Tri-iodothyronine Stimulates Food Intake via the Hypothalamic Ventromedial Nucleus Independent of Changes in Energy Expenditure

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Running title: T3 stimulates food intake via the VMN

Key words: hypothalamus, food intake, thyroid, energy expenditure, type 2 deiodinase

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This work is supported by a MRC programme grant.

Abbreviations

AgRP, agouti related protein; BAT, brown adipose tissue; D2, type 2 iodothyronine deiodinase; Egr-1, early growth response gene-1; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; RPA, ribonuclease protection assay; T3, tri-iodothyronine; T4, thyroxine; UCP-1, uncoupling protein-1; VMN, ventromedial nucleus; VO_2, oxygen consumption; WAT, white adipose tissue.

Abstract

Increased food intake is characteristic of hyperthyroidism, although this is presumed to compensate for a state of negative energy balance. However, here we show that the thyroid hormone tri-iodothyronine (T3) directly stimulates feeding at the level of the hypothalamus. Peripheral administration of T3 doubled food intake in *ad libitum* fed rats over 2 hours and induced expression of the immediate early gene, Egr-1, in the hypothalamic ventromedial nucleus (VMN), whilst maintaining plasma fT3 levels within the normal range. T3-induced feeding occurred without altering energy expenditure or locomotion. Injection of T3 directly into the VMN produced a four-fold increase in food intake in the first hour. The majority of T3 in the brain is reported to be produced by tissue-specific conversion of T4 to T3 by the enzyme type 2 iodothyronine deiodinase (D2). Hypothalamic D2 mRNA expression showed a diurnal variation, with a peak in the nocturnal feeding phase. Hypothalamic D2 mRNA levels also increased following a 12- and 24 hour fast, suggesting that local production of T3 may play a role in this T3 feeding circuit. Thus, we propose a novel hypothalamic feeding circuit in which T3, from the peripheral circulation or produced by local conversion, stimulates food intake via the VMN.

In hyperthyroidism, elevated plasma levels of thyroid hormones increase energy expenditure and decrease body weight (1). It is widely assumed that the characteristic increased appetite of hyperthyroidism is compensatory for this state of negative energy balance. Interestingly, about 5-10% of hyperthyroid individuals have a sufficiently increased appetite to gain weight in spite of the catabolic thyrotoxic process (2), suggesting that thyroid hormones may directly stimulate feeding.

T3 is the biologically active thyroid hormone. Tissue T3 concentrations are controlled at the cellular level and may not reflect plasma thyroid hormone concentrations (3). Thus, within the rat central nervous system (CNS), physiological levels of T3 are largely dependent on cellular uptake and intracellular deiodination of T4 to T3 by D2 (4,5). Within the hypothalamus, D2 mRNA (6,7) and activity (8) are concentrated in the periventricular region of the third ventricle, the arcuate nucleus (ARC) and median eminence. D2 mRNA is localised to tanycytes, specialised ependymal cells, lining the third ventricle (9). Tanycytes have long cytoplasmic processes projecting to several hypothalamic nuclei, including the ARC and the ventromedial nucleus (VMN) (10). The function of this local hypothalamic T3 production is unknown.

The hypothalamus plays an essential role in the regulation of energy homeostasis, integrating signals from other areas of the CNS and the periphery. Several hypothalamic nuclei have been implicated in the regulation of food intake and energy balance including the ARC, paraventricular nucleus (PVN) and VMN. Within the ARC two important neuronal populations have been identified: appetite inhibiting pro-opiomelanocortin (POMC) expressing neurons and appetite stimulating neuropeptide Y (NPY) and agouti related protein (AgRP) co-expressing neurons (11). Both of these

neuronal populations project to the PVN. The VMN was labelled as a satiety centre over 50 years ago when studies demonstrated that lesioning the VMN resulted in hyperphagia and weight gain (12). However more recent studies suggest that the role of the VMN in appetite regulation is more complex. The VMN receives and sends out extensive projections to other regions of the hypothalamus including the PVN and dorsomedial hypothalamus and may modulate the release of orexigenic signals from these hypothalamic nuclei (13).

The effects of overt hyperthyroidism and hypothyroidism on energy homeostasis and appetite have been well-established in rodents and man (14-16). However, such states are associated with marked effects on behavior and metabolism. Therefore it is not possible to infer from these studies a physiological role for thyroid hormones in the regulation of food intake. To investigate a role for T3 in the physiological regulation of food intake, we studied the effects of peripheral and CNS administration of T3, using doses of T3 which did not elevate plasma fT3 levels outside of the normal range (referred to as 'low-dose' T3 for the remainder of the paper). We examined the effects of low-dose T3 on food intake, energy expenditure and behavior. In addition, we studied diurnal variation and the effect of short-term fasting on hypothalamic D2 mRNA expression.

Methods

Animal Care and Maintenance

Male Wistar rats (ICSM, London, UK) (200-250g) aged 7-8 weeks were maintained under standardized barrier conditions (21-23^{o}C, lights on 07:00–19:00) and fed *ad libitum* RM1 diet (SDS Ltd., Witham, UK) unless described otherwise. All animal procedures were conducted under the British Home Office Animals (Scientific Procedures) Act (1986). All injections were administered in the early light phase (07:00-09:00).

T3 preparation for peripheral administration

For peripheral (subcutaneous (s.c.)) injections, T3 was prepared as 3, 3', 5-triiodo-$_{L}$-thyronine (T3) (Sigma, Dorset, UK) dissolved in absolute ethanol and emulsified in safflower oil (1:10) (volume 0.1ml). Controls received vehicle (emulsion alone). Prior to all studies, animals received 2 sham s.c. saline injections.

Peripheral administration of T3 and food intake

In the acute study of the effects of T3 on food intake, animals received either s.c. T3 (1.1, 2.3 or 4.5 nmol/kg) or s.c. vehicle (n = 12 per group). Food was weighed at 2, 4, 8 and 24 hours post-injection. In a separate study, animals received s.c. T3 (4.5 nmol/kg) or vehicle (controls) and were killed 2 hours post-injection by decapitation for collection of trunk blood as described (17). In the chronic study, animals (n = 12 per group) were injected with s.c. T3 (4.5, 9 or 75 nmol/kg) or s.c. vehicle daily for 5 days. On day 5 trunk blood was collected. Brains were removed and hypothalami dissected out and snap frozen for subsequent measurement of neuropeptide mRNA expression by RNase protection assay (RPA) (described below). Interscapular brown adipose tissue

(BAT) and epididymal white adipose tissue (WAT) were also collected, then weighed and frozen. BAT UCP-1 mRNA expression was measured by RPA.

Radioimmunoassay (RIA)

Plasma TSH levels were assayed using methods and reagents kindly provided by A. Parlow (NIDDK National Hormone and Pituitary Program, Baltimore, MD) as previously described (17). Plasma leptin (Linco Research, St. Charles, MO), fT3 and fT4 (Diagnostic Products Corp., Los Angeles, CA) were measured using commercial RIAs following manufacturer's instructions.

Behavioral study

Animals received a s.c. injection of T3 (4.5 nmol/kg) or vehicle at time zero (n = 16 per group). Behavioral patterns were monitored continuously from 30 to 120 minutes, by observers blinded to the experimental treatment. Behavior was classified into 3 different categories (adapted from Abbott (18)); feeding, active non-feeding behavior (drinking, grooming, burrowing, rearing, locomotion) and inactive non-feeding behavior (sleeping and still). These methods have previously been used to demonstrate abnormal behaviours following CNS administration of peptides (18). During the analysis, each rat was observed for 12 seconds every 5 minutes. This 12 second period was subdivided into 3 and the behavior of the rat during each section of the time period scored (816 total observations per rat).

Oxygen consumption (VO_2) studies

Indirect calorimetry was used to measure VO_2 as an indirect measurement of energy expenditure as previously described (19). VO_2 was determined in closed circuit respirometers maintained at thermoneutral temperature for rats (29°C). Animals were acclimatized to the calorimetry chamber for 2 hours prior to injection (n = 8 per group) and injected with T3 or vehicle at time zero. VO_2 was measured for 240 minutes after treatment. To study of the acute effects of T3 on VO_2, calorimetry was performed after a single s.c. injection of 4.5 nmol/kg T3 or vehicle. This was repeated following a single intraperitoneal (i.p.) injection of the β_3-adrenoceptor agonist BRL 35135 (40μg/kg) as a positive control (20). To study the effects of chronic T3 administration on VO_2, animals received 5 days of once daily s.c. injections of T3 (4.5 or 75 nmol/kg) or vehicle, and calorimetry was performed on day 5.

Ribonuclease Protection Assay (RPA)

Total RNA was extracted from hypothalami and BAT collected from animals after a single s.c. T3 injection or 5 days of once daily T3 injections (4.5 nmol/kg) (n = 12 per group) using Tri-Reagent (Helena Biosciences, Sunderland, UK) following the manufacturer's protocol. Hypothalamic AgRP, POMC, NPY and D2 (all 5μg) and BAT UCP-1 (0.25μg) mRNA were quantified by RPA (21) (Ambion RPA III kit, Ambion Inc. Austin, TX) using in-house probes (Accession numbers: UCP-1 M11814, AgRP U89484, POMC NM_139326, NPY NM_012614 and D2 NM_031720). Rat β-actin was used as an internal control (Ambion Inc). RNA was hybridised overnight and separated on a 5% polyacrylamide gel. The dried gel was exposed to a phosphoimager screen overnight and protected RNA hybrids quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For each neuropeptide, the ratio of the optical density of

the band of neuropeptide mRNA to that of β-actin was calculated and expressed in relative units (R.U.) (22).

Immunocytochemistry (ICC)

Egr-1 immunoreactivity (IR) was measured by ICC in paraffin embedded, paraformaldehyde fixed male Wistar rat brains collected 2 hours after s.c. administration 4.5 nmol/kg T3 or vehicle (23). Non-specific binding was blocked using normal donkey serum and sections incubated in rabbit anti Egr-1 antibody (Santa-Cruz Biotechnology, Santa-Cruz, CA) diluted 1:1000, at 4°C overnight. Slides were incubated for 30 minutes in biotinylated donkey anti-rabbit secondary antibody (1:50) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) followed by a 30 minute incubation in ABC-horseradish peroxidase (HRP) (1:100) (Dako Cytomation, Glostrup, Denmark). The antigen-antibody complex was visualised with 3,3'-Diaminobenzidine (DAB) in 0.01% hydrogen peroxide. Every 5th section was stained with hematoxylin and eosin to allow identification of the relevant hypothalamic nuclei. Egr-1-IR was counted by an observer blinded to the experimental treatment using a Nikon Eclipse E800 microscope and Image Pro-Plus v 4.5 software (MediaCybernetics, Silver Spring, MD).

Intranuclear cannulation and injection of T3

Animal surgical procedures and handling were carried out as previously described (18). Animals were anaesthetised by i.p. injection of a mixture of Ketalar (ketamine HCl 60mg/kg; Parke-Davis, Pontypool, UK) and Rompun (xylazine 12 mg/kg; Bayer UK Ltd, Bury St Edmonds, UK) and placed on a stereotaxic frame (David Kopf instruments, Tujunga, CA). Permanent 26 gauge stainless steel guide cannulae (Plastics

One Inc., Roanoke, VA) were stereotactically placed into the hypothalamic VMN or ARC as previously described (24). Due to its low solubility, T3 was dissolved in 5% ethanol. We have previously shown that CNS injection of up to 70% ethanol does not produce behavioral abnormalities (25). Animals received 0.5, 1, 5 and 50 pmol T3 or vehicle administered in 1µl. Food was weighed at 1, 2, 4, 8 and 24 hours post-injection. Cannula placement was verified at the end of the study by the injection of black ink (26). Data from an animal was excluded if its injection site extended more than 0.2mm outside the intended hypothalamic injection site or if any ink was detected in the cerebral ventricular system.

Measurement of D2 mRNA expression

For the fasting study, hypothalami were dissected and collected from rats either fed *ad libitum* (control) or fasted for 12 or 24 hours (n = 10 per group). For the diurnal variation study, rats were killed at 9 time points (n = 10 per group) throughout a 24 hour period. Hypothalamic D2 mRNA levels were measured using RPA (as described).

Statistical analysis

Statistical analyses were carried out in collaboration with David Stephens (Department of Mathematics, Imperial College London). Values are presented as the mean ± SEM unless otherwise stated. Behavioral data are presented as % of total observations ± estimated standard error. A Bayesian analysis was used to compare the relative probabilities of each behavioral profile in each group. For VO_2 studies, groups of data were compared using a two-way ANCOVA (S-plus, Seattle, WA). For Egr-1 studies (data expressed as median values with interquartile ranges), a Wilcoxon non-parametric test was used. In the remaining studies, comparisons were made using ANOVA, with

post hoc Fisher's Least Significant Difference method (Systat, Evanston, IL). Normal ranges (mean ± 2SD) for thyroid hormones were calculated within our laboratory from euthyroid control rats. Polynomial regression analysis was used for studying D2 mRNA diurnal rhythm (SigmaStat 2.03, Chicago, IL). P values < 0.05 were considered significant.

Results

Acute Effects of Peripheral T3 on Food Intake, Plasma Hormones and Behavior

Two hours post-s.c. injection of T3 (4.5 nmol/kg), food intake increased by 140% (1.2 ± 0.3 [T3] vs. 0.5 ± 0.1g [control], $P < 0.05$) (Fig. 1A). This stimulatory effect persisted for 8 hours (5-8 hours: 1.3 ± 0.4 [T3] vs. 0.6 ± 0.1g [control], $P < 0.05$) (Fig. 1B). Lower doses of T3 had no effect on feeding. Twenty four hours post-injection, there was no difference in food intake between T3-treated and control animals.

Plasma leptin levels were unchanged 2 hours following T3 treatment (4.5 nmol/kg) (2.8 ± 0.2 [T3] vs. 2.9 ± 0.3 ng/ml [control]). Plasma fT3 was 46% higher in the T3-treated group compared to controls (3.5 ± 0.2 [T3] vs. 2.4 ± 0.2 pmol/L [control], $P<0.001$), yet remained within the normal range (1.2 - 3.6 pmol/L). There was no difference in plasma fT4 (15.3 ± 0.6 [T3] vs. 15.7 ± 0.5 pmol/L [control]) or TSH (2.9 ± 0.3 [T3] vs. 3.5 ± 0.4 ng/ml [control]) in T3-treated animals compared to controls.

T3-treated (4.5 nmol/kg) animals spent over twice as much time feeding than control animals (5.3 ± 0.8 [T3] vs. 2.2 ± 0.5 % total observations [control], $P < 0.005$). Notably, there were no significant differences in active non-feeding behaviors (including locomotion) between the 2 groups (15.6 ± 1.3 [T3] vs. 15.3 ± 1.3 % total observations [control]). No adverse behaviors were observed at any time.

Chronic Effects of Peripheral T3 on Food Intake, Body Weight and Body Adiposity

T3 was administered (s.c) once daily for 5 days at a dose of 4.5, 9 or 75 nmol/kg. Mean daily food intake on day 5 was significantly greater in the 4.5 nmol/kg T3 and 9

nmol/kg T3 groups (30.1 ± 0.4 [4.5 nmol/kg T3], 29.1 ± 0.7 [9nmol/kg] and 27.3 ± 0.4g [control], ($P < 0.005$, 4.5 nmol/kg T3 vs. control), ($P < 0.05$, 9nmol/kg T3 vs. control) (Fig. 2A). 4.5 nmol/kg T3 also significantly increased cumulative food intake (days 0-5: 116.0 ± 1.7 [T3] vs. 107.6 ± 1.9g [control], $P < 0.05$) (Fig. 2B). The highest dose of T3, 75 nmol/kg, did not increase feeding. Consistent with the increased food intake, cumulative weight gain was 30% greater in the 4.5 nmol/kg T3 group, although this did not reach statistical significance (day 5: 23.3 ± 1.6 [T3] vs. 18.2 ± 2.1g [control], $P = 0.1$) (Fig. 2*C*).

The effects of chronic administration of T3 on body adiposity, plasma leptin and thyroid hormones are shown in Table 1. Chronic treatment with 4.5 nmol/kg T3 also increased plasma fT3 by 40%, although this remained within the normal range. Plasma TSH was lowered by 20% ($P < 0.05$) compared to controls but again remained within the normal range. Although 9 nmol/kg T3 had some stimulatory effects on feeding, this was associated with suppression of the thyroid axis (Table 1). The highest dose of T3, 75 nmol/kg, produced a five-fold increase in plasma fT3 ($P < 0.0005$), with marked suppression of TSH consistent with significant thyrotoxicosis. Chronic administration of 4.5 mol/kg T3 did not alter plasma leptin, interscapular BAT mass or interscapular BAT uncoupling protein -1 (UCP-1) mRNA levels (20.0 ± 2.3 [T3] vs. 19.0 ± 2.1 relative units [control]).

Effects of acute and chronic peripheral administration of T3 on energy expenditure

Following a single injection of T3 (4.5 nmol/kg), oxygen consumption (VO_2) was unchanged compared to control animals (Fig. 3A). However, an i.p. injection of the β_3-

adrenoreceptor agonist BRL 35135 significantly increased VO_2 as previously reported (20) (Fig. 3B). Similarly, once daily injection of 4.5 nmol/kg T3 for 5 days did not alter day 5 VO_2 (Fig. 4A). Following chronic daily administration of 75nmol/kg T3, basal VO_2 had significantly increased (day 5: 18.7 ± 0.5 [T3] vs. 15.8 ± 0.5ml/$kg^{0.75}$/min [control], $P < 0.005$) and it was further increased 30 minutes after T3 injection (21.1 ± 0.4 [T3] vs. 16.5 ± 0.7 ml/$kg^{0.75}$/min [control], $P < 0.0005$) (Fig. 4B). This elevation in VO_2 remained evident for the duration of VO_2 measurement (240 minutes).

Effects of acute and chronic peripheral T3 treatment on hypothalamic neuropeptide mRNA expression

Following a single injection of T3 (4.5 nmol/kg), hypothalamic POMC mRNA, AgRP mRNA and NPY mRNA levels were unchanged compared to control animals (data not shown). This was also true of 5 days of daily administration of T3 (4.5, 9, 75 nmol/kg) (Table 1).

Determination of neuronal activation following peripheral T3 injection.

Within the CNS, T3 induces expression of the early growth response (Egr) family of transcription factors (27, 28), yet may inhibit *c-fos* expression (29, 30). Therefore, to investigate a potential hypothalamic site of action for peripheral T3, Egr-1-IR was examined. Peripheral T3 injection was associated with a significant increase in the number of cells positive for Egr-1 in the VMN (median value and [interquartile range]: 1080 [879:1282] [T3] vs. 642 [620:664] immunoreactive cells [control], $P < 0.05$ (Figs. 5A and B). No change in Egr-1–IR was detected within the ARC (511 [367:656] [T3]

vs. 399 [378:420] immunoreactive cells [control]). No Egr-1-IR was observed in the PVN post-T3 administration. See Supplemental Data for representative images.

Effects of intranuclear administration of T3 on food intake

In view of the findings from these ICC studies, we studied the effects of injection of T3 (0.5, 1, 5 or 50 pmol) directly into the VMN. All doses of T3 significantly increased food intake 1 hour post-injection (Fig. 5C). Fifty picomoles T3 produced a four-fold stimulation in feeding (0-1h: 3.1 ± 0.5 [50 pmol T3] vs. 0.8 ± 0.2g [control], $P < 0.0001$). This stimulatory effect on food intake was short-lived. Food intake between 1-2 and 2-4 hours post-injection was unchanged compared to controls. In contrast, intra-ARC injection of T3 did not alter food intake at any dose ($P = 0.3$).

Hypothalamic D2 mRNA expression

Effect of fasting

In animal fasted for 12 and 24 hours, hypothalamic D2 mRNA expression was increased by approximately 50% compared to fed controls (5.9 ± 0.6 [12h fast] vs. 4.0 ± 0.5 relative units [fed], $P <0.05$), (6.1 ± 0.9 [24h fast] vs. 4.0 ± 0.5 relative units [fed], $P < 0.05$) (Fig. 6A).

Diurnal variation

Hypothalamic D2 mRNA expression exhibited a diurnal rhythm (Fig. 6B), reaching a nadir at 15:00 hours, 8 hours after lights on. Subsequently, D2 mRNA expression progressively increased, with maximal levels at 23:00 hours, 4 hours after lights out. This peak D2 mRNA expression was significantly greater than all other light-phase time-points ($P < 0.05$). A three-fold change in D2 mRNA expression was observed

through the 24 hour period. Trend analysis by polynomial fit revealed a cubic relationship between D2 mRNA expression and time of day ($P = 0.001$).

Discussion

We have demonstrated a novel role for T3 in the stimulation of food intake. This effect was seen using T3 doses substantially lower than those used by other groups (14, 31). In our study, plasma fT3 levels were increased by approximately 40%, but remained within the normal range following acute and chronic peripheral administration of 4.5 nmol/kg T3. In contrast, the higher doses of T3 produced up to a five-fold increase in plasma fT3 and were associated with a marked suppression of plasma fT4 and TSH. Supraphysiological levels of T3 are likely to result in metabolic and behavioral changes that would make the effects on food intake difficult to interpret. Therefore, in our experiments studying the effects of low-dose T3 on feeding, we were careful to adjust the dose of T3 to maintain fT3 levels within the normal range.

In our studies, we peripherally administered T3, the biologically active thyroid hormone, which readily crosses the blood brain barrier (32, 33). Our results support a direct effect of T3 on feeding, since this stimulation occurred in the absence of changes in energy expenditure, behavior or plasma leptin. The chronic orexigenic effect of T3 is unlikely to be a compensatory response to weight loss, since our data showed that the dose of T3 which stimulated feeding (4.5nmol/kg) was actually associated with a trend towards increased weight gain. The well characterised effects of thyroid hormones are mediated by nuclear hormone receptors via activation of gene transcription and occur over a period of hours to days (34). However, a number of thyroid hormone effects occur more rapidly and are independent of the cell nucleus (34, 35). These non-genomic effects of thyroid hormones have been described in a variety of tissues (36-39). The rapid increase in feeding following T3 injection observed in the current study suggests such a non-genomic effect.

In rodents, BAT is the major site of adaptive thermogenesis due to the expression of uncoupling proteins (UCPs). UCP-1 is exclusive to BAT and thyroid hormones are permissive for UCP-1 expression (40). We found that peripheral administration of low-dose T3 for five days increased food intake, without any alteration in BAT weight or UCP-1 mRNA levels. This suggests that the orexigenic effect of T3 is not secondary to changes in adaptive thermogenesis. Consistent with this, energy expenditure, measured as VO_2, was unchanged following both acute and chronic peripheral administration of low-dose T3. Thyrotoxicosis is associated with a significant increase in physical activity and this alteration in energy balance may contribute to the characteristic increased food intake. However, we found that administration of low-dose T3 increased feeding behavior without altering locomotor activity.

Our studies demonstrate a role for the VMN in the feeding response to T3. Although early studies demonstrated that lesioning of the VMN produces hyperphagia and obesity (12), more recent work suggests a more complex role for the VMN in appetite regulation (41, 42). The VMN has projections to a number of hypothalamic nuclei involved in appetite regulation (43) and disruption of the VMN alters the expression of NPY mRNA in the ARC and NPY peptide in the PVN (44). However, it is unlikely that either the ARC or PVN are the primary site of action for the stimulatory feeding effects of T3, since peripheral T3 did not increase Egr-1-IR in either of these nuclei. In addition, injection of T3 into the ARC, unlike the VMN, did not affect feeding. The observed increase in food intake produced by T3 may not be mediated by well characterised neuropeptide regulators of feeding, such as POMC, AgRP or NPY, since their hypothalamic mRNA expression was unaltered following chronic T3 administration. However, changes in peptide synthesis and release may occur in the absence of altered mRNA expression.

Other molecules involved in the regulation of food intake, such as dopamine (45), serotonin (45), and brain-derived neurotrophic factor (46) are expressed in the VMN and their expression is altered by nutritional status. Therefore, the possible involvement of such neuromodulators of feeding cannot be excluded.

Rats display a diurnal pattern in feeding and many neuropeptides involved in the regulation of food intake show a similar diurnal pattern in expression, for example, AgRP (47), NPY (48) and POMC (48). Campos-Barros *et al* (49) have shown that in rats, hypothalamic T3 concentrations peak just prior to the onset of the dark-phase, when feeding begins and hypothalamic D2 activity is maximal in the mid-dark phase. Consistent with this we demonstrated a diurnal variation in hypothalamic D2 mRNA, with levels greatest 4 hours into the dark phase. A previous study has shown that hypothalamic D2 mRNA expression increases following a prolonged, 72 hour fast (6). However, this duration of fasting was associated with pronounced suppression of the thyroid axis. We have shown that hypothalamic D2 mRNA expression is increased after only 12 hours of fasting, which is more relevant to the daily regulation of food intake. This increase in hypothalamic D2 mRNA levels with fasting may increase hypothalamic T3 levels and hence, stimulate appetite. One way to investigate this would be to inhibit the action of D2, for example with reverse T3, and examine the effects of this inhibition on food intake.

Studies suggest that T4 is taken up by tanycytes from the cerebrospinal fluid and capillaries, and converted by D2 to the active hormone T3 for use in various hypothalamic regions (50). Therefore, T3 may be transported, possibly by tanycytes, to the VMN, to stimulate food intake. These data have led us to propose a hypothalamic

circuit, involving T3, which is regulated by daily energy requirements. The source of T3 may be the peripheral circulation or locally derived in the hypothalamus by deiodination of T4 to T3 by D2.

Previously, the role of thyroid hormones in energy balance has been largely confined to pathological states, such as hyperthyroidism. The effects of thyroid hormones on appetite have been presumed to be secondary to increased metabolism. Here, we propose a novel role for T3 in the regulation of daily food intake via the hypothalamic VMN.

Acknowledgements

W.M.K and N.M.M are Wellcome Trust Clinical Training Fellows. K.L.S is supported by a MRC PhD studentship. W.S.D is supported by a Department of Health Clinician Scientist Fellowship. This work is supported by an MRC programme grant G7811974 (Bloom, Ghatei, Gardiner and Small).

	Treatment Group			
	Control	**4.5 nmol/kg T3**	**9 nmol/kg T3**	**75 nmol/kg T3**
fT3 (pmol/L)	1.8 ± 0.2	2.5 ± 0.1	2.9 ± 0.1†	9.4 ± 0.6‡
fT4 (pmol/L)	15.3 ± 0.3	14.4 ± 0.6	7.7 ± 0.6‡	1.2 ± 0.2‡
TSH (ng/ml)	1.9 ± 0.2	1.5 ± 0.1*	0.9 ± 0.1‡	0.8 ± 0.1‡
Leptin (ng/ml)	2.7 ± 0.3	1.9 ± 0.2	2.4 ± 0.3	2.6 ± 0.3
WAT (g)	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.7 ± 0.1
BAT (g)	0.3 ± 0.10	0.3 ± 0.02	0.3 ± 0.02	0.3 ± 0.02
POMC mRNA (R.U.)	46.4 ± 3.3	38.6 ± 2.7	45.0 ± 2.0	51.0 ± 3.8
AgRP mRNA (R.U.)	123.0 ± 9.8	148.3 ± 15.0	141.7 ± 24.3	145.0 ± 10.5
NPY mRNA (R.U.)	104.9 ± 10.4	91.9 ± 9.5	92.7 ± 11.7	101.8 ± 10.6

Table 1.

Table 1. Chronic T3 treatment: plasma thyroid hormones, body adiposity and hypothalamic neuropeptide expression.

Ad libitum fed rats were injected once daily with s.c. T3 at doses indicated (nmol/kg) or vehicle for 5 days (n = 12/group). R.U. = relative units. $^{*}P < 0.05$, $^{\dagger}P < 0.005$, $^{\ddagger}P < 0.0005$ vs controls. Results are mean ± S.E.M.

Fig. 1. Peripheral administration of T3 increases food intake.

Ad libitum fed rats received a single s.c. injection of T3 at the doses indicated (nmol/kg) or vehicle (V; control) (n = 12/group). Food intake **A** 0 to 2 hours and **B** 5 to 8 hours post-injection. $^{*}P < 0.05$ vs control. Results are mean ± S.E.M.

Fig. 2. Chronic peripheral administration of T3 increases food intake and body weight.

Ad libitum fed rats were injected once daily with s.c. T3 at the doses indicated (nmol/kg) or vehicle (V; control) for 5 days (n = 12/group). Food intake and body weight were measured daily. **A** Mean daily food intake, **B** Cumulative food intake and **C** Cumulative body weight gain. **A, B** and **C** show values on day 5 of the study. $^{\dagger}P < 0.005$ and $^{*}P < 0.05$ vs control. Results are mean ± S.E.M.

Fig. 3. Peripheral T3 (4.5nmol/kg) stimulates feeding but does not alter energy expenditure.

Oxygen consumption (VO_2) at thermoneutrality ($29^{o}C$) following a single s.c. injection of **A** 4.5 nmol/kg T3 (open circles) or vehicle (filled circles) or **B** BRL 35135 (open squares) or vehicle (filled squares); or 5 once daily injections of **C** 4.5 nmol/kg T3 (open circles) or vehicle (filled circles) and **D** 75 nmol/kg T3 (open triangles) or vehicle (filled triangles).

Animals were acclimatized to the chambers for 120 minutes prior to injection with T3 or vehicle at time zero (n = 8/group). Results are mean VO_2 ± S.E.M. for each 30 minute period *P < 0.05, †P < 0.005 and ‡P < 0.0005 vs control.

Fig. 4. Peripheral T3 (4.5nmol/kg) administration induces Egr-1 IR in the VMN and injection of T3 into the VMN stimulates feeding

Representative example (bregma, -2.56mm) of Egr-1-IR in response to a s.c. injection of **A** vehicle and **B** T3 (4.5 nmol/kg), x100 magnification. 3V = 3rd ventricle and dotted line delineates the VMN. **C** Food intake in *ad libitum* fed rats 1 hour post-injection of T3 (at the doses indicated (pmol)) or vehicle (V; control) into the VMN (n = 11 – 14/group), results are mean ± S.E.M. *P < 0.05 and ‡P < 0.0001 vs control.

Fig. 5. Hypothalamic D2 mRNA increases with fasting and has a diurnal variation.

A Hypothalamic D2 mRNA expression in rats fasted for 12 and 24 hours compared to *ad libitum* fed rat (n = 10/group). *P < 0.05 vs fed group. **B** Hypothalamic D2 mRNA expression over a 24 hour period (n = 10/group). Black line denotes duration of dark phase. *P < 0.05 vs D2 mRNA expression at all other light-phase time points. Results are mean ± S.E.M. RU = relative units.

Reference List

1. **Alton S, O'Malley BP** 1985 Dietary intake in thyrotoxicosis before and after adequate carbimazole therapy; the impact of dietary advice. Clin Endocrinol (Oxf) 23:517-520

2. **Gurney C, Hall R, Harper M, Owen SG, Roth M, Smart GA** 1970 Newcastle thyrotoxicosis index. Lancet 2:1275-1278

3. **de Jesus LA, Carvalho SD, Ribeiro MO, Schneider M, Kim SW, Harney JW, Larsen PR, Bianco AC** 2001 The type 2 iodothyronine deiodinase is essential for adaptive thermogenesis in brown adipose tissue. J Clin Invest 108:1379-1385

4. **Crantz FR, Silva JE, Larsen PR** 1982 An analysis of the sources and quantity of 3,5,3'-triiodothyronine specifically bound to nuclear receptors in rat cerebral cortex and cerebellum. Endocrinology 110:367-375

5. **van Doorn J, Roelfsema F, van der HD** 1985 Concentrations of thyroxine and 3,5,3'-triiodothyronine at 34 different sites in euthyroid rats as determined by an isotopic equilibrium technique. Endocrinology 117:1201-1208

6. **Diano S, Naftolin F, Goglia F, Horvath TL** 1998 Fasting-induced increase in type II iodothyronine deiodinase activity and messenger ribonucleic acid levels is not reversed by thyroxine in the rat hypothalamus. Endocrinology 139:2879-2884

7. **Diano S, Leonard JL, Meli R, Esposito E, Schiavo L** 2003 Hypothalamic type II iodothyronine deiodinase: a light and electron microscopic study. Brain Res 976:130-134

8. **Riskind PN, Kolodny JM, Larsen PR** 1987 The regional hypothalamic distribution of type II 5'-monodeiodinase in euthyroid and hypothyroid rats. Brain Res 420:194-198

9. **Tu HM, Kim SW, Salvatore D, Bartha T, Legradi G, Larsen PR, Lechan RM** 1997 Regional distribution of type 2 thyroxine deiodinase messenger ribonucleic acid in rat hypothalamus and pituitary and its regulation by thyroid hormone. Endocrinology 138:3359-3368

10. **Flament-Durand J, Brion JP** 1985 Tanycytes: morphology and functions: a review. Int Rev Cytol 96:121-155

11. **Schwartz MW, Woods SC, Porte D, Jr., Seeley RJ, Baskin DG** 2000 Central nervous system control of food intake. Nature 404:661-671

12. **Brobeck JR** 1946 Mechanisms of the development of obesity in animals with hypothalamic lesions. Physiol Rev 26:541-559

13. **Kalra SP, Dube MG, Pu S, Xu B, Horvath TL, Kalra PS** 1999 Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. Endocr Rev 20:68-100

14. **Luo L, MacLean DB** 2003 Effects of thyroid hormone on food intake, hypothalamic Na/K ATPase activity and ATP content. Brain Res 973:233-239

15. **Lovejoy JC, Smith SR, Bray GA, DeLany JP, Rood JC, Gouvier D, Windhauser M, Ryan DH, Macchiavelli R, Tulley R** 1997 A paradigm of experimentally induced mild hyperthyroidism: effects on nitrogen balance, body composition, and energy expenditure in healthy young men. J Clin Endocrinol Metab 82:765-770

16. **Pijl H, de Meijer PH, Langius J, Coenegracht CI, van den Berk AH, Chandie Shaw PK, Boom H, Schoemaker RC, Cohen AF, Burggraaf J, Meinders AE** 2001 Food choice in hyperthyroidism: potential influence of the autonomic nervous system and brain serotonin precursor availability. J Clin Endocrinol Metab 86:5848-5853

17. **Kim MS, Small CJ, Stanley SA, Morgan DG, Seal LJ, Kong WM, Edwards CM, Abusnana S, Sunter D, Ghatei MA, Bloom SR** 2000 The central melanocortin system affects the hypothalamo-pituitary thyroid axis and may mediate the effect of leptin. J Clin Invest 105:1005-1011

18. **Abbott CR, Rossi M, Wren AM, Murphy KG, Kennedy AR, Stanley SA, Zollner AN, Morgan DG, Morgan I, Ghatei MA, Small CJ, Bloom SR** 2001 Evidence of an orexigenic role for cocaine- and amphetamine-regulated transcript after administration into discrete hypothalamic nuclei. Endocrinology 142:3457-3463

19. **Connoley IP, Liu YL, Frost I, Reckless IP, Heal DJ, Stock MJ** 1999 Thermogenic effects of sibutramine and its metabolites. Br J Pharmacol 126:1487-1495

20. **Liu YL, Stock MJ** 1995 Acute effects of the beta 3-adrenoceptor agonist, BRL 35135, on tissue glucose utilisation. Br J Pharmacol 114:888-894

21. **Lee JJ, Costlow NA** 1987 A molecular titration assay to measure transcript prevalence levels. Methods Enzymol 152:633-648

22. **Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes AE, Low MJ, Ghatei MA, Cone RD, Bloom SR** 2002 Gut hormone PYY(3-36) physiologically inhibits food intake. Nature 418:650-654

23. **Ghanem MA, Van der Kwast TH, den Hollander JC, Sudaryo MK, Oomen MH, Noordzij MA, Van den Heuvel MM, Nassef SM, Nijman RM, Van Steenbrugge GJ** 2000 Expression and prognostic value of Wilms' tumor 1 and early growth response 1 proteins in nephroblastoma. Clin Cancer Res 6:4265-4271

24. **Kim MS, Rossi M, Abusnana S, Sunter D, Morgan DG, Small CJ, Edwards CM, Heath MM, Stanley SA, Seal LJ, Bhatti JR, Smith DM, Ghatei MA,**

Bloom SR 2000 Hypothalamic localization of the feeding effect of agouti-related peptide and alpha-melanocyte-stimulating hormone. Diabetes 49:177-182

25. **O'Shea D, Morgan DG, Meeran K, Edwards CM, Turton MD, Choi SJ, Heath MM, Gunn I, Taylor GM, Howard JK, Bloom CI, Small CJ, Haddo O, Ma JJ, Callinan W, Smith DM, Ghatei MA, Bloom SR** 1997 Neuropeptide Y induced feeding in the rat is mediated by a novel receptor. Endocrinology 138:196-202

26. **Abbott CR, Kennedy AR, Wren AM, Rossi M, Murphy KG, Seal LJ, Todd JF, Ghatei MA, Small CJ, Bloom SR** 2003 Identification of hypothalamic nuclei involved in the orexigenic effect of melanin-concentrating hormone. Endocrinology 144:3943-3949

27. **Mellstrom B, Pipaon C, Naranjo JR, Perez-Castillo A, Santos A** 1994 Differential effect of thyroid hormone on NGFI-A gene expression in developing rat brain. Endocrinology 135:583-588

28. **Mercier G, Turque N, Schumacher M** 2001 Rapid effects of triiodothyronine on immediate-early gene expression in Schwann cells. Glia 35:81-89

29. **Perez P, Schonthal A, Aranda A** 1993 Repression of c-fos gene expression by thyroid hormone and retinoic acid receptors. J Biol Chem 268:23538-23543

30. **Perez P, Palomino T, Schonthal A, Aranda A** 1994 Determination of the promoter elements that mediate repression of c-fos gene transcription by thyroid hormone and retinoic acid receptors. Biochem Biophys Res Commun 205:135-140

31. **Ishii S, Kamegai J, Tamura H, Shimizu T, Sugihara H, Oikawa S** 2003 Hypothalamic neuropeptide Y/Y1 receptor pathway activated by a reduction in circulating leptin, but not by an increase in circulating ghrelin, contributes to hyperphagia associated with triiodothyronine-induced thyrotoxicosis. Neuroendocrinology 78:321-330

32. **Pardridge WM** 1979 Carrier-mediated transport of thyroid hormones through the rat blood-brain barrier: primary role of albumin-bound hormone. Endocrinology 105:605-612

33. **Dratman MB, Crutchfield FL, Schoenhoff MB** 1991 Transport of iodothyronines from bloodstream to brain: contributions by blood:brain and choroid plexus:cerebrospinal fluid barriers. Brain Res 554:229-236

34. **Bassett JH, Harvey CB, Williams GR** 2003 Mechanisms of thyroid hormone receptor-specific nuclear and extra nuclear actions. Mol Cell Endocrinol 213:1-11

35. **Davis PJ, Davis FB, Lawrence WD** 1989 Thyroid hormone regulation of membrane Ca2(+)-ATPase activity. Endocr Res 15:651-682

36. **Warnick PR, Davis PJ, Davis FB, Cody V, Galindo J, Jr., Blas SD** 1993 Rabbit skeletal muscle sarcoplasmic reticulum Ca(2+)-ATPase activity: stimulation in vitro by thyroid hormone analogues and bipyridines. Biochim Biophys Acta 1153:184-190

37. **Lakatos P, Stern PH** 1991 Evidence for direct non-genomic effects of triiodothyronine on bone rudiments in rats: stimulation of the inositol phosphate second messenger system. Acta Endocrinol (Copenh) 125:603-608

38. **Yin Y, Vassy R, Nicolas P, Perret GY, Laurent S** 1994 Antagonism between T3 and amiodarone on the contractility and the density of beta-adrenoceptors of chicken cardiac myocytes. Eur J Pharmacol 261:97-104

39. **Davis PJ, Davis FB** 1996 Nongenomic actions of thyroid hormone. Thyroid 6:497-504

40. **Branco M, Ribeiro M, Negrao N, Bianco AC** 1999 3,5,3'-Triiodothyronine actively stimulates UCP in brown fat under minimal sympathetic activity. Am J Physiol 276:E179-E187

41. **Guan XM, Yu H, Van der Ploeg LH** 1998 Evidence of altered hypothalamic pro-opiomelanocortin/ neuropeptide Y mRNA expression in tubby mice. Brain Res Mol Brain Res 59:273-279

42. **Guan XM, Yu H, Trumbauer M, Frazier E, Van der Ploeg LH, Chen H** 1998 Induction of neuropeptide Y expression in dorsomedial hypothalamus of diet-induced obese mice. Neuroreport 9:3415-3419

43. **ter Horst GJ, Luiten PG** 1987 Phaseolus vulgaris leuco-agglutinin tracing of intrahypothalamic connections of the lateral, ventromedial, dorsomedial and paraventricular hypothalamic nuclei in the rat. Brain Res Bull 18:191-203

44. **Kalra PS, Dube MG, Xu B, Kalra SP** 1997 Increased receptor sensitivity to neuropeptide Y in the hypothalamus may underlie transient hyperphagia and body weight gain. Regul Pept 72:121-130

45. **Meguid MM, Fetissov SO, Blaha V, Yang ZJ** 2000 Dopamine and serotonin VMN release is related to feeding status in obese and lean Zucker rats. Neuroreport 11:2069-2072

46. **Xu B, Goulding EH, Zang K, Cepoi D, Cone RD, Jones KR, Tecott LH, Reichardt LF** 2003 Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. Nat Neurosci 6:736-742

47. **Lu XY, Shieh KR, Kabbaj M, Barsh GS, Akil H, Watson SJ** 2002 Diurnal rhythm of agouti-related protein and its relation to corticosterone and food intake. Endocrinology 143:3905-3915

48. **Xu B, Kalra PS, Farmerie WG, Kalra SP** 1999 Daily changes in hypothalamic gene expression of neuropeptide Y, galanin, proopiomelanocortin, and adipocyte leptin gene expression and secretion: effects of food restriction. Endocrinology 140:2868-2875

49. **Campos-Barros A, Musa A, Flechner A, Hessenius C, Gaio U, Meinhold H, Baumgartner A** 1997 Evidence for circadian variations of thyroid hormone concentrations and type II 5'-iodothyronine deiodinase activity in the rat central nervous system. J Neurochem 68:795-803

50. **Guadano-Ferraz A, Obregon MJ, St Germain DL, Bernal J** 1997 The type 2 iodothyronine deiodinase is expressed primarily in glial cells in the neonatal rat brain. Proc Natl Acad Sci U S A 94:10391-10396

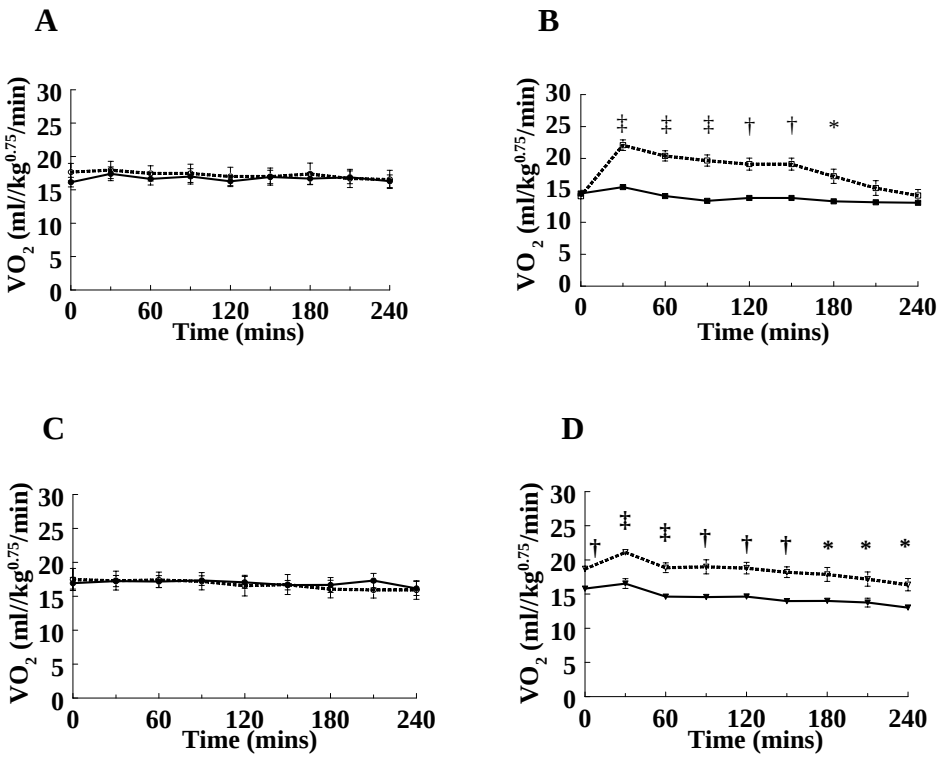
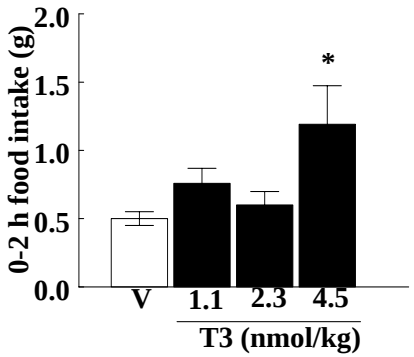


Figure 3

A


0-2 h food intake (g)
2.0
1.5
1.0
0.5
0.0
*
V
1.1
2.3
4.5
T3 (nmol/kg)

B

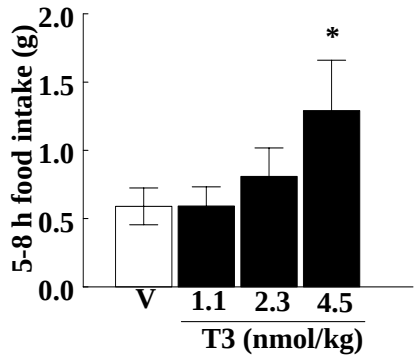

5-8 h food intake (g)
2.0
1.5
1.0
0.5
0.0
*
V
1.1
2.3
4.5
T3 (nmol/kg)

Figure 1

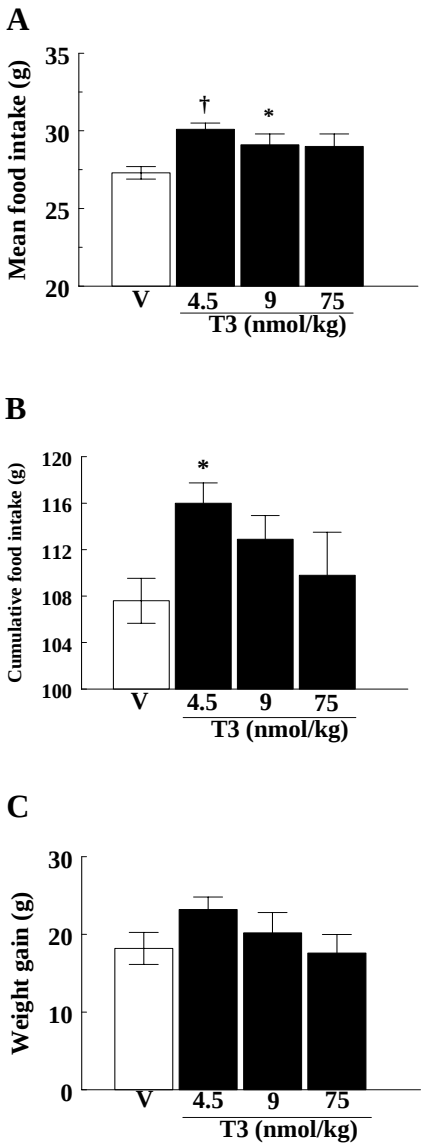
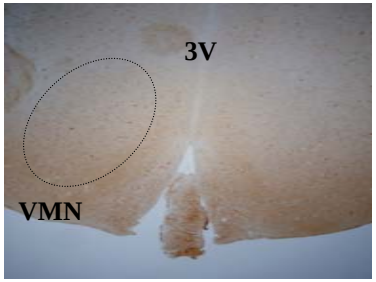
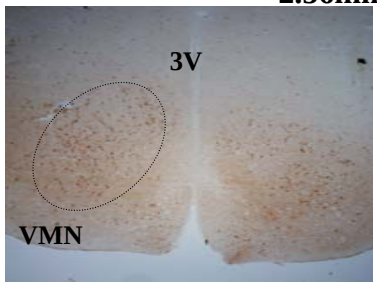

A
Mean food intake (g)
35
30
25
20
†
*
V
4.5
9
75
T3 (nmol/kg)
B
Cumulative food intake (g)
120
116
112
108
104
100
*
V
4.5
9
75
T3 (nmol/kg)
C
Weight gain (g)
30
20
10
0
V
4.5
9
75
T3 (nmol/kg)

Figure 2

-2.56mm



A



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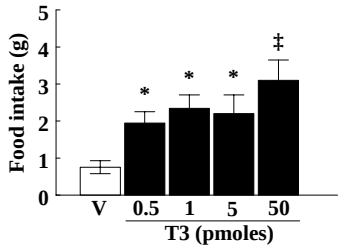

C

Figure 4

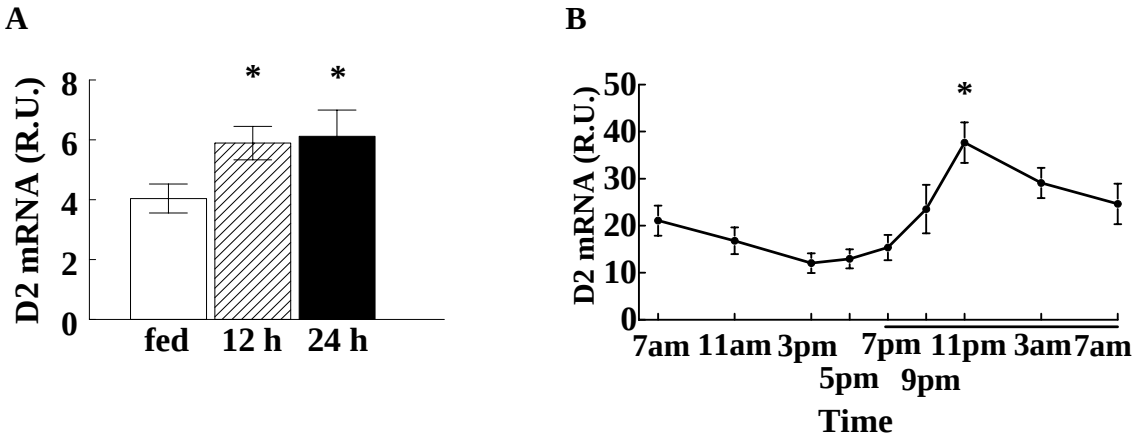
A
B
D2 mRNA (R.U.)
8
6
4
2
0
*
*
fed
12 h
24 h
D2 mRNA (R.U.)
50
40
30
20
10
0
*
7am
11am
3pm
5pm
7pm
9pm
11pm
3am
7am
Time

Figure 5