Central effects of thyronamines on glucose metabolism in rats

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Abstract

Thyronamines are naturally occurring, chemical relatives of thyroid hormone. Systemic administration of synthetic 3-iodothyronamine (T₁AM) and – to a lesser extent – thyronamine (T₀AM), leads to acute bradycardia, hypothermia, decreased metabolic rate, and hyperglycemia. This profile led us to hypothesize that the central nervous system is among the principal targets of thyronamines. We investigated whether a low dose i.c.v. infusion of synthetic thyronamines recapitulates the changes in glucose metabolism that occur following i.p. thyronamine administration. Plasma glucose, glucoregulatory hormones, and endogenous glucose production (EGP) using stable isotope dilution were monitored in rats before and 120 min after an i.p. (50 mg/kg) or i.c.v. (0.5 mg/kg) bolus infusion of T₁AM, T₀AM, or vehicle. To identify the peripheral effects of centrally administered thyronamines, drug-naïve rats were also infused intravenously with low dose

(0.5 mg/kg) thyronamines. Systemic T₁AM rapidly increased EGP and plasma glucose, increased plasma glucagon, and corticosterone, but failed to change plasma insulin. Compared with i.p.-administered T₁AM, a 100-fold lower dose administered centrally induced a more pronounced acute EGP increase and hyperglucagonemia while plasma insulin tended to decrease. Both systemic and central infusions of T₀AM caused smaller increases in EGP, plasma glucose, and glucagon compared with T₁AM. Neither T₁AM nor T₀AM influenced any of these parameters upon low dose i.v. administration. We conclude that central administration of low-dose thyronamines suffices to induce the acute alterations in glucoregulatory hormones and glucose metabolism following systemic thyronamine infusion. Our data indicate that thyronamines can act centrally to modulate glucose metabolism.

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Introduction

Thyronamines are a group of naturally occurring, chemical relatives of thyroid hormone (TH) with pronounced and rapid physiologic effects (Scanlan *et al.* 2004). Two representatives of the thyronamines, 3-iodothyronamine (T₁AM) and thyronamine (T₀AM), have been extracted from rat and mouse brain, heart, liver, and blood. T₁AM and T₀AM can theoretically be derived from iodothyronines thyroxine (T₄), 3,3',5-triiodothyronine (T₃), and/or 3,3',5'-triiodothyronine (reverse T₃) by removal of the carboxylate group on the β-alanine side chain in addition to deiodination. Indeed, thyronamines have recently been identified as isoenzyme-specific substrates of the iodothyronine deiodinases type 1, 2, and 3 (Piehl *et al.* 2008). T₁AM and, to a lesser extent, T₀AM are potent *in vitro* agonists of the trace amine-associated receptor type 1 (TAAR1; Scanlan *et al.* 2004, Hart *et al.* 2006), a Gs protein-coupled membrane receptor with a broad expression profile (Grandy 2007). In rodents and humans, high levels of TAAR1 expression are found in liver,

kidney, gastrointestinal tract, pancreas, heart, and many areas of the brain (Borowsky *et al.* 2001, Bunzow *et al.* 2001). Moreover, T₁AM has the potential to act as an adrenergic receptor α₂ (ARα₂) agonist in the mouse, explaining in part the decrease in insulin secretion by pancreatic β-cells exposed to thyronamines (Regard *et al.* 2007).

When administered to rodents, T₁AM and T₀AM have striking effects on physiology. Within minutes after systemic administration, profound hypothermia, bradycardia, and decreased cardiac output occur. In addition, thyronamines rapidly induce metabolic alterations such as decreased metabolic rate and a dramatic shift to preferential lipid fuelling at the cost of carbohydrate oxidation (Scanlan *et al.* 2004, Braulke *et al.* 2007). These apparently non-genomic effects are thought to occur via binding to and activating membrane-bound G protein-coupled receptors (GPCRs) such as TAAR1 and ARα₂ (Liggett 2004, Scanlan *et al.* 2004). Furthermore, it has been proposed but not yet demonstrated that THs can be converted to thyronamines by enzymatic deiodination and decarboxylation. Since most

actions of T₁AM and T₀AM are opposite in direction to the bioactive TH T₃, thyronamines have been hypothesized to play a role in fine-tuning and/or antagonizing T₃ actions on a moment-to-moment timescale (Liggett 2004, Weatherman 2007).

The brain, in particular the hypothalamus, regulates most of the processes affected by thyronamines (body temperature, cardiac function, and energy metabolism). Moreover, a principal role in regulating hepatic glucose metabolism has recently emerged for the hypothalamus (Obici *et al.* 2002, Kalsbeek *et al.* 2004, Pocai *et al.* 2005). As T₁AM and T₀AM are present in rat brain, we hypothesized that these novel compounds could affect glucose metabolism via actions in the central nervous system (CNS).

In the present study, we tested the hypothesis that thyronamines act centrally to induce changes in glucose metabolism using stable isotope dilution and three different routes of administration: systemic (i.p.), central (i.c.v.), and i.v. in rats. Our results are consistent with the interpretation that T₁AM and T₀AM can act centrally to recapitulate the changes in glucose metabolism that occur following systemic thyronamine administration.

Materials and Methods

Animals

Male Wistar rats (Harlan, Horst, The Netherlands) between 350 and 400 g body weight (BW), housed under constant conditions of temperature ($21 \pm 1^\circ\text{C}$) and humidity ($60 \pm 2\%$) with a 12 h light:12 h darkness schedule (lights on at 0700 h), were used in all experiments. Food and drinking water were available *ad libitum*. All of the following experiments were conducted with the approval of the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences.

T₁AM and T₀AM

T₁AM-HCl (391 g/mol) and T₀AM-HCl (264 g/mol) were synthesized as previously reported (Hart *et al.* 2006) and dissolved in 20% DMSO and 80% saline (vehicle) at a concentration of 40 mg/ml.

Experimental groups

Two independent studies were performed. For the first study, permanent jugular vein and carotid artery cannulae were placed in rats ($n=22$) under anesthesia (see below). Animals were allowed to recover from the surgery for 8 days prior to any further manipulations. Each rat thus cannulated received an i.p. bolus infusion of 50 mg/kg of T₁AM ($n=7$), 50 mg/kg of T₀AM ($n=8$), or an equal volume (500 μl) of vehicle ($n=7$). For the second study, rats ($n=31$) were equipped with a guide cannula placed

into the left lateral cerebral ventricle in addition to the carotid artery and jugular vein cannulae. Rats thus cannulated received an i.c.v. 100-fold lower dose (0.5 mg/kg) of either T₁AM ($n=9$), T₀AM ($n=8$), or DMSO-saline vehicle ($n=8$) in a volume of 4 μl . To control for the possibility that any observed effect of the i.c.v.-infused thyronamines was somehow due to spillover into the circulation, an additional group of cannulated rats was infused intravenously with 0.5 mg/kg T₁AM ($n=3$) and T₀AM ($n=3$) in a volume of 500 μl . In both of these experiments, before and 120 min after i.p. or i.c.v. bolus infusion, isotope dilution and blood sampling were conducted to permit measurement of endogenous glucose production (EGP), and the concentration of plasma glucose, insulin, glucagon, corticosterone, thyroid stimulating hormone (TSH), T₃ and T₄ concentrations.

Surgery

Animals were anaesthetized using a mixture of Hypnorm (Janssen; 0.05 ml/100 g BW, i.m.) and Dormicum (Roche; 0.04 ml/100 g BW, s.c.). Vascular and i.c.v. cannulae were fixed with dental cement to four stainless-steel screws inserted into the skull. Post-operative care was provided by s.c. injection of 0.01 ml/100 g BW of Temgesic (Schering-Plough, Utrecht, The Netherlands). In all animals, an intra-atrial silicone cannula was implanted through the right jugular vein and a second silicone cannula was placed in the left carotid artery for isotope infusion and blood sampling as described previously (Klieverik *et al.* 2008). For the second study, stainless-steel i.c.v. cannulae were implanted into the left cerebral ventricle using the following stereotaxic coordinates: anteroposterior: -0.8 mm; lateral: $+2.0$ mm; ventral: -3.2 mm, with the toothbar set at -3.4 mm. Guide cannula placement was confirmed by dye (4 μl of ethylene blue) injection and inspection post-mortem. Only animals that showed staining of the left lateral cerebral ventricle and third cerebral ventricle were included in the final analysis.

Stable isotope dilution and systemic versus central thyronamine administration

Eight days post-surgery, stable isotope dilution was performed in combination with the administration of synthetic thyronamines. Animals weighed between 335 and 380 g. BW increased in all groups during the 3 days preceding the experimental infusions, indicating recovery from surgery and a positive energy balance. One day before the experimental infusions, rats were connected to a metal collar to which polyethylene tubing (for blood sampling and infusion) was attached and kept out of reach of the animals by a counterbalanced beam. This permitted all subsequent manipulations to be performed outside the cages without handling the animals (Klieverik *et al.* 2008). For determining basal plasma concentrations of TSH, T₃, and T₄, a blood sample was obtained at 1400 h. On the day

of thyronamine administration, food was removed from the cages 4 h (~ 0830 h) before the first basal measurements. At ~ 1100 h, a blood sample was taken (200 μ l, $t = -110$ min) for determination of background isotopic enrichment. Subsequently, a primed (8.0 μ mol in 5 min) continuous (16.6 μ mol/h) infusion of the stable isotope tracer [6,6- $^2\text{H}_2$]-glucose ($>99\%$ enriched; Cambridge Isotope Laboratories, Cambridge, MA, USA) was started using an infusion pump (Harvard Apparatus, Holliston, MA, USA). After an equilibration period of 90 min, additional blood samples (200 μ l) were obtained for the determination of basal plasma glucose, isotopic enrichment ($t = -20$, -10 and 0 min), plasma corticosterone ($t = -20$ and 0 min), insulin, and glucagon ($t = 0$ min) concentrations.

After the $t = 0$ blood sample, in study 1, animals received an i.p. bolus of either T₁AM, T₀AM (50 mg/kg in 500 μ l), or vehicle. In study 2, again after the $t = 0$ blood sample, animals received an i.c.v. bolus infusion of either T₁AM, T₀AM (0.5 mg/kg in 4 μ l), or vehicle delivered through the i.c.v. cannula in 105 s using a Hamilton syringe. After i.p. or i.c.v. bolus infusion, blood samples were obtained for measurement of glucose concentration, isotopic enrichment (5, 10, 20, 30, 45, 60, 75, 90, and 120 min), plasma corticosterone ($t = 10$, 20, 30, 60, and 120 min), plasma insulin, glucagon ($t = 10$, 60 and 120 min), and plasma TSH, T₃, and T₄ concentrations ($t = 120$ min).

Plasma hormone and isotope analyses

Plasma glucose concentration was determined in triplicate by a glucose oxidase method (Boehringer Mannheim). Plasma glucagon and corticosterone were measured using a commercially available RIA (LINCO Research, St Charles, MO, USA and ICN Biomedicals, Costa Mesa, CA, USA respectively). Plasma concentrations of T₃ and T₄ were determined by an in-house RIA (Kalsbeek *et al.* 2000), with inter- and intra-assay variation coefficients (CV) of 7–8 and 3–4% (T₃), and 3–6 and 2–4% (T₄) respectively. Detection limits for T₃ and T₄ were 0.3 and 5 nmol/l respectively. Plasma TSH concentrations were determined by a chemiluminescent immunoassay (Immulite 2000, Diagnostic Products Corp., Los Angeles, CA, USA) using a rat-specific standard. The inter- and intra-assay CV's for TSH were <4 and 2% at ± 3.5 mU/l respectively with a detection limit of 0.40 mU/l. Plasma insulin was measured by a commercially available ELISA (Mercodia, Uppsala, Sweden; Ackermans *et al.* 2008). The inter- and intra-assay CV's were 4 and 2%, detection limit 13 pmol/l. All samples were measured in duplicate, i.e. two tubes were analyzed per plasma sample. Glucose enrichment was measured as previously described (Ackermans *et al.* 2001). The [6,6- $^2\text{H}_2$]-glucose enrichment (tracer/tracee ratio) inter-assay CV was 1%, the intra-assay CV 1%, and the detection limit 0.04%.

Calculations and statistical analysis

EGP was calculated from isotope enrichment and plasma glucose concentration using modified forms of steady-state (basal) and non-steady-state (after thyronamine infusion) Steele equations (Steele 1959). Data were analyzed by two-way ANOVA with repeated measurements, with treatment group (T₁AM, T₀AM, or Veh) and time as dependent factors. Significance was defined at $P < 0.05$ using paired t -tests (i.e. within treatment groups) and independent t -tests (i.e. between treatment groups) to identify experimental groups that differed significantly. The SPSS statistical software program version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data are presented as mean $\pm$ S.E.M.

Results

In two independent studies, eight groups of rats were investigated. In the first study, rats received an i.p. bolus infusion of either T₁AM (50 mg/kg, $n = 7$), T₀AM (50 mg/kg, $n = 8$), or vehicle ($n = 7$). In the second study, rats were intracerebroventricularly infused with a 100-fold lower dose (i.e. 0.5 mg/kg) of either T₁AM ($n = 9$), T₀AM ($n = 8$), or vehicle ($n = 8$). To address the possibility that any physiologic response observed following i.c.v. infusion of the thyronamines was due to the peripheral action of drug that spilled over into the circulation, two additional groups of animals were intravenously infused with 0.5 mg/kg of T₁AM ($n = 3$) or 0.5 mg/kg of T₀AM ($n = 3$).

Study #1: systemic thyronamine infusion

Rats injected i.p. with 50 mg/kg T₁AM or 50 mg/kg T₀AM exhibited a behavioral phenotype as described previously (Scanlan *et al.* 2004). Interestingly, the animals injected with T₁AM displayed the more robust phenotype even though the dose (on a per mole basis) was approximately half that of T₀AM.

Glucose homeostasis: plasma concentration and endogenous production

Systemic infusion of either T₁AM or T₀AM by the i.p. route of administration induced a rapid and significant increase in plasma glucose concentration (Fig. 1a). The onset and magnitude of this effect was similar for the two compounds until 45 min post-infusion when the effect of T₀AM apparently plateaued, while the T₁AM-induced hyperglycemia continued to develop eventually reaching a maximum $371 \pm 27\%$ of basal values 120 min after infusion.

Within 10 min of receiving an i.p. bolus of T₁AM, the EGP increased to $143 \pm 3\%$ of basal values ($P = 0.001$ versus Veh; Fig. 1b), which was sustained for the duration of the experiment. Similarly, i.p. administration of T₀AM rapidly increased EGP, reaching a maximum of $158 \pm 16\%$ of basal values after 20 min ($P = 0.032$ versus Veh). Forty-five minutes after injection with T₀AM, EGP gradually returned to basal values by $t = 120$ min.

Figure 1 (a) Plasma glucose concentrations before and after i.p. bolus infusion of T₁AM, T₀AM, or vehicle. Note that from $t=10$ min, glucose concentration is higher in T₁AM- and T₀AM-treated animals when compared with vehicle rats ($P<0.05$). From $t=60$, glucose concentration is higher in T₁AM when compared with T₀AM-treated rats ($P<0.05$). ANOVA RM factor time $P<0.0001$, time $\times$ group $P<0.0001$, and group $P<0.0001$. (b) Endogenous glucose production (EGP) before and after i.p. bolus infusion of T₁AM, T₀AM, or vehicle. From $t=10$ and $t=20$, EGP is higher in T₁AM- and T₀AM-infused rats respectively, when compared with vehicle ($P<0.05$). From $t=60$, EGP is lower in T₀AM relative to T₁AM-treated animals ($P<0.05$). From $t=90$, EGP in T₀AM-treated rats is not different from vehicle rats. ANOVA RM factor time $P<0.0001$, time $\times$ group $P<0.0001$, and group $P=0.001$.

Glucoregulatory hormones Given the profound effect of T₁AM and T₀AM on plasma glucose and EGP, we characterized the status of three glucoregulatory hormones: insulin; glucagon; and corticosterone (Fig. 2). Surprisingly, even though T₁AM and T₀AM (50 mg/kg, i.p.) produced hyperglycemia and elevated EGP, plasma insulin concentrations were unchanged relative to plasma from vehicle-injected rats (Fig. 2a). By contrast, plasma glucagon concentrations were significantly increased within 10 min of either T₁AM or T₀AM administration (Fig. 2b). However, by 60 min post-injection, the time-effect profiles of the two compounds had begun to diverge with T₀AM's effect reaching a plateau at 240% ($P=0.007$ T₀AM versus Veh, $t=60$) of basal levels, while T₁AM's effect continued to

develop for the duration of the experiment ($447 \pm 44\%$ of basal values at 120 min, $P<0.0001$ T₁AM versus Veh; Fig. 2b). Of note, the time-course profiles of plasma glucagon (Fig. 2b) and plasma glucose (Fig. 1a) in response to i.p. T₁AM and T₀AM were essentially superimposable.

Plasma corticosterone displayed a significant increase in response to both T₁AM- and T₀AM-injected i.p., compared with vehicle-injected rats (Fig. 2c). T₁AM infusion induced a maximal increase at $t=60$ min (482 ± 106 vs $179 \pm 71\%$ of basal levels at $t=60$ min, T₁AM versus Veh; $P=0.022$). T₀AM infusion increased plasma corticosterone to a similar extent (393 ± 59 vs $172 \pm 102\%$ of basal levels at $t=120$ min, T₀AM versus Veh; $P=0.022$). At no time point was there a difference in the corticosterone response between T₁AM- and T₀AM-infused groups.

Plasma T₃, T₄, and TSH concentrations before and 120 min after i.p. T₁AM, T₀AM, and vehicle infusion are depicted in Table 1. Within 120 min, both T₄ and TSH levels were significantly decreased in response to i.p. T₁AM or, to a larger extent, i.p. T₀AM (50 mg/kg). Intriguingly, T₃ levels were also decreased 120 min after i.p. T₀AM (50 mg/kg) when compared with vehicle-injected rats.

Study #2: central thyronamine infusion

I.c.v. infusion of 0.5 mg/kg T₁AM or T₀AM did not induce any of the phenotypical alterations observed after systemic thyronamine administration.

Glucose homeostasis: plasma concentration and endogenous production With an i.c.v. bolus infusion of 0.5 mg/kg, T₁AM plasma glucose concentration began to increase immediately (Fig. 3a) until it reached a maximum $199 \pm 13\%$ of basal levels 30 min after infusion ($P<0.0001$ versus Veh). During the next 90 min, plasma glucose decreased slightly stabilizing at $\sim 163\%$ of basal values. T₀AM (0.5 mg/kg, i.c.v.) significantly elevated plasma glucose as well, but to a lesser degree than T₁AM (maximum $134 \pm 6\%$ at $t=45$, $P<0.0001$ versus Veh; Fig. 3a).

T₁AM (0.5 mg/kg, i.c.v.) induced a rapid and significant increase in EGP (Fig. 3b) by 10 min post-infusion reaching a maximum $178 \pm 16\%$ of basal values at 30 min ($P<0.0001$ versus Veh), which gradually decreased with time to $113 \pm 5\%$ when the experiment was terminated at $t=120$ min. Although T₀AM (0.5 mg/kg, i.c.v.) significantly increased EGP above basal levels ($P<0.0001$; $t=0$ vs $t=10$), its maximum effect ($20 \pm 9\%$ increase at $t=20$; $P=0.069$ versus Veh) was approximately one-third of T₁AM's maximal effect (Fig. 3b).

Importantly, when T₁AM or T₀AM were infused intravenously at the dose that was used in the i.c.v. infusion experiments (0.5 mg/kg, T₁AM $n=3$, T₀AM $n=3$) neither thyronamine had any effect on plasma glucose concentrations, EGP, plasma insulin, nor glucagon at any time point when compared with basal values (data not shown).

When the absolute changes in plasma glucose concentration produced by 0.5 mg/kg T₁AM i.c.v. and 50 mg/kg T₁AM i.p. are compared over time, their profiles are practically superimposable for the first 30 min of exposure (Fig. 4a). Thereafter, they diverged as the systemic effect of T₁AM continued to develop. Plotting the absolute values for EGP in response to 0.5 mg/kg T₁AM i.c.v. and 50 mg/kg T₁AM i.p. revealed both routes of administration-produced identical profiles during the initial 20 min post-exposure

(Fig. 4b). However, at 30 min thereafter, the magnitude of the EGP effect elicited by T₁AM i.c.v. was significantly greater than the effect of T₁AM i.p. (Fig. 4b).

Glucoregulatory hormones Similar to T₁AM i.p. (Fig. 2a), neither T₁AM- nor T₀AM-administered i.c.v. induced a significant change in plasma insulin content (Fig. 5a). Although there was a trend for insulin to decrease 10 min after i.c.v. infusion of T₁AM, this response failed to achieve statistical significance ($P=0.063$; Fig. 5a).

Plasma glucagon increased by 155% (from 69 ± 10 to 176 ± 20 pg/ml) 10 min after i.c.v. T₁AM infusion ($P<0.0001$ versus Veh). During the same time period, T₀AM i.c.v. also significantly increased plasma glucagon, but only by 58% ($P=0.004$ versus Veh). Interestingly, the magnitude of T₁AM's impact on circulating glucagon levels was dependent on the route of administration with i.c.v. infusion producing a greater effect in the first 10 min than i.p. administration (108 ± 21 vs 50 ± 11 pg/ml respectively; $P=0.044$). Unlike the sustained elevation that followed i.p. administration of either T₁AM or T₀AM, plasma glucagon returned to basal levels within 60 min of infusing either compound i.c.v. (Fig. 5b).

Plasma corticosterone concentrations were significantly increased following i.c.v. infusion of either 0.5 mg/kg T₁AM or T₀AM (Fig. 5c) with both treatments producing nearly equivalent maximum effects by $t=20$ min post-injection (delta corticosterone $t=20$ vs $t=0$; 6 ± 44 ng/ml Veh i.c.v., 206 ± 58 ng/ml T₁AM i.c.v., 296 ± 49 ng/ml T₀AM i.c.v., $P=0.02$ T₁AM versus Veh, $P<0.0001$ T₀AM versus Veh).

Plasma T₃, T₄, and TSH concentrations before and after central (i.c.v.) infusion of either T₁AM, T₀AM, or vehicle (saline-DMSO) infusion are shown in Table 2. Although plasma T₃, T₄, and TSH were found to decrease in all treatment groups by 120 min post-i.c.v. bolus infusion, neither i.c.v. T₁AM nor T₀AM had a statistically significant effect on plasma T₃, T₄, or TSH compared with vehicle i.c.v.

Figure 2 (a) Plasma insulin concentration before ($t=0$) and after i.p. injection of T₁AM, T₀AM, or vehicle. ANOVA indicates no effects of time and no differences in insulin concentration between groups (ANOVA RM factor *time* $P=0.556$, *time*×*group* $P=0.735$, and *group* $P=0.213$). (b) Plasma glucagon concentration before ($t=0$) and after i.p. injection of T₁AM, T₀AM, or vehicle. From $t=10$, glucagon is higher in both T₁AM- and T₀AM-treated rats relative to vehicle rats ($*P<0.05$ versus Veh). At $t=60$, glucagon concentration is higher in T₁AM when compared with T₀AM-treated rats ($*P=0.05$ T₀AM versus T₁AM). ANOVA RM factor *time* $P<0.0001$, *time*×*group* $P<0.0001$, and *group* $P=0.001$. (c) Plasma corticosterone concentration before ($t=-20$ and $t=0$ min) and after ($t=5-120$ min) i.p. injection of T₁AM, T₀AM, or vehicle. T₀AM-treated animals have higher plasma corticosterone from $t=30$ and T₁AM-treated animals only on $t=60$ when compared with vehicle ($*P<0.05$ versus Veh). Note that at no time-point, plasma corticosterone between T₁AM- and T₀AM-injected animals differs. ANOVA RM factor *time* $P<0.0001$, *time*×*group* $P=0.050$, and *group* $P=0.030$.

Table 1 Plasma thyroid hormone concentrations before (basal) and after (2 h) i.p. vehicle, T₁AM, and T₀AM infusion

	Veh i.p. n=7		T ₁ AM i.p. n=7		T ₀ AM i.p. n=8		ANOVA RM		
	Basal	2 h	Basal	2 h	Basal	2 h	Time	Time×group	Group
T ₃ (nmol/l)	0.82±0.05	0.78±0.05	0.88±0.04	0.83±0.05	1.12±0.07 [†]	0.64±0.07* [§]	0.0001	0.0001	0.515
T ₄ (nmol/l)	86±3	70±4*	91±4	48±3* [‡]	80±3	42±2* [‡]	0.0001	0.002	0.0001
TSH (mU/l)	1.46±0.27	2.03±0.31	1.79±0.25	1.09±0.29 [‡]	1.68±0.16	0.40±0.04* ^{‡,§}	0.028	0.004	0.014

*P≤0.05 versus basal value within the same group, [†]P<0.05 versus Veh basal, [‡]P<0.05 versus Veh 2 h, [§]P<0.05 versus T₁AM 2 h.

Discussion

In an effort to determine whether the thyronamines T₁AM and T₀AM can affect glucose homeostasis by acting directly on the brain, we compared their physiologic consequences following systemic and central administration. The major finding of our study is that central administration of low-dose (i.e. 1% of the systemic dose) T₁AM acutely increases EGP and plasma glucose concentration to a similar – or even greater – extent compared with systemic T₁AM, concomitant with an increase in plasma glucagon and corticosterone concentrations. Similar effects were observed following central T₀AM infusion, albeit to a lesser extent. When administered intravenously, the same low dose of T₁AM and T₀AM, which was effective centrally, had no detectable effect on plasma glucose or EGP; thus excluding the possibility that the observed responses were the result of leakage of the centrally administered compound into the circulation and acting peripherally.

Rats infused intraperitoneally with T₁AM, and to a lesser extent T₀AM, exhibited a behavioural phenotype within minutes of administration, which was remarkably similar to the fully reversible behavioural changes reported earlier in mice (Scanlan *et al.* 2004, Doyle *et al.* 2007). In short, animals exhibited a decrease in overall locomotor activity and responsiveness to external stimuli (visual and auditory), while reflexes were preserved. Furthermore, the hyperglycemia that develops in mice (Regard *et al.* 2007) following thyronamine exposure also is seen in rats (Fig. 1a). Moreover, we show for the first time that the hyperglycemia induced by the thyronamines T₁AM and (to a lesser extent) T₀AM occurs simultaneously with a rapid (i.e. within 10 min), ~50% increase in EGP that was maintained for the duration of the experiment (Fig. 1b).

With respect to the systemic administration of thyronamines, there are several mechanisms that may contribute to the alterations in glucose metabolism we observed. First, plasma glucagon increases rapidly in response to systemic T₁AM and T₀AM administration, concomitant with the increase in plasma glucose and EGP. It was expected that the thyronamine-induced hyperglycemia (up to 22 mmol/l; Fig. 1a) would provoke a considerable insulin response. However, plasma insulin did not change in spite of the overt

hyperglycemia produced by either thyronamine. The plasma glucagon increase together with this inadequate insulin response is likely to be causal factors in the increase in plasma glucose and EGP induced by thyronamines. These effects on

Figure 3 (a) Plasma glucose concentration before and after i.c.v. bolus infusion of T₁AM, T₀AM, or vehicle. From *t*=5 and *t*=10, glucose concentration in T₁AM- and T₀AM-infused rats respectively is higher compared with vehicle rats (*P*<0.05). From *t*=10, glucose concentration in T₁AM-infused rats is higher relative to T₀AM-infused rats (*P*<0.05). ANOVA RM factor *time* *P*<0.0001, *time*×*group* *P*<0.0001, and *group* *P*<0.0001. (b) Endogenous glucose production (EGP) before and after i.c.v. bolus infusion of T₁AM, T₀AM, or vehicle. There is no significant difference between basal samples of any group. From *t*=10, EGP in T₁AM-infused rats is higher compared with vehicle rats (*P*<0.05). In T₀AM rats, EGP at *t*=30, 45, 90, 105, and 120 min is higher relative to vehicle-infused rats (*P*<0.05). From *t*=20 to *t*=90, EGP is higher in T₁AM relative to T₀AM-infused rats (*P*<0.05). ANOVA RM factor *time* *P*<0.0001, *time*×*group* *P*<0.0001, and *group* *P*=0.006.

Figure 4 (a) Changes in plasma glucose concentration (Δ) before and after i.p. versus i.c.v. bolus infusion of T₁AM (T₁AM i.p. and T₁AM i.c.v.) or vehicle (Veh i.p. and Veh i.c.v.). From $t=5$, glucose concentration in T₁AM i.p. and T₁AM i.c.v. groups is increased relative to the respective vehicle group. Note that on $t=20$, 30, and 45 min, glucose concentration in T₁AM i.p. and T₁AM i.c.v. rats is not different. The changes in plasma glucose concentration are expressed as the difference compared to the plasma glucose concentration at $t=0$ min for each individual animal. ANOVA RM factor time $P<0.0001$, time $\times$ group $P<0.0001$, and group $P<0.0001$. * $P<0.05$; T₁AM i.p. versus T₁AM i.c.v. (b) Changes in endogenous glucose production (EGP) before and after i.p. versus i.c.v. bolus infusion of T₁AM (T₁AM i.p. and T₁AM i.c.v.) or vehicle (Veh i.p. and Veh i.c.v.). From $t=5$ and $t=10$ min, EGP in T₁AM i.c.v.- and T₁AM i.p.-infused rats respectively is increased when compared with respective vehicle groups ($P<0.05$). Note that on $t=30$, EGP is higher in T₁AM i.c.v. than in T₁AM i.p.-treated rats (* $P<0.05$). The changes in EGP are expressed as the difference compared to EGP at $t=0$ min for each individual animal. ANOVA RM factor time $P<0.0001$, time $\times$ group $P<0.0001$, and group $P<0.0001$.

plasma glucagon and insulin might be explained by direct actions of T₁AM and T₀AM on the pancreatic α and β -cells, supposedly by binding to GPCRs such as TAAR1 or AR α 2 (Liggett 2004, Scanlan *et al.* 2004). Indeed, pharmacological stimulation of AR α 2 has been shown to induce hyperglycemia and inhibit insulin release (Angel *et al.* 1990). Second, given the rapid onset of thyronamine-induced changes, it is possible that T₁AM and T₀AM activation of GPCRs expressed in hepatocytes underlies the stimulation of EGP we observed, analogous to the stimulation of β -ARs by norepinephrine.

With regard to the possible mechanisms underlying the effects of centrally administered thyronamines on glucose metabolism, it is interesting that concomitant with the rapid increase in EGP (Fig. 3b), plasma insulin levels tended to decrease acutely after central administration of 0.5 mg/kg T₁AM (Fig. 5a) in contrast to systemically (i.e. i.p.) administered drug. In addition, after i.c.v. infusion of 0.5 mg/kg T₁AM, there was a rapid increase in plasma glucagon (Fig. 5b), which was more pronounced than the early glucagon increase after systemic thyronamine infusion. No change in EGP, plasma insulin, and glucagon levels was observed after i.v. infusion of 0.5 mg/kg T₁AM, confirming that T₁AM-imposed actions on the CNS are causal to these phenomena. This dependence of thyronamine effects on the route of administration point to neural or (neuro)transmitter type, rather than humoral type of actions. Indeed, it has been demonstrated that T₁AM modulates synaptosomal transport of neurotransmitters such as dopamine and norepinephrine (Snead *et al.* 2007), supposedly by behaving as endogenous monoamine reuptake inhibitors (Weatherman 2007). In addition, low-dose T₁AM administration in the lateral cerebral ventricles and in the arcuate nucleus was recently reported to rapidly increase food intake (Dhillon *et al.* 2008). The effects of thyronamines on plasma insulin and glucagon in the present study may be explained by increased sympathetic tone in the pancreas, mediated via central thyronamine actions. In addition, centrally administered thyronamines might stimulate autonomic outflow from the hypothalamus to the liver thereby elevating EGP. In support of this conjecture is accumulating evidence demonstrating the brain's important role, particularly the hypothalamus, in regulating hepatic glucose metabolism via sympathetic and parasympathetic projections to the liver (Obici *et al.* 2002, Kalsbeek *et al.* 2004, Pocai *et al.* 2005, Klieverik *et al.* 2008).

As thyronamines have been hypothesized to constitute a novel aspect of TH biology (Liggett 2004, Scanlan *et al.* 2004), it was of interest to assess how thyroid-related parameters in euthyroid animals responded to synthetic thyronamines. Systemic infusion of these compounds, in particular T₀AM, depressed plasma TSH, T₄, and T₃ levels, whereas central administration had no such effect. These responses could represent a state reminiscent of the non-thyroidal illness syndrome (Flies *et al.* 1997, Boelen *et al.* 2004, Adler & Wartofsky 2007). Although it is conceivable that the thyronamines altered TH secretion by decreasing TSH release from the pituitary, the observation that central thyronamine administration does not induce plasma TH alterations relative to vehicle, argues against this possibility. Finally, an effect of thyronamines on plasma concentrations of iodothyronines via interaction with the deiodinase enzymes seems less likely as T₁AM does not interfere with D1-mediated iodothyronine deiodination *in vitro* (Piehl *et al.* 2008).

Systemic thyronamine administration produced significant increases in plasma corticosterone levels that were similar in T₁AM- and T₀AM-infused rats (Fig. 2c), and could be recapitulated by low-dose (0.5 mg/kg) i.c.v. infusion of

synthetic T₁AM or T₀AM (Fig. 5c), suggesting that these represent central effects of thyronamines on the hypothalamus–pituitary–adrenal axis. The elevated corticosterone could contribute in a limited way to the hyperglycemic state but, more importantly, because in both experiments the hyperglycemia was much more pronounced in T₁AM when compared with T₀AM-infused animals, it is unlikely to account for the major glucose increase induced by systemic and central T₁AM.

There are several mechanisms by which circulating thyronamines might exert their actions in the CNS. First, there could be passive or active transport of circulating thyronamines across the blood–brain barrier, the latter analogous to iodothyronines (Dratman *et al.* 1991). Second, circulating thyronamines might bind cell-surface receptors in the plasma membrane of neurons located in circumventricular nuclei such as the arcuate nucleus where the blood–brain barrier is absent. The arcuate nucleus is known to mediate central actions of the peptide hormones like insulin and leptin via locally expressed leptin and insulin receptors (Niswender & Schwartz 2003), and TAAR1 is expressed in the arcuate nucleus (Borowsky *et al.* 2001). However, our finding that the EGP increase is not as robust following central administration of thyronamines as it is after systemic administration suggests their central actions alone are insufficient to account for the persistent EGP increase and hyperglycemia. Consistent with this interpretation are the results from a recent study in which mice pre-treatment with 6-hydroxydopamine still developed hyperglycemia and hypoinsulinemia following i.p. administration of T₁AM, suggesting that these T₁AM-induced alterations can occur in the absence of sympathetic signalling (Regard *et al.* 2007). Another possibility is that thyronamines impose both peripheral and central actions on glucose metabolism, occurring independently. In this intriguing scenario, central actions could be mediated by thyronamines formed locally in the brain, by conversion from iodothyronines such as T₄, T₃, and/or reverse T₃.

The rapid and pronounced metabolic effects produced by central administration of T₁AM and T₀AM suggest that one or more receptors mediate their actions. Indeed, T₁AM and T₀AM dose dependently activate the Gs protein-coupled TAAR1 receptor (Borowsky *et al.* 2001, Bunzow *et al.* 2001, Grandy 2007, Wainscott *et al.* 2007). TAAR1 belongs to a large family of related receptors (Borowsky *et al.* 2001, Grandy 2007), and this receptor's mRNA is expressed in a wide variety of tissues including many areas of the brain

Figure 5 (a) Plasma insulin concentrations before ($t=0$) and after i.c.v. bolus infusion of T₁AM, T₀AM, or vehicle. Note that at $t=10$ min, there is a trend for insulin concentrations to be depressed in animals receiving 0.5 mg/kg T₁AM i.c.v. compared with vehicle-treated rats ($^*P=0.063$). ANOVA RM factor *time* $P=0.01$, *time*×*group* $P=0.161$, and *group* $P=0.758$. (b) Plasma glucagon concentrations before ($t=0$) and after i.c.v. bolus infusion of T₁AM, T₀AM, or vehicle. At $t=10$ min, glucagon is significantly higher in T₁AM i.c.v. and, to a lesser extent, T₀AM i.c.v. when compared with vehicle i.c.v.-treated rats. $^*P<0.01$ versus vehicle i.c.v., $^{\wedge}P<0.01$ T₁AM versus T₀AM i.c.v. ANOVA RM factor *time* $P<0.0001$, *time*×*group* $P<0.0001$, and *group* $P<0.0001$. (c) Plasma corticosterone concentration before ($t=-20$ and 0 min) and after ($t=5$ to $t=120$ min) i.c.v. bolus infusion of T₁AM, T₀AM, or vehicle. Circulating corticosterone levels rapidly increase following the infusion of T₁AM or T₀AM at $t=20$ and $t=10$ min when compared with vehicle respectively. Note that T₁AM- and T₀AM i.c.v.-infused groups do not differ at any time point, except for $t=5$ min. $^*P<0.05$ versus vehicle i.c.v., $^{\wedge}P<0.01$ T₁AM versus T₀AM i.c.v. ANOVA RM factor *time* $P<0.0001$, *time*×*group* $P=0.002$, and *group* $P=0.001$.

Table 2 Plasma thyroid hormone concentrations before (basal) and after (2 h) i.c.v. vehicle, T₁AM, and T₀AM infusion

	Veh i.c.v. n=8		T ₁ AM i.c.v. n=9		T ₀ AM i.c.v. n=8		ANOVA RM		
	Basal	2 h	Basal	2 h	Basal	2 h	Time	Time× group	Group
T ₃ (nmol/l)	1.27±0.08	0.77±0.05*	1.39±0.15	0.86±0.09*	1.00±0.05	0.73±0.05*	P	P	P
T ₄ (nmol/l)	81±7	53±4*	79±7	64±7*	87±4	62±4*	0.0001	0.184	0.578
TSH (mU/l)	1.64±0.33	0.77±0.16*	1.36±0.26	0.64±0.19	1.65±0.25	0.58±0.10*	0.0001	0.665	0.734

* $P \leq 0.05$ versus basal value within the same group.

(Borowsky *et al.* 2001, Bunzow *et al.* 2001). The fact that the rank order of potency as a TAAR1 agonist *in vitro*, T₁AM being more potent than T₀AM (Scanlan *et al.* 2004), is also reflected in the metabolic responses described in the present study, fits with the notion that TAAR1 may mediate some actions of T₁AM and T₀AM. In addition, Regard *et al.* (2007) have recently shown that whereas T₁AM induces hyperglycemia after systemic administration in wild-type mice, this effect is lost in AR α 2-deficient mice as well as mice pre-treated with the AR α 2 antagonist yohimbine. Moreover, by using a transgenic approach, they provided strong evidence that the hyperglycemia and concurrent hypoinsulinemia following T₁AM infusion in mice was dependent upon pancreatic Gi protein-coupled receptor expression. Collectively, these data suggest that, at least for the effects of systemically administered T₁AM on glucose metabolism, AR α 2 is important. Interestingly, AR α 2 are highly expressed in the hypothalamus as well and contribute to the hypothalamic regulation of sympathetic outflow (Li *et al.* 2005), supporting their possible involvement in mediating effects of centrally administered thyronamines.

To date, every published metabolic and physiologic study involving thyronamines, including this one, has relied on the administration of synthetic material (Bräulke *et al.* 2007, Chiellini *et al.* 2007, Doyle *et al.* 2007, Regard *et al.* 2007). Therefore, the biological significance of endogenous thyronamines remains to be addressed. In this context, it will be important to establish how and where these compounds are synthesized. Although there is currently no direct evidence in the literature for *in vivo* conversion of thyronamines from precursor iodothyronines (i.e. T₃, T₄, and rT₃), the enzymes indispensable for such conversion such as aromatic amino acid decarboxylase for decarboxylation and iodothyronine deiodinases type 2 and 3 for deiodination are widely distributed in the CNS, and indeed within the hypothalamus (Zhu & Juorio 1995, Tu *et al.* 1997, Alkemade *et al.* 2005, Lechan & Fekete 2005). An important remaining question is whether the metabolic effects in the present and other studies represent physiological or pharmacological effects of thyronamines. The systemic dose of T₁AM used in our study has been shown to induce a tenfold increase in plasma T₁AM concentration within 3 h after infusion in Siberian hamsters (Bräulke *et al.* 2007). However, there are currently no data on

the pharmacokinetic characteristics (distribution volume, clearance, and binding to plasma proteins) of thyronamines, and at present it is unknown how thyronamine tissue concentrations during experimental manipulations compare to their concentrations under more physiologic conditions.

We conclude that central administration of a low dose of either T₁AM or T₀AM can acutely induce increased EGP and hyperglycemia, concomitant with increased plasma glucagon, corticosterone, and a deficient insulin response. These changes are very similar to the acute changes observed after systemic T₁AM and T₀AM administration. Our data indicate that thyronamines can act centrally in order to modulate glucose metabolism.

Declaration of interest

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

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