

RESEARCH PAPER

3-Iodothyronamine: a modulator of the hypothalamus-pancreas- thyroid axes in mice

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BACKGROUND AND PURPOSE

Preclinical pharmacology of 3-iodothyronamine (T1AM), an endogenous derivative of thyroid hormones, indicates that it is a rapid modulator of rodent metabolism and behaviour. Since T1AM undergoes rapid enzymatic degradation, particularly by MAO, we hypothesized that the effects of T1AM might be altered by inhibition of MAO.

EXPERIMENTAL APPROACH

We investigated the effects of injecting T1AM (i.c.v.) on (i) feeding behaviour, hyperglycaemia and plasma levels of thyroid hormones and (ii) T1AM systemic bioavailability, in overnight fasted mice, under control conditions and after pretreatment with the MAO inhibitor clorgyline. T1AM (1.3, 6.6, 13, 20 and 26 $\mu\text{g}\cdot\text{kg}^{-1}$) or vehicle were injected i.c.v. in fasted male mice not pretreated or pretreated i.p. with clorgyline (2.5 $\text{mg}\cdot\text{kg}^{-1}$). Glycaemia was measured by a glucofractometer, plasma triiodothyronine (fT3) by a chemiluminescent immunometric assay, *c-fos* activation immunohistochemically and plasma T1AM by HPLC coupled to tandem-MS.

KEY RESULTS

T1AM, 1.3 $\mu\text{g}\cdot\text{kg}^{-1}$, produced a hypophagic effect (-24% vs. control) and reduced *c-fos* activation. This dose showed systemic bioavailability (0.12% of injected dose), raised plasma glucose levels and reduced peripheral insulin sensitivity (-33% vs. control) and plasma fT3 levels. These effects were not linearly related to the dose injected. Clorgyline pretreatment strongly increased the systemic bioavailability of T1AM and prevented the hyperglycaemia and reduction in fT3 induced by T1AM.

CONCLUSIONS AND IMPLICATIONS

T1AM induces central and peripheral effects including hyperglycaemia and a reduction in plasma fT3 levels in fasted mice. These effects critically depend on the concentration of T1AM or its metabolites in target organs.

Abbreviations

fT3, triiodothyronine; fT4, thyroxine; NPY, neuropeptide Y; T1AM, 3-iodothyronamine; TARR, trace amine-associated receptor; TSH, thyroid stimulating hormone

Introduction

3-Iodothyronamine (T1AM) is an endogenous primary amine related to thyroid hormones (Scanlan *et al.*, 2004; Phiel *et al.*,

2011). T1AM circulates in healthy rodents and humans but is also present in most tissues, and the highest concentrations in rats occur in the liver and the brain (Saba *et al.*, 2010; Hoefig *et al.*, 2011). The physiological role of T1AM is still

under investigation, and recent data suggest that it may have metabolic and endocrine effects (Hoefig *et al.*, 2011). Different pharmacological responses have been elicited after administration of exogenous T1AM. T1AM injected i.c.v. into rodents rapidly induces metabolic effects, which are not linearly related to the dosage and are dependent on the animal species and route of administration. In particular, Klieverik *et al.* (2009) observed that i.c.v. injection of T1AM ($0.5 \text{ mg}\cdot\text{kg}^{-1}$) in rats rapidly induced hyperglycaemia, whereas after i.p. administration of T1AM, the hyperglycaemia observed and was also associated with decreased plasma levels of triiodothyronine (T3).

Dhillon *et al.* (2009) reported a transient increase in food intake in rats and mice treated with T1AM (1.2 and $4 \text{ nmol}\cdot\text{kg}^{-1}$, respectively), an effect that was not conserved at lower and higher doses. The same authors demonstrated that exposure of hypothalamic slices to T1AM *in vitro* induced neuropeptide Y (NPY) release, suggesting that this potent orexigenic peptide is involved in the hyperphagic effect. However, in a recent preliminary study, Hettinger *et al.* (2010) observed that chronic systemic administration of T1AM ($31 \text{ }\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) reduced food intake in mice.

These discrepancies might derive, at least in part, from the complex metabolism of T1AM, that includes amine oxidation by mitochondrial (MAO) and/or semicarbazide-sensitive amine oxidases, yielding metabolites that have functional effects, if any, that are still unknown. Rapid amine degradation might contribute to the bell-shaped dose-response relationships observed following T1AM treatment of rodents. In addition, T1AM has been found to interfere with the response to other biogenic amines (Snead *et al.*, 2007).

The molecular target(s) responsible for T1AM metabolic effects are largely unknown. *In vitro* studies provided evidence that T1AM can activate amine GPCRs with high affinity, including trace amine-associated receptors (TAARs) (Scanlan *et al.*, 2004; Zucchi *et al.*, 2006) and possibly α_2 -adrenoceptors (Regard *et al.*, 2007). Other putative molecular targets are membrane transporters, including vesicular monoamine transporter 2, which suggests a possible neuromodulatory role for T1AM (Snead *et al.*, 2007; Ianculescu *et al.*, 2009). Recently, functional evidence of mitochondrial targets has been obtained (Venditti *et al.*, 2011).

The present investigation was planned to explore the consequences of injecting T1AM (from 1.3 to $26 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$, i.c.v.) on feeding behaviour, hyperglycaemia and plasma levels of thyroid hormones in overnight fasted mice, under control conditions and after pretreatment with clorgyline, an inhibitor of MAO activity. Moreover, we also aimed to verify whether T1AM injected i.c.v. had any systemic bioavailability. The rationale for using fasting animals is that fasting causes activation of hypothalamic orexigenic systems (Coppola *et al.*, 2007) and pancreatic secretion, making it easier to detect a potential inhibitory effect.

Methods

Animals

Male mice (CD1 strain; $25.2 \pm 3.2 \text{ g}$ mean weight) from Harlan-Nossan (Italy) were used. Five mice were housed per

cage. The cages were placed in the experimental room 24 h before the test for adaptation and, unless otherwise stated, the mice were deprived of food (fasted) overnight before the experiments. The animals were kept at $23 \pm 1^\circ\text{C}$ with a 12 h light-dark cycle (light on at 07h00min) and were fed a standard laboratory diet with water *ad libitum*.

All animal care and experimental procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for experimental animal care. All efforts were made to minimize animal suffering and to reduce the number of animals used.

I.c.v. injection technique

I.c.v. administration was performed under light ether anaesthesia according to the method described by Haley and McCormick (1957) with certain modifications. The depth of anaesthesia was checked by monitoring respiratory rate (which was reduced within 2 min) and testing the lack of pain response to gentle pressure on the hind paws. The head of the anaesthetized mouse was grasped firmly, and the needle of a $10 \text{ }\mu\text{L}$ microsyringe was inserted perpendicularly 2 mm through the skull into the brain. Ten microlitres of solution were then injected slowly, in 20 s, into a lateral ventricle. The injection site was 1 mm to the left from the midpoint on a line drawn through to the anterior base of the ears. Immediately after needle removal, the animal remained quiet for approximately 1 min and then resumed its normal activity. To ascertain that solutions were administered exactly into the cerebral ventricle, some mice were injected with $10 \text{ }\mu\text{L}$ of diluted 1:10 India ink, and their brains were examined macroscopically after sectioning, showing that 95% of injections were correct.

Evaluation of food consumption

Mice (five per cage) were deprived of food for 16 h (from 17 h to 09 h), but water was available *ad libitum*.

A weighed amount of food (standard laboratory pellets) was then given, and the amount consumed (evaluated as the difference between the original amount and the food left in the cage without littermate, including spillage) was measured at 30, 60, 90, 120 min after i.c.v. (second ventricle) administration to anaesthetized mice of $10 \text{ }\mu\text{L}$ of 0.5% dimethyl sulfoxide $\text{w}\cdot\text{v}^{-1}$ in saline solution (vehicle) or T1AM (1.3 , 6.6 , 13.2 , 20 and $26 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ prepared in vehicle), with an accuracy of 0.1 g . Both the T1AM and vehicle-injected mice were treated i.p. with saline solution or clorgyline ($2.5 \text{ mg}\cdot\text{kg}^{-1}$; Sigma-Aldrich, St Louis, MO, USA) 30 min before the T1AM i.c.v. injections. The results presented are the mean \pm SEM of two experiments; in each one 10 animals were used for each injection.

c-fos determination

Mice fasted for 16 h were pretreated with saline i.p., 30 min before an injection of T1AM $1.3 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ ($n = 3$), T1AM $20 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ ($n = 3$) or vehicle i.c.v. ($n = 3$) under ether anaesthesia, as described above. Animals were then killed by decapitation, within 60 min after i.c.v. injection. The skull was opened with scissors and the brain surface was cooled down, by exposing it to liquid nitrogen vapours for 1–2 s *in situ*. The brain was then removed and the forebrain was

divided into two parts: the cortex and the diencephalon. The hypothalamus was quickly separated from the thalamus, flash-frozen at -80°C and embedded in Killik cryostat embedding medium (Bio-Optica, Milan Italy). Cryosections, $10\text{ }\mu\text{m}$ thick, were collected on polylysine-coated slides and fixed in paraformaldehyde vapour for 10 min.

In order to quench endogenous peroxidase activity, the slides were treated with 3% H_2O_2 in H_2O and then pre-incubated with 1% BSA (Sigma Aldrich, Milan, Italy) in PBS (pH 7.8) for 20 min to saturate non-specific sites. Then, the slides were incubated overnight at 4°C with rabbit polyclonal anti-*c-fos* antibodies (Santa Cruz Biotechnology, CA, USA) at a final dilution of 1:100. In the negative controls carried out, the primary antibody was omitted. Immunoreactivity was revealed by goat anti-rabbit IgG conjugated with biotin (Vector Lab, Burlingame, CA, USA) at a final dilution of 1:1000 followed by incubation with ABC complex (Vector Lab) and then with Sigma FAST 3-3'-diaminobenzidine (Sigma Aldrich).

Staining was performed in a single session, to minimize artificial differences. Photomicrographs were randomly taken using a digital photomicroscopy apparatus (Jenoptik, Jena, Germany) with a $\times 25$ objective. Each microscopic field corresponded to a test area of $24.2\text{ }\mu\text{m}^2$. The optical density of *c-fos* immunostained tissue was measured on the digitized images (10 per animal) using the Image J image analysis programme (by the National Institute of Health, Bethesda, MD, USA). After evaluation of the optical density, sections were counterstained with haematoxylin for 90 s.

Glycaemia and collection of samples for T1AM plasma level determination

Blood samples were collected from the tail vein of 16 h fasted mice pretreated i.p. with clorgyline ($2.5\text{ mg}\cdot\text{kg}^{-1}$) or with saline, 30 min before i.c.v. vehicle ($n = 15$) or T1AM 1.3 ($n = 15$) or $20\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ ($n = 15$). Five mice in each group were killed by decapitation 30 min after T1AM injection to collect the blood and the brains in order to measure plasma T1AM tissue levels.

In another set of experiments, glycaemia was monitored in the blood collected from the tail vein of 4 h (from 8 h to 12 h) fasted mice who had received T1AM ($1.3\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ i.c.v.) and saline (i.p.), or T1AM ($1.3\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ i.c.v.) and exenatide ($0.25\text{ mg}\cdot\text{mL}^{-1}$ i.p.), or vehicle i.c.v. and exenatide ($0.25\text{ mg}\cdot\text{mL}^{-1}$ i.p.) ($n = 5$ in each group). Glycaemia was evaluated by a glucorefractometer at 0, 15, 30 and 60 min after the i.c.v. injections.

T1AM plasma and brain levels

T1AM levels were measured in plasma and brain samples by HPLC coupled to tandem-MS, as described previously (Saba *et al.*, 2010).

Insulin tolerance test

An i.p. insulin tolerance test was performed in 4 h fasted mice (from 8 h to 12 h), a condition chosen to avoid hypoglycaemia following insulin treatment.

Three experimental groups were considered ($n = 5$ mice per group): one group was injected i.c.v. with vehicle, one with $1.3\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ T1AM and one with T1AM $20\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ T1AM under light ether anaesthesia. All these animals received

insulin ($1\text{ U}\cdot\text{kg}^{-1}$ Humulin R Regular, Ely Lilly, Indianapolis, IN, USA) i.p. 10 min after the i.c.v. injection.

Small blood samples from the lateral tail vein were collected to measure glycaemic levels at time 0, 15, 30, 60, 90 and 120 min after i.p. insulin injection. A glucose solution ($33\%\text{ w}\cdot\text{v}^{-1}$) was prepared in order to correct symptomatic hypoglycaemia. Results are shown as glucose concentration ($\text{mg}\cdot\text{L}^{-1}$) and as the difference from the baseline value.

Plasma levels of thyroid hormones

To evaluate free plasma triiodothyronine (fT3), thyroxine (fT4) and thyroid stimulating hormone (TSH) levels, three sets of experiments were performed on 4 h fasted mice ($n = 5$ mice in each experimental group):

Exp. 1: mice pretreated with saline i.p., 30 min before i.c.v. injection with vehicle, $1.3\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ T1AM, or $20\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ T1AM.

Exp. 2: mice pretreated with clorgyline ($2.5\text{ mg}\cdot\text{kg}^{-1}$) i.p., 30 min before i.c.v. injection with vehicle or $1.3\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ T1AM.

Exp. 3: mice treated with exenatide ($0.25\text{ mg}\cdot\text{mL}^{-1}$) i.p., 30 min before i.c.v. injection of vehicle or $1.3\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ T1AM.

All the animals were killed by decapitation within 60 min after i.c.v. injections. The blood was collected and centrifuged at $3000\times g$ for 20 min at room temperature to prepare plasma.

Circulating fT3, fT4 and TSH levels were quantified in $100\text{ }\mu\text{L}$ of mouse plasma, by a solid-phase, two-site chemiluminescent immunometric assay (Immunolite 2000; Siemens Corp., Tarrytown, NY, USA). The manufacturer's reference limit were $2.3\text{--}4.2\text{ pg}\cdot\text{mL}^{-1}$, $0.5\text{ nmol}\cdot\text{L}^{-1}$ and $0.40\text{ mU}\cdot\text{L}^{-1}$, respectively, fT3, fT4 and TSH. The assay was validated by unchanged plasma fT3 and fT4 levels recovered over 1:10 plasma. Intra-assay variability was in the range of 8%.

Statistical analysis

Data are expressed as mean \pm SEM of independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Student–Newman–Keuls multiple comparison *post hoc* test; the threshold of statistical significance was set at $P < 0.05$. Data analysis was performed by GraphPad Prism 5.0 statistical program (GraphPad software, San Diego, CA, USA).

Results

T1AM injected i.c.v. in fasted mice induced hypophagia and hyperphagia: the effect of clorgyline pretreatment

Increasing doses of T1AM (1.3 , 6.5 , 13 , 20 and $20\text{ }\mu\text{g}\cdot\text{kg}^{-1}$; $n = 10$ mice for each dose) produced opposite effects on feeding. In particular, after 120 min, the total amount of food eaten (g per mouse) by mice receiving 1.3 and $20\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ T1AM was significantly ($P < 0.05$) lower (2.74 ± 0.084) and higher (3.83 ± 0.02), respectively, than the control value (3.29 ± 0.08). The hypophagic effect of T1AM $1.3\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ derived from increased satiety occurring within the first 30 min after injection. In fact, mice injected with T1AM $1.3\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ almost stopped feeding within the first 30 min, while those injected

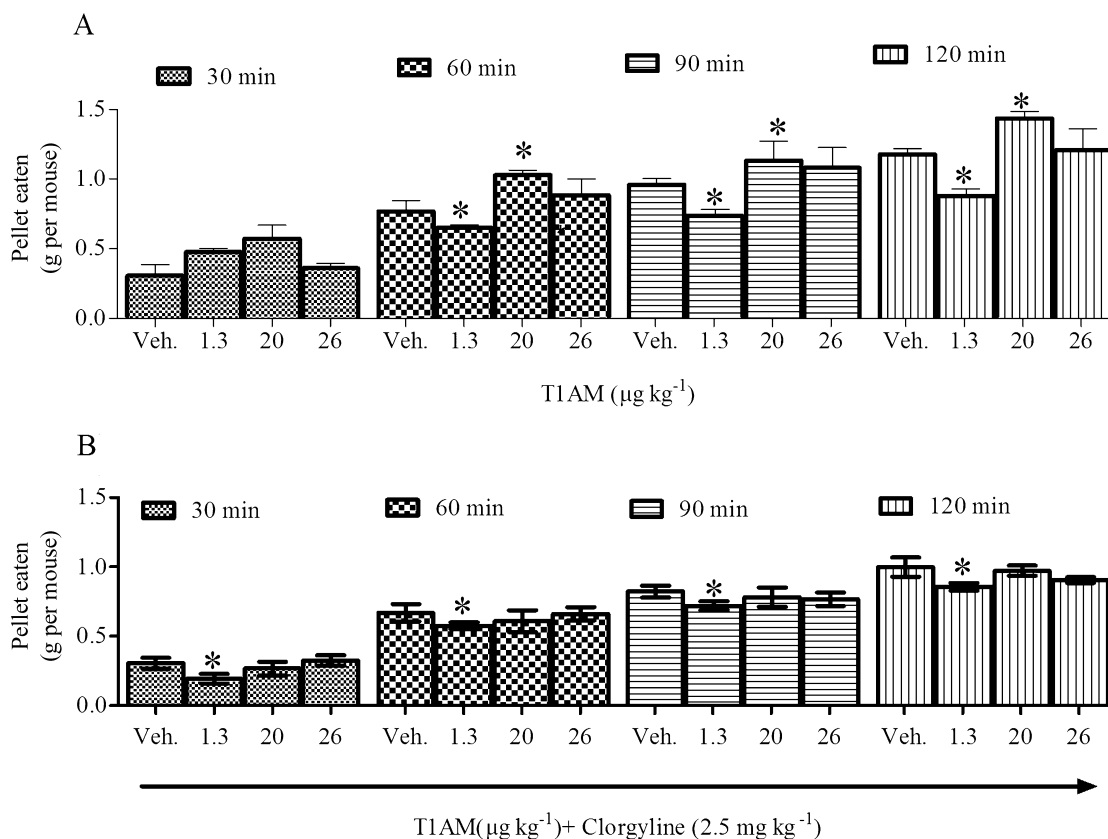


Figure 1

The i.c.v. administration of T1AM resulted in hypo- and hyperphagia: the effect of clorgyline pretreatment. Mice fasted for 16 h were pretreated i.p. with saline solution (A) or with clorgyline 2.5 mg·kg⁻¹ (B). After 30 min, mice ($n = 10$) were injected i.c.v. with vehicle or with T1AM (from 1.3 to 26 $\mu\text{g kg}^{-1}$; $n = 10$ mice for each dose), mice were then allowed food and water *ad libitum*. The amount of food consumed after 30, 60, 90 and 120 min following T1AM or vehicle injection was evaluated as described in the Methods section. Results are expressed as means \pm SEM of two experiments. Clorgyline pretreatment did not affect mice feeding behaviour compared to vehicle-injected mice. * $P < 0.05$ vs. vehicle.

with T1AM 20 $\mu\text{g kg}^{-1}$ and with vehicle reached satiety within 60 min. Both effects on feeding were significantly different from vehicle-injected mice 60 min after injection, and the difference persisted for the whole duration of the experiment (120 min).

With T1AM doses of 6.5, 13 or 26 $\mu\text{g kg}^{-1}$, a slight increase in feeding versus vehicle-injected mice was observed, but the effect did not reach the threshold of statistical significance (data not shown). T1AM effects on feeding behaviour of fasted mice did not show a linear dose–effect relationship but, rather, a biphasic response.

In mice pretreated with clorgyline (2.5 mg·kg⁻¹; Ilani *et al.*, 2000), the hypophagic effect of T1AM 1.3 $\mu\text{mol kg}^{-1}$ was conserved, and it reached the threshold of statistical difference versus vehicle-injected mice within the first 30 min after injection (Figure 1B), while satiety occurred within 60 min. Conversely, the hyperphagia induced by 20 $\mu\text{mol kg}^{-1}$ T1AM was completely prevented (Figure 1B). Clorgyline did not affect the food intake of fasted mice injected with vehicle.

All further investigations were performed using T1AM dosages able to produce effects on feeding, namely 1.3 and 20 $\mu\text{mol kg}^{-1}$.

Hypothalamic c-fos expression in fasted mice is reduced by T1AM injection

In the hypothalamus of 16 h fasted mice injected i.c.v. with vehicle, strong *c-fos* activation was observed. A significantly lower activation was found in hypothalamic slices derived from mice that had been injected i.c.v. with either 1.3 or 20 $\mu\text{g kg}^{-1}$ T1AM (Figure 2).

T1AM injected i.c.v. presented systemic bioavailability

T1AM given i.c.v. showed systemic bioavailability, suggesting spillover from the central nervous system. As shown in Table 1, the percentage of amine recovered was around 0.2% of the amount injected at both the low and the high dose (1.3 and 20 $\mu\text{g kg}^{-1}$, respectively). This result suggested that only a small amount of the injected T1AM can reach the periphery and that the mechanism responsible for its transport into the blood is not simple diffusion but rather a carrier-mediated mechanism. In mice with MAO blockade, systemic bioavailability of endogenous and pharmacological injected T1AM increased markedly (Table 1). Under these experimental

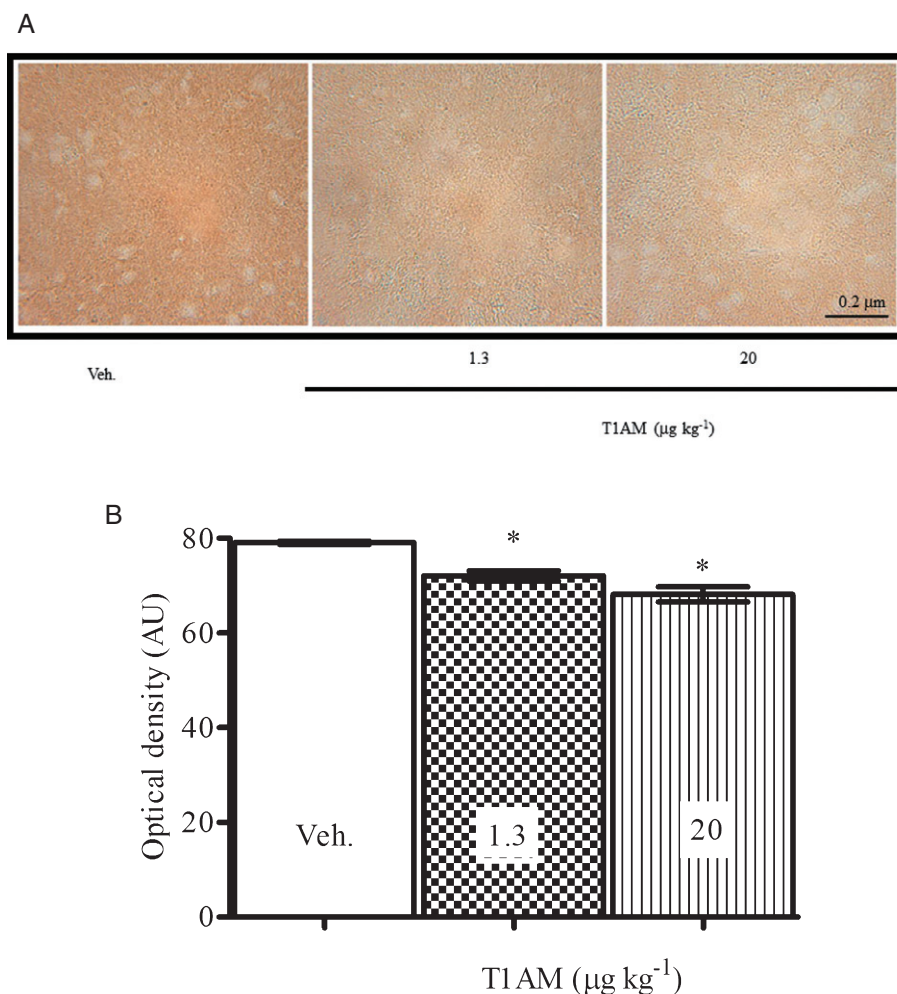


Figure 2

T1AM reduces *c-fos* expression in the hypothalamus of fasted mice. Hypothalamus was isolated from the brain of animals killed within 60 min after i.c.v. injection of vehicle (Veh.) or T1AM 1.3 and 20 $\mu\text{g kg}^{-1}$. Sections were prepared for immunohistochemistry (A) and evaluation of optical density (B) was performed as described in the Methods section. * $P < 0.05$ vs. vehicle.

Table 1

T1AM levels in the systemic circulation

T1AM ($\mu\text{g}\cdot\text{kg}^{-1}$)	pmol·mL ⁻¹	Total pmol	Yield (%)
Vehicle	ND		
1.3	0.19	0.28	0.215
20	1.5	2.25	0.15
Veh +clorgyline	0.05	0.082	0.082
1.3+clorgyline	0.35	0.52	0.52
20+clorgyline	57.05	85.58	5.7

T1AM plasma levels were assayed as described in the Methods section following i.c.v. injection of vehicle or 1.3 and 20 $\text{mg}\cdot\text{kg}^{-1}$.

Results were obtained from pooled plasma samples (five mice for each group) collected from 16 h-fasted mice. Veh, vehicle. The dose of clorgyline used was 2.5 $\text{mg}\cdot\text{kg}^{-1}$.

conditions, the bioavailability of 1.3 $\mu\text{g}\cdot\text{kg}^{-1}$ T1AM increased only twice, while with 20 $\mu\text{g}\cdot\text{kg}^{-1}$ T1AM, a 40-fold increase was observed. The yield of T1AM recovery was calculated in a volume of 1.5 mL of blood for each mouse (Lee and Blaurox, 1985).

T1AM injected i.c.v. rapidly induced hyperglycaemia: the effect of clorgyline pretreatment

Despite opposite effects on feeding, both 1.3 and 20 $\mu\text{g}\cdot\text{kg}^{-1}$ T1AM rapidly (within 15 min) induced hyperglycaemia (Figure 3) in fasted mice, which persisted up to 60 min after injection (data not shown).

Clorgyline (2.5 $\text{mg}\cdot\text{kg}^{-1}$) pretreatment strongly affected T1AM-induced hyperglycaemia, since both doses of T1AM became ineffective (Figure 3). Interestingly, 60 min after the administration of 20 $\mu\text{g}\cdot\text{kg}^{-1}$ T1AM, the animals actually showed very low levels of glycaemia ($47.7 \pm 5.6 \text{ mg}\cdot\text{dL}^{-1}$).

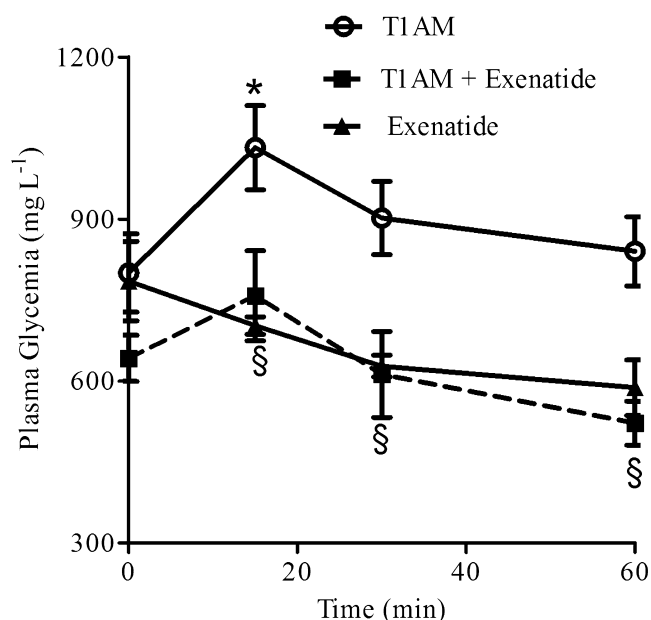


Figure 3

T1AM injected i.c.v. increases plasma glycaemia: the effect of clorgyline pretreatment. The blood was collected from the tail vein of two groups ($n = 5$ each) of mice (fasted for 16 h) pretreated i.p. with clorgyline ($2.5 \text{ mg} \cdot \text{kg}^{-1}$) or with saline, 30 min before i.c.v. injection of vehicle or T1AM 1.3 and $20 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$. Glycaemia was evaluated by a glucorefractometer at 0, 15, 30 and 60 min after i.c.v. injections. Results are the means \pm SEM of two experiments carried out on five mice for each experimental setting. The value of glycaemia measured 15 min after T1AM injection is shown. * $P < 0.05$ vs. vehicle i.c.v. and saline i.p.; § and ° $P < 0.05$ vs. T1AM 1.3 and $20 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$, respectively, in the absence of clorgyline pretreatment.

T1AM-induced hyperglycaemia increased insulin resistance

We also tested whether the hyperglycaemia induced by T1AM was associated with a reduction in insulin peripheral sensitivity. To do this, we performed an i.p. insulin tolerance test in mice deprived of food for 4 h, as described in the Methods section. The condition of 4 h fasting was chosen to avoid severe hypoglycaemia. Peripheral insulin sensitivity was significantly reduced after the injection of $1.3 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$ T1AM, while at the dose of $20 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$, the response to insulin was not different from that obtained in vehicle-injected animals (Figure 4A and B).

T1AM $1.3 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$ -induced hyperglycaemia was prevented by exenatide treatment

In order to evaluate whether the hyperglycaemia induced by $1.3 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$ T1AM involved glucagon secretion, we repeated the previous experiments in mice treated i.p. with exenatide ($0.25 \text{ mg} \cdot \text{mL}^{-1}$; Byetta, Ely Lilly; $n = 5$), a glucagon-like peptide-1 analogue which prevents glucagon release with a low risk of hypoglycaemia. As shown in Figure 5, the hyperglycaemia induced by T1AM ($1.3 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$) was prevented in mice treated with exenatide.

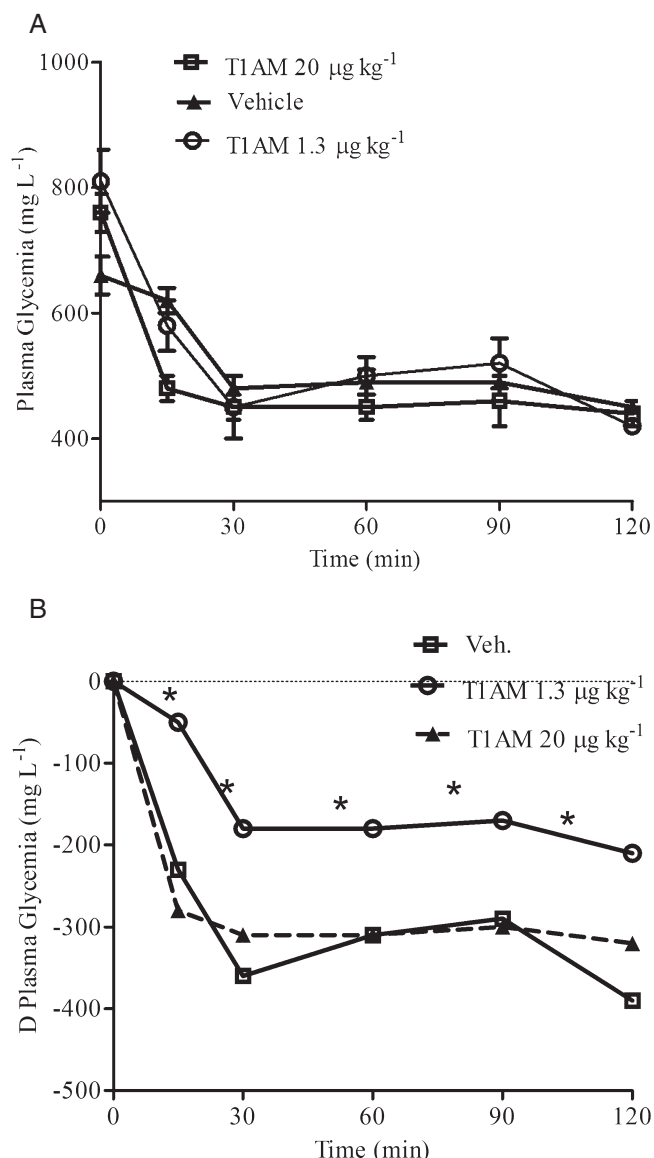


Figure 4

T1AM $1.3 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$ reduces peripheral insulin sensitivity. Fifteen mice fasted for 4 h, were divided into three groups (five mice each). One group was injected i.c.v. with T1AM $1.3 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$, one with T1AM $20 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$, and both i.p. with insulin ($1 \text{ U} \cdot \text{kg}^{-1}$). The remaining group was injected i.c.v. with vehicle and i.p. with insulin ($1 \text{ U} \cdot \text{kg}^{-1}$). Small blood samples from the lateral tail vein were collected to measure glycaemic levels at 0, 15, 30, 60, 90 and 120 min after i.p. insulin treatment. Results are shown as glucose levels ($\text{mg} \cdot \text{L}^{-1}$) and as the difference between glycaemia at one time point and the value at $T = 0$ (Δ glycaemia $\text{mg} \cdot \text{L}^{-1}$). Results represent the means \pm SEM of the values of two experiments carried on five mice for each time point. * $P < 0.05$ vs. vehicle.

T1AM injection reduced fT3 plasma levels: the effect of exenatide and clorgyline pretreatment

Subsequently, we verified whether the T1AM doses active on food intake and on glucose homeostasis were able to modify

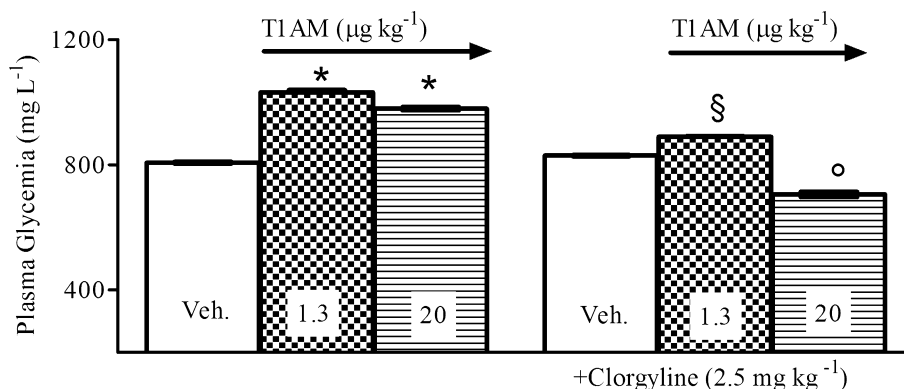


Figure 5

Exenatide treatment prevented T1AM ($1.3 \mu\text{g kg}^{-1}$)-induced increase in plasma glycaemia. Glycaemia was also monitored in the blood collected from the tail vein of 4 h fasted mice ($n = 5$ mice for each injection) treated as described in the Methods section. Mice received T1AM $1.3 \mu\text{g kg}^{-1}$ i.c.v and saline i.p., or T1AM $1.3 \mu\text{g kg}^{-1}$ i.c.v and exenatide (0.25 mg mL^{-1}) i.p., or vehicle (Veh.) i.c.v and exenatide (0.25 mg mL^{-1}) i.p. Results represent the means \pm SEM of the values of two experiments carried out on five mice for each time point. * $P < 0.05$ vs. basal glycaemia level ($T = 0$); § $P < 0.05$ vs. T1AM in the absence of exenatide.

plasma T3 levels. Following an injection of $1.3 \mu\text{g kg}^{-1}$ T1AM, the ft3 plasma concentration was significantly reduced, from 2.34 ± 0.09 in vehicle treated mice to $1.82 \pm 0.11 \text{ pmol L}^{-1}$ ($P < 0.05$ vs. vehicle), while no change was observed following $20 \mu\text{g kg}^{-1}$ T1AM ($2.37 \pm 0.20 \text{ pmol L}^{-1}$). This T1AM effect on ft3 was not detected in mice treated with exenatide i.p. or in those pretreated with clorgyline 30 min before T1AM injection (2.41 ± 0.09 and $2.20 \pm 0.18 \text{ pmol L}^{-1}$, respectively).

Plasma ft4 levels averaged $33.05 \pm 1.55 \text{ pmol L}^{-1}$ in the vehicle group, and were not significantly modified in T1AM 1.3 and $20 \mu\text{g kg}^{-1}$ injected mice (30.67 ± 1.96 and 31.66 ± 3.75 , respectively) as well as in exenatide (37.00 ± 2.88) and clorgyline (29.66 ± 1.45) i.p. treated mice. TSH levels measured in vehicle and T1AM-injected mice stabilized closed to the limit of the method ($4 \pm 2 \text{ mU mL}^{-1}$).

Discussion

We observed that i.c.v. injection of $1.3 \mu\text{g kg}^{-1}$ T1AM in fasted mice produced central and peripheral effects, including hypophagia, hyperglycaemia and a reduction in plasma ft3 levels. All these effects were not linearly dependent on T1AM dosage. In fact, the hypophagia vanished at doses higher than $1.3 \mu\text{g kg}^{-1}$, and it was replaced by hyperphagia at $20 \mu\text{g kg}^{-1}$. The latter dose was also ineffective on plasma ft3 levels. This biphasic response might account for the different results reported in the literature in *ad libitum* fed mice (Dhillon *et al.*, 2009; Hettinger *et al.*, 2010).

It is well known that fasting induces *c-fos* expression and that activation of NPY-ergic neurons plays an important role in this response (Yang *et al.*, 1995; Becskei *et al.*, 2010). Accordingly, in the hypothalamus of our fasted mice, we observed a strong positivity for *c-fos* immunostaining, which was reduced by injecting T1AM (either 1.3 or $20 \mu\text{g kg}^{-1}$). This is consistent with the proposed anti-adrenergic features of T1AM, and might be due either to TAAR1-mediated inhibi-

tion of cAMP production, or to activation of presynaptic α_2 -adrenoceptors (Gompfa *et al.*, 2010), causing a reduction of *c-fos* activation and of the 'functional' anorexigenic effect resulting from fasting-induced NPY overflow (Murphy *et al.*, 2009). Instead, the hyperphagia observed with $20 \mu\text{g kg}^{-1}$ T1AM could be the consequence of desensitization of α_2 -adrenergic receptors located on NPY-ergic neurons or of α_2 -mediated reduction of the release of some anorexigenic signals, including catecholamines and/or 5-HT. Both mechanisms could occur simultaneously but, depending on T1AM dosage, one would prevail over the other.

After clorgyline pretreatment, the hypophagic effect was preserved, while hyperphagia was prevented. This observation might imply that: (i) increased adrenergic drive due to amine oxidase inhibition can overcome the anti-adrenergic effect of T1AM; (ii) since T1AM itself is a substrate of amine oxidases, T1AM catabolites like 3-iodoacetic acid or thyroacetic acid might be involved in neuronal effects; or (iii) increased T1AM levels might induce a rapid desensitization of the target (s).

Our finding that T1AM injected i.c.v. had systemic bioavailability is novel and interesting. Even though we only tested two T1AM doses, our results suggest that T1AM entry into the blood is mediated by a transporter rather than representing simple diffusion. Whatever the mechanism may be, the occurrence of T1AM in the systemic circulation suggests that peripheral effects should be expected following i.c.v. T1AM administration. Systemic bioavailability increased markedly under conditions of MAO inhibition, indicating intracellular deamination as a prominent metabolic pathway of T1AM. Therefore, in the presence of the MAO inhibitor clorgyline, T1AM central and peripheral levels increase, mimicking the effects of higher T1AM doses. This is another possible reason why after clorgyline pretreatment, $20 \mu\text{g kg}^{-1}$ T1AM was ineffective at modifying feeding as seen with higher doses of T1AM ($26 \mu\text{g kg}^{-1}$) in the absence of clorgyline.

While T1AM has been detected in rat and human serum (Saba *et al.*, 2010; Hoefig *et al.*, 2011), endogenous T1AM was not detectable in mouse plasma. This is probably due to the fact that our extraction procedure is much less effective in plasma than in serum (Saba *et al.*, unpublished results); the endogenous plasma concentration is probably below the limit of detection.

Peripheral actions are the likely cause for the effects of T1AM on plasma glucose, since, in pancreatic cells, T1AM has been reported to induce glucagon secretion and decrease insulin secretion (Regard *et al.*, 2007). The hyperglycaemic effect of $1.3 \mu\text{g}\cdot\text{kg}^{-1}$ was associated with a transient reduction in peripheral insulin sensitivity, and was prevented by exenatide, suggesting a major role for glucagon secretion, while at $20 \mu\text{g}\cdot\text{kg}^{-1}$, reduced insulin secretion might also be involved. Notably, our results indicate that T1AM has a high affinity for the pancreas, and hyperglycaemia was achieved at plasma concentrations of T1AM in the order of 0.35 nM. With regard to the hyperphagic effect, T1AM was unable to produce hyperglycaemia in mice pretreated with clorgyline. Again, this might imply that increased sympathetic drive counteracting the effect of T1AM depends on glucagon release, since a biphasic effect of T1AM on insulin secretion has been described: low concentrations inhibited insulin secretion, possibly through α_2 -adrenoceptors, but high concentration had a stimulating effect, possibly mediated by TAAR1 (Regard *et al.*, 2007; Winzell and Ahren, 2007). An alternative possibility is that glucagon release may be stimulated by T1AM catabolites produced through oxidative deamination. The latter hypothesis seems unlikely, however, since such derivatives could not be detected in plasma, at least within limits of our assay.

T1AM ($1.3 \mu\text{g}\cdot\text{kg}^{-1}$) also reduced systemic fT3 levels. This effect was prevented by exenatide, supporting a role for glucagon. Consistent with this hypothesis, inhibition of liver deiodinase activities by glucagon has been reported (Mitchell and Raza, 1986; Kabadi and Premachandra, 1988). No change in fT3 was observed after $20 \mu\text{g}\cdot\text{kg}^{-1}$ T1AM, which might be explained by increased insulin secretion, since insulin is known to stimulate liver deiodinase activities (Sato *et al.*, 1984). Another hint regarding the role of the pancreas is the observation that changes in fT3 were prevented by clorgyline pretreatment. Glucagon might represent the factor linking T3 plasma levels with hyperglycaemia and might even contribute to changes in mice feeding patterns (Valassi *et al.*, 2008).

In summary, we have demonstrated that a single low dose of T1AM is able to decrease mice feeding and plasma fT3 levels while increasing plasma glucose and raising insulin resistance. T1AM appears to be a potent amine stimulating the CNS, the pancreas and, in turn, the liver where it might affect fT3 production and/or degradation. The picture we have drawn indicates that T1AM is a molecule with a very interesting non-genomic profile. Since these effects cluster in endocrine disorders, including thyroid dysfunctions and diabetes, the assay of tissue and plasma T1AM levels in experimental models of thyroid diseases and diabetes might open new paths for diagnosis and/or therapy of such diseases. In addition, prevention of the metabolic effects of T1AM might be included among the therapeutic, or side effects, of MAO inhibitors.

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Conflict of interest

The authors state the absence of any conflict of interest.

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