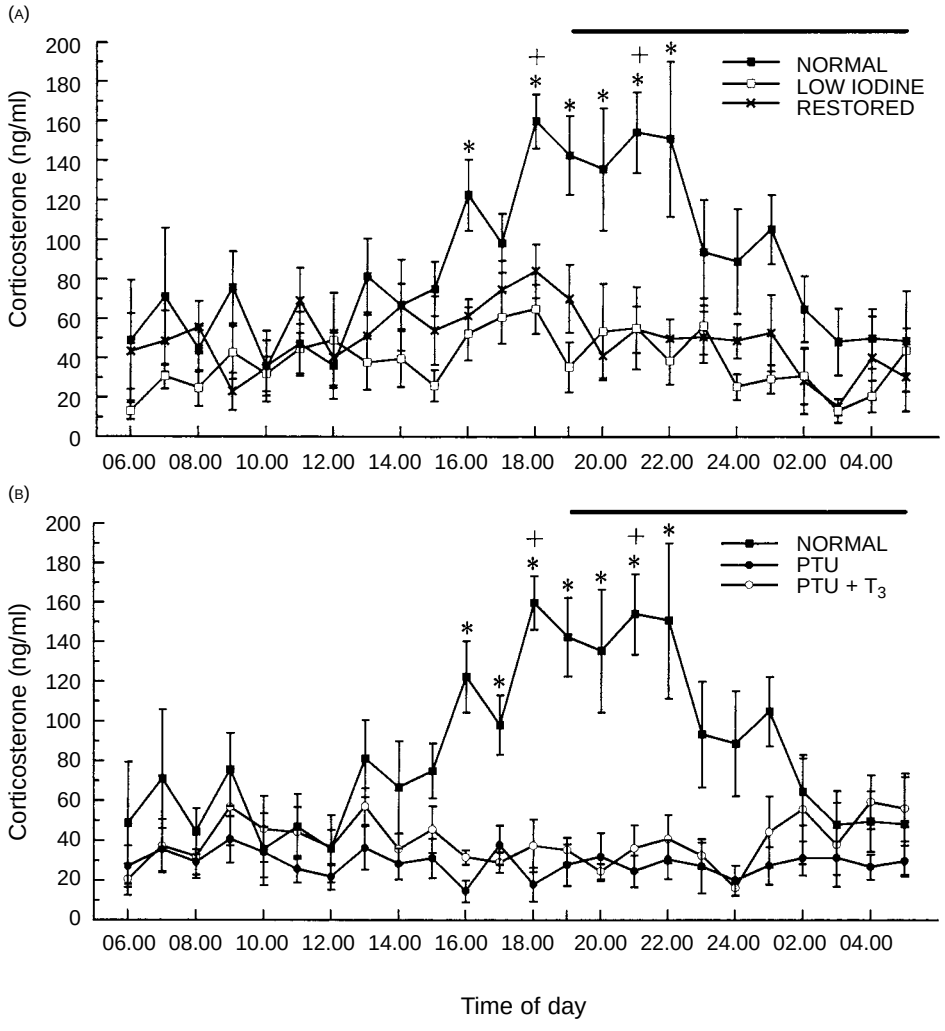


FIG. 4. Twenty-four hour profiles of plasma corticosterone concentrations in female Wistar rats fed (A) a low iodine diet for 6 months (LOW IODINE) or a low iodine diet followed by restoration of a normal diet for one month (RESTORED), and (B) propylthiouracil (PTU) or PTU + $T_3$ for 6 months compared with control animals receiving a normal diet throughout (NORMAL). Means ± SE are shown; n = 5–9. + $P < 0.05$ compared with morning samples in control animals; * $P < 0.05$ compared with the same time point from different dietary groups. Solid bar represents the dark period.

changes in transcript levels for these peptides does not correlate with actual changes in protein concentration. Restoration of normal iodine-containing diet (without further iodine supplementation), returned hypothalamic TRH and pituitary TSH$\beta$ transcript prevalence to control levels, but was insufficient to restore the diurnal pattern of corticosterone secretion or to fully restore the HPA response to stress.

This study has also demonstrated important differences between experimental models of hypothyroidism induced by chronic iodine deficiency and hypothyroidism induced by PTU, a type 1 5′-iodothyronine deiodinase inhibitor. In general, the effects of PTU administration are more profound than those of iodine deprivation in terms of growth retardation, serum $T_3$ and CBG concentration, CRH transcript downregulation and corticosterone secretory response to acute noise stress. The amount of chow consumed by the rats was not measured and it is therefore possible that part of the effects observed are due to low calorie intake as opposed to hypothyroidism alone. However, the body weights of animals receiving concurrent $T_3$ replacement in the PTU-containing diet were significantly higher than those receiving PTU alone, an effect that is unlikely to be due to variances in palatability of the diets. Animals receiving the low iodine diet grew at the same rate as control rats after the age of 7 weeks which suggests that the effects of diet on bodyweight were manifest during a restricted time period only. Furthermore, low calorie intake would result in central downregulation of TRH (and TSH) transcription (16), rather than upregulation of TRH transcript prevalence as was seen here. Both chronic iodine deficiency and PTU treatment have a similar effect on the early evening rise in corticosterone secretion characteristic of normal diurnal variation, together with a small reduction in the mean basal corticosterone levels seen throughout the rest of the 24 h cycle.

In contrast to animals fed PTU alone, concurrent $T_3$ treatment decreased the degree of growth retardation, prevented downregulation of CRH transcripts and limited the reduction in corticosterone secretory response to acute

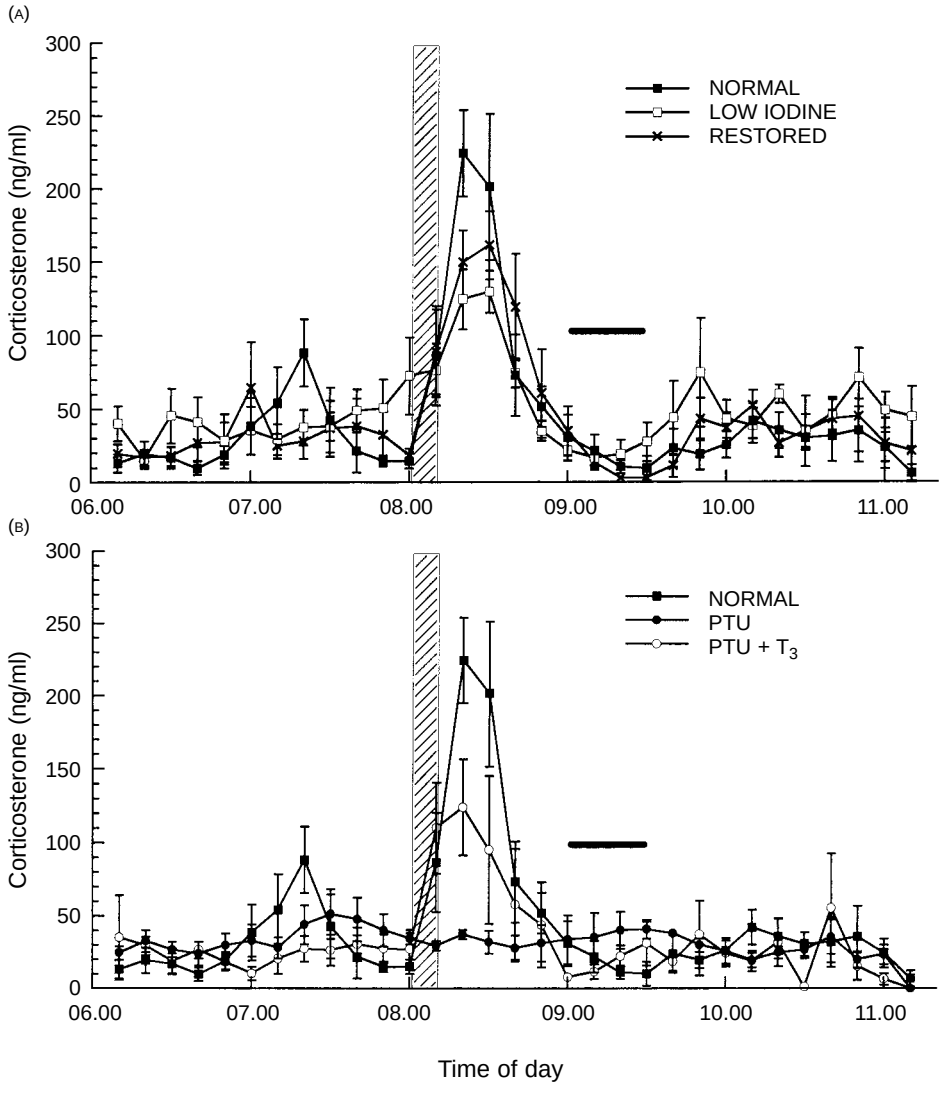


FIG. 5. The effect of noise stress (114 dB × 10 min commencing at 08.00 h; hatched bar) on plasma corticosterone concentrations in female Wistar rats fed (A) a low iodine diet for six months (LOW IODINE) or a low iodine diet followed by restoration of a normal diet for one month (RESTORED), and (B) propylthiouracil (PTU) or PTU + $T_3$ for 6 months compared with control animals receiving a normal diet throughout (NORMAL). Means ± SE are shown; n = 5–6. The period 60–90 min after the onset of noise (indicated by the bar) represents the period of maximum inhibition.

noise stress seen in animals fed PTU alone. With the exception of the hypothalamic CRH transcript prevalence, however, none of the parameters was returned completely to normal. This also suggests that there is a dissociation between the effects of PTU with or without $T_3$ on CRH transcript prevalence and basal and stress-induced HPA activity. These findings corroborate the observation made by others that maintenance of normal serum $T_3$ concentration by itself is not sufficient to maintain tissue euthyroidism at least at the pituitary level (17–19) and indicate that the maintenance of euthyroidism in PTU-treated rats requires $T_4$ replacement with or without $T_3$, as has been found to be the case following surgical thyroidectomy and thiouracil administration (5, 20). It remains possible, nevertheless, that PTU (a drug derived from immunomodulatory thiourea antihypertensives that were incidentally found to be goitrogens) has other actions unrelated to the induction of hypothyroidism (21).

The loss of the normal diurnal variation in corticosterone secretion observed in both of the models of long-term hypothyroidism used in this study and the gradual loss seen five weeks following surgical thyroidectomy in the study by Murakami *et al.* (1), suggests that thyroid hormones have an important role to play in the pathway responsible for the instigation of the early evening rise in corticosterone levels. CRH has also been implicated as one of a number of factors involved in the neuroendocrine control of this pathway (22). Muglia *et al.* (23) have shown that in adult CRH knockout mice, the diurnal variation in plasma corticosterone is absent in males and severely attenuated in females, but is restored to normal by constant infusion of CRH in both sexes even with the underlying presence of a dysregulated rhythm in serum ACTH production in these mice. Bagdy *et al.* (24) have also demonstrated that CRH immunoneutralization abolished the evening rise in corticosterone in male Sprague-Dawley rats.

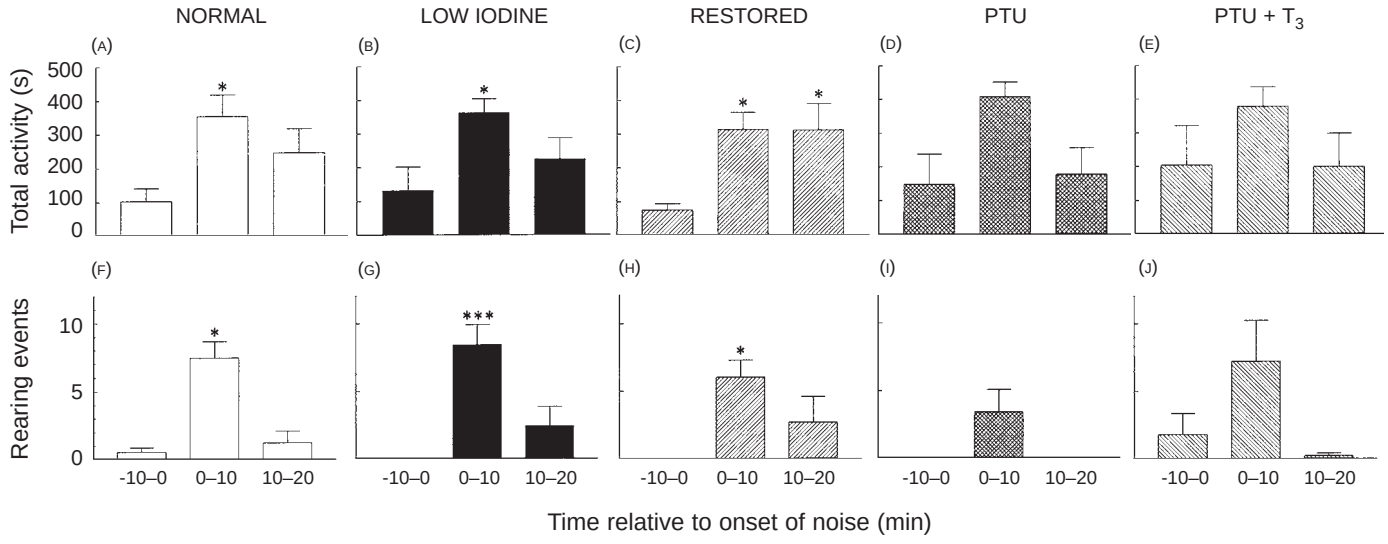


FIG. 6. The effect of hypothyroidism on the behavioural response to noise stress. Time spent engaged in total activity (A–E) and the total number of rearing events observed (F–J) are shown for each dietary group. Means ± SE are shown; n = 5–6. *P < 0.05, ***P < 0.001 compared with prenoise values in each group.

While a deficit in CRH may explain the loss of a diurnal rhythm in the PTU-treated rats, there appears to be no such deficit in iodine-deficient animals to explain the loss of rhythmicity, although more subtle changes to CRH synthesis and release may have occurred.

Interactions between the generation of the hypothyroid state and disturbances in the HPA axis of rats have been extensively reported (1–7, 25, 26). The almost complete loss of detectable CRH transcripts found after only 14 days of PTU treatment (4) was mirrored in the current study following six months of PTU treatment, although one further study reported no such decrease in hypothalamic CRH detected by Northern analysis following 7 weeks of PTU treatment (26). The loss of CRH transcripts observed in PTU-treated animals in this study occurred in the presence of significantly reduced plasma CBG levels which may lead to increased free corticosterone concentration and a marked negative feedback on CRH gene transcription. Although CRH transcripts have also been shown to be reduced at both 28 and 50 days after surgical thyroidectomy (3), they were not found to be significantly reduced following 6 months of iodine deprivation in either this study or that reported by Kondo *et al.* (7), perhaps reflecting a less severe degree of hypothyroidism in these animals (compared to those treated with PTU) associated with normal circulating levels of CBG. PTU treatment completely abolished and chronic iodine deficiency significantly attenuated the amplitude of corticosterone secretory response to an acute noise stress compared to controls. These data provide further evidence of the less severe nature of the effects of hypothyroidism induced by iodine-deprivation compared to that induced by PTU treatment. Others have found that the corticosterone secretory response to a 3-h restraint stress is also significantly reduced after 4 weeks of thiouracil treatment (despite increased plasma concentrations of ACTH) and that $T_4$ treatment partly restored the response (5).

The presence of a CRH release-inhibiting factor termed preproTRH178–199 and encoded within the TRH precursor sequence has also been identified, thus providing further evidence of the linkage between the HPA and hypothalamo-

TABLE 2. The Effects of Iodine Deprivation and Propylthiouracil (PTU) on Corticosterone Secretory Response to a White Noise Stress.

| | Circulating corticosterone (increase over pre-noise baseline) | |
|---|---|---|
| Group (n ≥ 5) | Level 20 min after the onset of the white noise stress (ng/ml) | Δ area under the curve for the 60 min from the end of the noise stress (arbitary units) |
| Normal diet | 191 ± 36 | 445 ± 88 |
| Low iodine diet | 84 ± 20* | 215 ± 77 |
| Iodine restored | 120 ± 22 | 422 ± 108 |
| PTU | 4 ± 2* | −12 ± 11* |
| PTU + $T_3$ | 140 ± 53 | 286 ± 190 |

Means ± SEM are shown. ***P < 0.05 and ***P < 0.01 compared with controls.

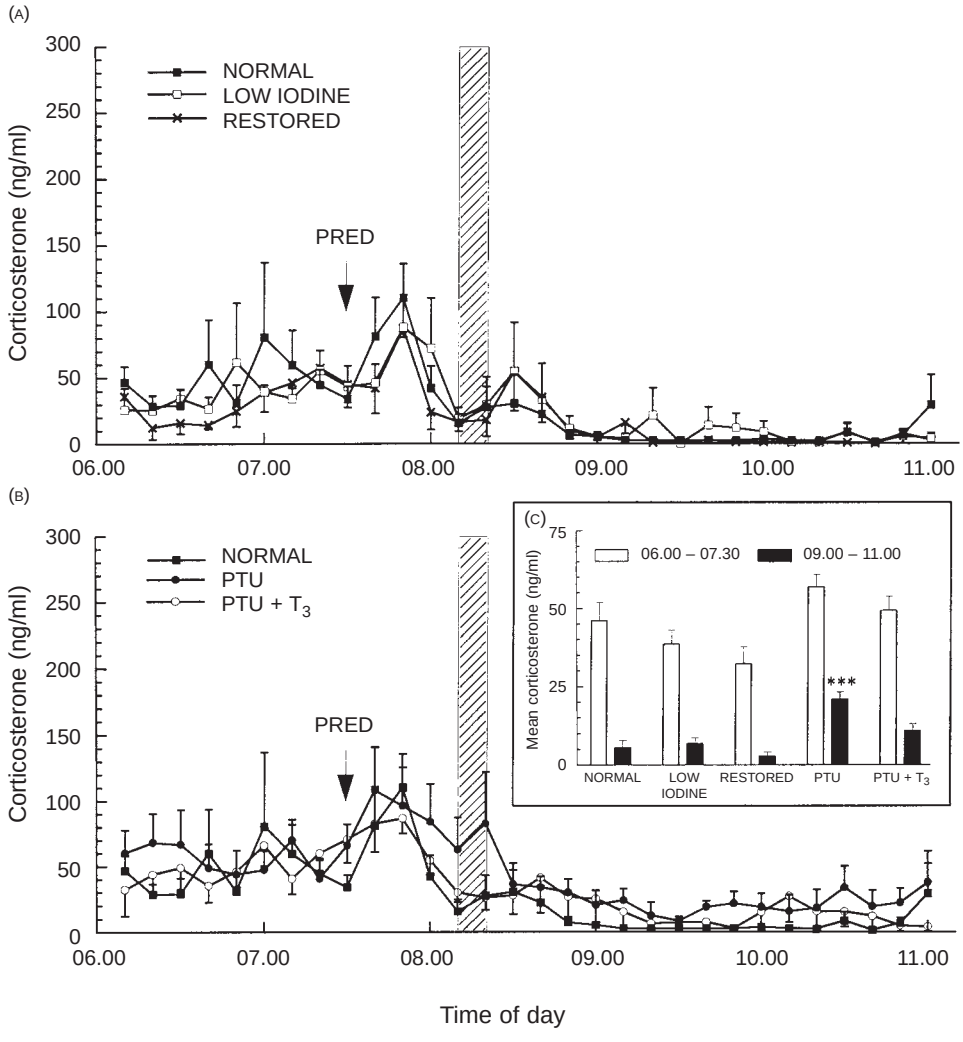


FIG. 7. The effect of prednisolone injection (arrow) given 40 min prior to the application of noise stress (114 dB × 10 min commencing at 08.10 h; hatched bar) on plasma corticosterone concentrations in female Wistar rats fed (A) a low iodine diet for 6 months (LOW IODINE) or a low iodine diet followed by restoration of a normal diet for one month (RESTORED) and (B) propylthiouracil (PTU) or PTU + $T_3$ for six months compared with control animals receiving a normal diet throughout (NORMAL). Means ± SE are shown; n = 5–7. Insert (C) shows a comparison of mean basal plasma corticosterone concentrations immediately prior to prednisolone administration (06.00–07.30 h) with that from 09.00 to 11.00 h following noise stress. ***$P < 0.001$ *vs* 0900–1100 control value (NORMAL).

pituitary-thyroid axes (25–28). The upregulation of TRH which occurs during hypothyroidism may therefore also result in an upregulation of preproTRH178–199 which has been demonstrated to result in the inhibition of pituitary ACTH synthesis and secretion both *in vitro* and *in vivo* (25–28). Administration of preproTRH178–199 to normal rats immediately prior to restraint stress significantly attenuated both the stress-induced rise in plasma ACTH seen in untreated controls, and the duration of the subsequent elevated plasma corticosterone concentration, but had no effect on the magnitude of the early secretory corticosterone response to stress (25). In the present study, the maximum amplitude of the corticosterone response to noise was still reduced 1 month after restoration of a normal diet to iodine-deprived rats despite a return to euthyroidism in terms of hypothalamic TRH and pituitary TSH$\beta$ transcripts, which suggests that increased CRH release-inhibiting peptide derived from increased TRH transcription is not solely responsible for the extended abnormalities in HPA axis function seen in the current study. In view of the chronicity and relatively early onset of iodine deficiency in the present study, the complete return of HPA axis responses given a longer period of recovery from iodine deprivation cannot be assumed.

The behavioural response to noise in terms of the total activity time and exploratory behaviour measured as the total number of rearings made by the rats did not significantly differ between the different groups of animals studied. Although it remains possible that a degree of auditory impairment was responsible for the lack of HPA response to a noise stress (29–32), rats from all the dietary groups displayed an increase in activity during the period of noise with a reduction beginning immediately after cessation of noise, thus indicating that PTU-treated animals do respond to noise despite the concurrent lack of an HPA response.

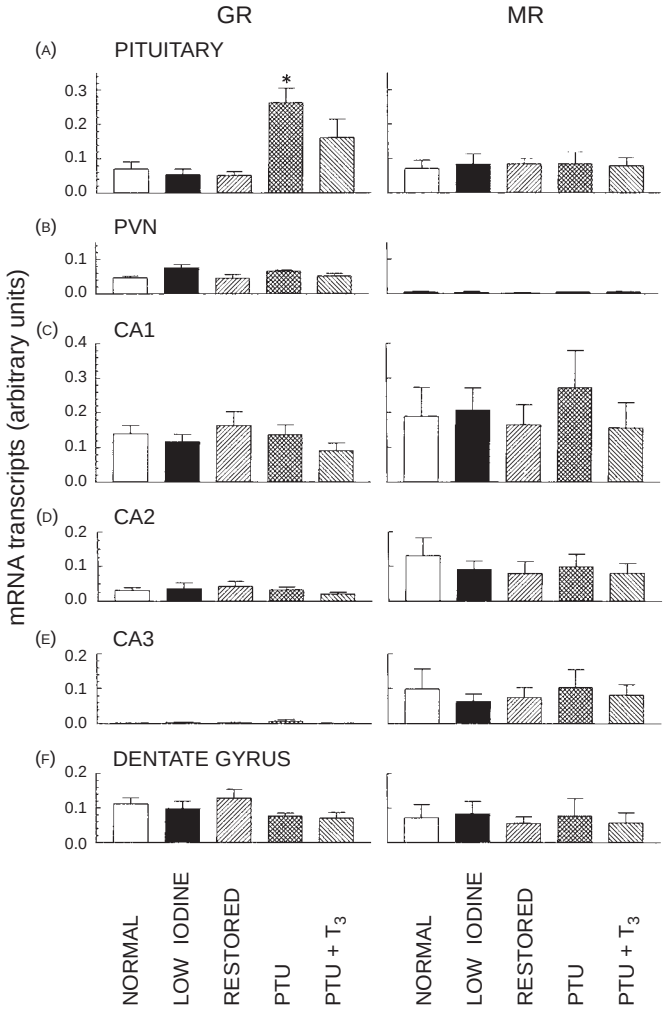


FIG. 8. The effects of a normal powdered diet for 6 months (NORMAL), a low iodine diet for 6 months (LOW IODINE), a low iodine diet for 6 months followed by restoration of a normal diet for one month (RESTORED), dietary propylthiouracil (PTU) for 6 months (PTU) and dietary PTU + $T_3$ for 6 months (PTU + $T_3$) on the prevalence of glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) mRNA transcripts in (A) pituitary (B) PVN and (C–F) hippocampal areas as indicated. Means ± SE are shown (optical density expressed in arbitrary units); n = 3–6. *$P < 0.05$.

The changes in CRH gene transcription, the change in diurnal corticosterone secretion and the attenuation of stress-induced HPA activity in PTU-treated animals may be explained by an increase in the efficacy of glucocorticoid negative feedback. Changes in negative feedback may either involve an increase in the effective free corticosterone concentration due to the very marked reduction in CBG levels or to a change in receptor-effector coupling of GR or MR in targets for negative feedback. Prior administration of methylprednisolone reduced the corticosterone secretory response to noise stress in control animals and further reduced the already attenuated corticosterone secretory response seen in iodine deficient rats. Mean plasma corticosterone levels measured in PTU-treated rats following the period of noise stress remained significantly higher than those measured in either normally fed animals or those receiving a low iodine diet. These data indicate that the feedback mechanisms operating on the HPA axis are still functional in chronically iodine deficient rats. However, PTU treatment reduced the efficacy of this rapid negative feedback on basal HPA activity. The mechanism underlying this effect is unclear as hippocampal and hypothalamic GR and MR expression was unaffected while pituitary GR mRNA in fact increased. This region-specific change in GR expression may have been the result of increased free corticosterone due to the very marked reduction in plasma CBG levels. Chronic (6 days) corticosterone treatment of intact female rats has been shown to have no effect on total hippocampal GR mRNA, but to increase anterior pituitary GR mRNA (33). Thus while chronic iodine deficiency appears to have no effect on glucocorticoid inhibition of HPA activity, PTU appears to disrupt negative feedback by a mechanism independent of any change in GR and MR gene transcription. This further emphasizes the marked difference in characteristics of these two models of hypothyroidism.

In conclusion, female rat HPA axis function in terms of corticosterone diurnal variation and the corticosterone secretory response to stress are downregulated by chronic iodine deprivation from weaning. Full recovery of the HPA axis lags behind recovery of the thyroid axis. The effects of PTU-induced hypothyroidism are more marked than those induced by iodine deprivation and result in virtually no detectable hypothalamic CRH transcripts, complete loss of the diurnal variation in plasma corticosterone pattern, abolition of the secretory corticosterone response to noise stress and an abnormal feedback to exogenously administered prednisolone. These findings indicate that chronic iodine deficiency can result in dysregulation of HPA axis secretory function that may persist in the presence of apparently normal thyroid axis function, and also suggest that long-term clinical use of PTU either alone or as part of high dose block and replace regimens designed to clamp thyroid function within the normal range may have unexpected and unwanted effects on the HPA axis and stress responses.

## Acknowledgements

This study was supported by The Wellcome Trust.

Accepted 15 June 2000

## References

1 Murakami N, Hayafuji C, Takahashi K. Thyroid hormone maintains normal circadian rhythm of blood corticosterone levels in the rat by restoring the release and synthesis of ACTH after thyroidectomy. *Acta Endocrinol (Copenh)* 1984; **107:** 519–524.

2 Kamilaris TC, DeBold CR, Johnson EO, Mamalaki E, Listwak SJ, Calogero AE, Kalogeras KT, Gold PW, Orth DN. Effects of short and long duration hypothyroidism and hyperthyroidism on the plasma adrenocorticotropin and corticosterone responses to ovine corticotropin-releasing hormone in rats. *Endocrinology* 1991; **128:** 2567–2576.

3 Ceccatelli S, Giardino L, Calza L. Response of hypothalamic peptide mRNAs to thyroidectomy. *Neuroendocrinology* 1992; **56:** 694–703.

4 Shi Z-X, Levy A, Lightman SL. Thyroid hormone mediated regulation of corticotrophin-releasing hormone mRNA in the rat. *Endocrinology* 1994; **134:** 1577–1580.

5 Tohei A, Akai M, Tomabechi T, Mamada M, Taya K. Adrenal and

gonadal function in hypothyroid adult male rats. *J Endocrinol* 1997; **152:** 147–154.
6 Tohei A, Watanabe G, Taya K. Hypersection of corticotrophin-releasing hormone and arginine vasopressin in hypothyroid male rats as estimated with push–pull perfusion. *J Endocrinol* 1998; **156:** 395–400.
7 Kondo K, Levy A, Lightman S. Effects of maternal iodine deficiency and thyroidectomy on basal neuroendocrine function in rat pups. *J Endocrinol* 1997; **152:** 423–430.
8 Windle RJ, Wood SA, Shanks N, Lightman SL, Ingram CD. Ultradian rhythm of basal corticosterone release in the female rat: dynamic interaction with the response to acute stress. *Endocrinology* 1998; **139:** 443–450.
9 Windle RJ, Wood S, Shanks N, Perks P, Condé GL, da Costa AP, Ingram CD, Lightman SL. Endocrine and behavioural responses to noise stress: comparison of virgin and lactating female rats during non-disrupted maternal activity. *J Neuroendocrinol* 1997; **9:** 407–414.
10 Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates.* San Diego: Academic Press, 1998.
11 Young WSI, Bonner TI, Brann MR. Mesencephalic dopamine neurons regulate the expression of neuropeptide mRNAs in the rat forebrain. *Proc Natl Acad Sci USA* 1986; **83:** 9827–9831.
12 Levy A, Lightman SL. Quantitative in-situ hybridization histochemistry in the rat pituitary gland: effect of bromocriptine on prolactin and proopiomelanocortin gene expression. *J Endocrinol* 1988; **118:** 205–210.
13 Ivell R, Richter D. Structure and comparison of the oxytocin and vasopressin genes from rat. *Proc Natl Acad Sci USA* 1984; **81:** 2006–2010.
14 Fukuda H, Greer MA, Roberts L, Allen CF, Critchlow V, Wilson M. Nyctohemeral and sex-related variations in plasma thyrotropin, thyroxine and triiodothyronine. *Endocrinology* 1975; **97:** 1424–1431.
15 Ottenweller JE, Hedge GA. Thyroid hormones are required for daily rhythms of plasma corticosterone and prolactin concentration. *Life Sci* 1981; **28:** 1033–1040.
16 Shi Z-X, Levy A, Lightman SL. The effect of dietary protein on thyrotrophin-releasing hormone and thyrotrophin gene expression. *Brain Res* 1993; **606:** 1–4.
17 Santisteban P, Obregon MJ, Rodriguez-Pena A, Lamas L, Del Rey FE, De Escobar GM. Are iodine-deficient rats euthyroid? *Endocrinology* 1982; **110:** 1780–1790.
18 Escobar del Rey F, Ruiz de Ona C, Bernal J, Obregon MJ, Morreale de Escobar G. Generalized deficiency of 3,5,3′–triiodo–L–thyronine (T3) in tissues from rats on a low iodine intake, despite normal circulating T3 levels. *Acta Endocrinol (Copenh)* 1989; **120:** 490–498.
19 Silva JE. Pituitary–thyroid relationships in hypothyroidism. *Bailliere's Clin Endocrinol Metabol* 1988; **2:** 541–565.
20 Escobar-Morreale HF, del Rey FE, Obregon MJ, De Escobar GM. Only the combined treatment with thyroxine and triiodothyronine ensures euthyroidism in all tissues of the thyroidectomized rat. *Endocrinology* 1996; **137:** 2490–2502.
21 Lo M-J, Kau M-M, Chen Y-H, Tsai S-C, Chiao Y-C, Chen J-J, Liaw C, Lu C-C, Lee B-P, Chen S-C, Fang V-S, Ho L-T, Wang P-S. Acute effects of thyroid hormones on the production of adrenal cAMP and corticosterone in male rats. *Am J Physiol* 1998; **274:** E238–E245.
22 Venihaki M, Majzoub JA. Animal models of CRH deficiency. *Front Neuroendocrinol* 1999; **20:** 122–145.
23 Muglia LJ, Jacobson L, Weninger SC, Luedke CE, Bae DS, Jeong KH, Majzoub JA. Impaired diurnal adrenal rhythmicity restored by constant infusion of corticotropin-releasing hormone in corticotropin-releasing hormone-deficient mice. *J Clin Invest* 1997; **99:** 2923–2929.
24 Bagdy G, Chrousos GP, Calogero AE. Circadian patterns of plasma immunoreactive corticotropin, beta-endorphin, corticosterone and prolactin after immunoneutralization of corticotropin-releasing hormone. *Neuroendocrinology* 1991; **53:** 573–578.
25 McGivern RF, Rittenhouse P, Aird F, Van de Kar LD, Redei E. Inhibition of stress-induced neuroendocrine and behavioral responses in the rat by prepro-thyrotropin-releasing hormone 178–199. *J Neurosci* 1997; **17:** 4886–4894.
26 Rittenhouse PA, Redei E. Thyroxine administration prevents streptococcal cell wall-induced inflammatory responses. *Endocrinology* 1997; **138:** 1434–1439.
27 Redei E, Hilderbrand H, Aird F. Corticotropin release-inhibiting factor is preprothyrotropin-releasing hormone-(178–199). *Endocrinology* 1995; **136:** 3557–3563.
28 Redei E, Hilderbrand H, Aird F. Corticotropin release inhibiting factor is encoded within prepro-TRH. *Endocrinology* 1995; **136:** 1813–1816.
29 Eastman CJ, Phillips DI. Endemic goitre and iodine deficiency disorders – aetiology, epidemiology and treatment. *Baillieres Clin Endocrinol Metab* 1988; **2:** 719–735.
30 Rajatanavin R, Chailurkit L, Winichakoon P, Mahachoklertwattana P, Soranasataporn S, Wacharasin R, Chaisongkram V, Amatyakul P, Wanarata L. Endemic cretinism in Thailand: a multidisciplinary survey. *Eur J Endocrinol* 1997; **137:** 349–355.
31 Goldey ES, Kehn LS, Rehnberg GL, Crofton KM. Effects of developmental hypothyroidism on auditory and motor function in the rat. *Toxicol Appl Pharmacol* 1995; **135:** 67–76.
32 Hebert R, Langlois JM, Dussault JH. Permanent defects in rat peripheral auditory function following perinatal hypothyroidism: determination of a critical period. *Brain Res* 1985; **355:** 161–170.
33 Sheppard KE, Roberts JL, Blum M. Differential regulation of type II corticosteroid receptor messenger ribonucleic acid expression in the rat anterior pituitary and hippocampus. *Endocrinology* 1990; **127:** 431–439.