Distribution of exogenous [125 I]-3-iodothyronamine in mouse *in vivo*: relationship with trace amine-associated receptors

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Abstract

3-Iodothyronamine (T₁AM) is a novel chemical messenger, structurally related to thyroid hormone, able to interact with G protein-coupled receptors known as trace amine-associated receptors (TAARs). Little is known about the physiological role of T₁AM. In this prospective, we synthesized [125 I]-T₁AM and explored its distribution in mouse after injecting in the tail vein at a physiological concentration (0.3 nM). The expression of the nine TAAR subtypes was evaluated by quantitative real-time PCR. [125 I]-T₁AM was taken up by each organ. A significant increase in tissue vs blood concentration occurred in gallbladder, stomach,

intestine, liver, and kidney. Tissue radioactivity decreased exponentially over time, consistent with biliary and urinary excretion, and after 24 h, 75% of the residual radioactivity was detected in liver, muscle, and adipose tissue. TAARs were expressed only at trace amounts in most of the tissues, the exceptions being TAAR1 in stomach and testis and TAAR8 in intestine, spleen, and testis. Thus, while T₁AM has a systemic distribution, TAARs are only expressed in certain tissues suggesting that other high-affinity molecular targets besides TAARs exist.

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Introduction

The term thyroid hormone (TH) refers to 3,5,3',5'-tetraiodothyronine (thyroxine (T₄)) and 3,5,3'-triiodothyronine (T₃). The former is the main product released by thyrocytes while the latter is largely produced in the peripheral tissues and shows the highest affinity for the nuclear TH receptors, which act as transcriptional activators and control a wide range of physiological processes. 3-Iodothyronamine (T₁AM) is structurally related to THs as it can be potentially produced from T₃ or T₄ by decarboxylation and deiodination (Ianculescu & Scanlan 2010, Zucchi *et al.* 2010, Piehl *et al.* 2011). Administration of exogenous T₁AM determined significant physiological and behavioral effects in mammals, which were often opposite to those elicited on a longer time scale by THs, e.g. decreased body temperature (Scanlan *et al.* 2004), reduced heart rate and cardiac contractility (Scanlan *et al.* 2004, Chiellini *et al.* 2007), and modulation of insulin and glucagon secretion (Regard *et al.* 2007, Klieverik *et al.* 2009). As T₁AM was detected as an endogenous compound, it was proposed as a novel chemical messenger (Scanlan *et al.* 2004). This concept was supported by the observation that T₁AM does not interact with nuclear TH receptors, while it is the most powerful activator of trace amine-associated receptor 1 (TAAR1), the prototype of a novel family of G protein-coupled receptors that include nine different subtypes (Zucchi *et al.* 2006, Grandy 2007). While

the physiological role of T₁AM is still uncertain, this compound has recently been detected also in human blood (Saba *et al.* 2010, Hoefig *et al.* 2011, Galli *et al.* 2012).

When assaying endogenous T₁AM in rat tissues (Saba *et al.* 2010), we observed that T₁AM concentration was higher in each tested organ (i.e. liver, kidney, muscle, heart, lung, and brain) than in blood. This observation suggests that some tissues may be able to accumulate T₁AM. Determining whether T₁AM can be specifically taken up by certain organs *in vivo* and comparing T₁AM uptake among different organs is a crucial issue to understand the physiological role of this messenger. Therefore, in the present work, radiolabeled T₁AM was injected i.v. in mice at a concentration within the physiological range, and its distribution was evaluated and correlated with TAAR expression.

Materials and Methods

Chemical and radionuclides

T₁AM and its precursor *tert*-butyl-4-(4'-methoxymethoxy)-phenoxy-3-(trimethylstannyl) phenethyl carbamate were kindly provided by Tom Scanlan (Oregon Health and Science University, Portland, OR, USA). [125 I]-sodium iodine (specific activity 2200 Ci/mmol) was purchased from Perkin Elmer (Monza, Italy). Unless otherwise specified, all other

reagents were obtained from Sigma–Aldrich or from Invitrogen Life Technologies.

Synthesis of [125 I]-T₁AM

[125 I]-T₁AM was synthesized as described elsewhere (Miyakawa & Scanlan 2006). Briefly, chloramine-T (20 μ l, 4 mg/ml in water, 0.21 μ mol), 5% HCl (5 μ l), and [125 I] sodium iodine (1 mCi, carrier free) were added to a solution of *tert*-butyl-4-(4'-methoxymethoxy)phenoxy-3-(trimethylstannyl) phenethyl carbamate (100 μ g, 0.19 μ mol) in ethanol (10 μ l) in vial. The reaction was allowed to proceed at room temperature for 30 min. The reaction mixture was then diluted with brine and extracted with ether. The combined organic layer was passed through a MgSO₄ column and concentrated *in vacuo*. The mixture was dissolved in a 3 M HCl solution in ethyl acetate (200 μ l, anhydrous) and the reaction was allowed to proceed at room temperature for 3 h and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, ethyl acetate/methanol 1:0 to 2:1). The radioactive purity of the final compound was checked by exposing a thin layer chromatography plate to X-ray film. The total radiochemical yield of [125 I]-T₁AM after silica gel flash chromatography purification was 20%.

In vivo biodistribution studies

This investigation conforms to the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals. The project was approved by the Animal Care and Use committee of the University of Pisa.

[125 I]-T₁AM (about 100 μ Ci, corresponding to about 0.45 pmol), in a final volume of 0.1 ml (no carrier added), was administered via tail vein injection in normal BALB-c mice. Mice were killed by CO₂ administration followed by cervical fracture at 30, 60, 120, 240, and 1440 min after injection. Organs and tissues, including adipose tissue, blood, bone, brain, gallbladder, heart, intestine, kidney, liver, lung, muscle, pancreas, skin, spleen, stomach, and thyroid, were removed. Samples of organs and tissues were weighed, and the radioactivity was measured using an automated γ -counter (1282 CompuGamma CS Universal Gamma Counter; LKB-Wallac, Mt Waverley, Vic., Australia). The concentration of radiolabeled material was expressed as percentage of the injected dose per gram of wet tissue (% ID/g). Total tissue radioactivity was calculated as the product of the above variable and tissue weight.

In parallel experiments, the specificity of [125 I]-T₁AM uptake was investigated by injecting an over 2000-fold excess of unlabeled T₁AM (25 μ g/kg, corresponding to about 1200 pmol, in a final volume of 0.1 ml) 5 min before the radioligand. Mice were killed 60 min after administration of [125 I]-T₁AM, and tissue radioactivity was measured as described earlier.

Gene expression studies

The expression of TAARs was evaluated in different mouse tissue samples (brain, heart, intestine, kidney, liver, lung, spleen, stomach, testis, and thyroid) by absolute quantitative RT-PCR. Mice were killed after chloroform inhalation and tissue samples were immediately excised and treated with RNAlater buffer (Qiagen GmbH) to prevent RNA degradation. A portion of liver was flash frozen and used for DNA extraction with DNeasy kit (Qiagen) according to the manufacturer's manual. Similar experiments were also carried out on tissue samples obtained from Wistar rats.

RNAlater-treated samples were homogenized in RNeasy lysis reagent and total RNA was extracted following the manufacturer's protocol. RNA was treated with DNase I and purified again with RNeasy system. Nucleic acids were finally quantified with Qubit fluorometer and RNA was quality tested on 2100 Bio Analyzer (Agilent Technologies, Waldbronn, Germany). Then, 1 μ g total RNA was retro-transcribed using Quantitect RT Kit (Qiagen) according to the manufacturer's protocol. The same reactions were performed without reverse transcriptase to check for contamination by genomic DNA.

The cDNA was then used for absolute quantitative real-time PCR using genomic DNA as an external standard. For each TAAR, a standard curve was constructed with six threefold serial dilutions of mouse liver genomic DNA, starting from 9 ng (2745 gene copies). Absolute cDNA copy numbers were calculated from standard curves and normalized vs total RNA. Reactions were performed in a total volume of 20 μ l containing cDNA equivalent to 100 ng total RNA, 0.2 μ M each oligonucleotide, and 10 μ l iQ SYBR Green Supermix (Bio-Rad). Real-time PCR was conducted on an iQ5 Optical System (Bio-Rad) with the following cycle program: 30 s at 95 °C, followed by 45 two-step amplification cycles consisting of 10 s denaturation at 95 °C and 30 s annealing/extension at 60 °C. A final dissociation stage was run to generate a melting curve to verify amplicon specificity and primer dimer formation. All samples, including nontemplate controls, external standards, and no retrotranscription control, were run in duplicate.

Oligonucleotide sequences for mouse TAARs (TAAR1, TAAR2, TAAR3, TAAR4, TAAR5, TAAR6, TAAR7a–f, TAAR8a–c, and TAAR9) and hypoxanthine guanine phosphoribosyl transferase (HPRT), the control gene chosen to verify the system efficiency, are shown in Table 1. Sequences were designed on the basis of coding sequences published in Gene Bank using Beacon Designer 4 Software (Premier Biosoft International, Palo Alto, CA, USA). Owing to the high homology between TAAR7 and TAAR8 paralogs, we decided to design a single primer pair to amplify all members of each group. HPRT primers were found on RT-primer DB public database (<http://medgen.ugent.be/rtpimerdb>, ID: 45). The selectivity of each TAAR- and HPRT-specific primer pair was verified by amplicon

Table 1 Primers used for RT-PCR assays in mouse tissues

Gene	Accession no.	Forward primer	Reverse primer	Amplicon length (bp)
<i>Taar1</i>	NM_053205	5'-AGGACAAGCAAGGTCAATCAATCG-3'	5'-AGAACGGGGACCAGCATACG-3'	143
<i>Taar2</i>	NM_001007266	5'-GGATCTTGCCCAGAGAATGAAAGG-3'	5'-GGATCTTGCCCAGAGAATGAAAGG-3'	156
<i>Taar3</i>	NM_001008429	5'-AGGACAGGAAAGCAGCTAAGAC-3'	5'-GAAGTACCCGAGCCATACCAGAAG-3'	152
<i>Taar4</i>	NM_001008499	5'-GGCTACCACAGACTTCTGTTGAG-3'	5'-AAAGGGTCGCAGACGGCATAG-3'	192
<i>Taar5</i>	NM_001009574	5'-ACCAACTTCTGCTGCTCTCC-3'	5'-ACAGCGTGTCCAGATAGGTATGC-3'	145
<i>Taar6</i>	NM_001010828	5'-TCTCCGCCACCGTCCTG-3'	5'-GATGCCACACCAAAGTCAGC-3'	234
<i>Taar7a</i>	NM_001010829	5'-CCCTCGCTCATCTCTATGC-3'	5'-AGCCCTCCACAGACCTCACC-3'	200
<i>Taar7b</i>	NM_001010827			
<i>Taar7d</i>	NM_001010838			
<i>Taar7e</i>	NM_001010835			
<i>Taar7f</i>	NM_001010839			
<i>Taar8a</i>	NM_001010830	5'-TGTAAGTGGCAACAGAGGTGAATC-3'	5'-GGAGTGATGAAGCCCATGAAAGC-3'	171
<i>Taar8b</i>	NM_001010837			
<i>Taar8c</i>	NM_001010840			
<i>Taar9</i>	NM_001010831	5'-CCTTCTGTTTTGCGTCTCTTGTTC-3'	5'-CCTTCTCATTGGCTCCCCGTG-3'	186
<i>Hprt</i>	NM_013556	5'-CCTAAGATGAGCGCAAGTTGAA-3'	5'-CCACAGGACTAGAACACCTGCTAA-3'	86

Accession no., GenBank accession number; bp, base pairs; HPRT, hypoxanthine guanine phosphoribosyl transferase.

sequencing. When analyzing rat tissue samples, we used primers previously described (Chiellini *et al.* 2007).

Statistical analysis

Results are expressed as mean ± s.e.m. Differences between groups were evaluated as follows. One-way ANOVA was used as a global test for differences between means. If between-group variance was significantly ($P < 0.05$) higher than within-group variance, individual groups were compared with the control group by Dunnett's test. Regression analysis of decay curves was performed by a one-phase exponential model, namely $y = Ae^{Kx}$, where K represents the rate constant and A is referred to as 'span' (it corresponds to extrapolated radioactivity at time zero). Half-life is defined as $\ln 2/K$ and represents time needed for y to reach a value equal to $A/2$. GraphPad Prism version 4.1 for Windows (GraphPad Software, San Diego, CA, USA) was used for data processing and statistical analysis.

Results

[¹²⁵I]-T₁AM concentration in different organs

Figure 1 shows the tissue distribution of [¹²⁵I]-T₁AM at the five different time points in the 16 organs and tissues that were evaluated. The figure shows the concentration of tissue radioactivity, expressed as percentage of the injected dose per gram of tissue (% ID/g). After 30 min, the highest levels were detected in biliary system, liver, kidney, and gastrointestinal tract. The peak concentration occurred in the gallbladder and averaged 23.2% ID/g, while the radioactivity detected in the liver, kidney, stomach, and intestine was in the order of 6–10% ID/g. At later times, radioactivity decreased in all

organs, and the distribution between organs was similar, although at the latest time points a change in the pattern was observed. Gallbladder still showed the highest radioactivity concentration up to 240 min, while at 1440 min, liver radioactivity exceeded gallbladder radioactivity. Statistical analysis showed that differences among tissues were highly significant at each time point ($P < 0.001$). Comparison of tissue radioactivity vs blood radioactivity revealed a significant increase in gallbladder (at 30–240 min), liver (at 30, 60, and 1440 min), kidney (at 30–60 min), intestine (at 30 min), and stomach (at 30 min).

[¹²⁵I]-T₁AM displacement by unlabeled T₁AM

The effect of the coadministration of an over 2000-fold excess of unlabeled T₁AM together with [¹²⁵I]-T₁AM is shown in Fig. 2. In most of the organs, radioactivity levels decreased remarkably as > 90% of the radioactivity was displaced by the unlabeled compound, with the exception being represented by the skin. This observation suggests that most of the radioactivity was located in specific and saturable binding sites. The low levels of thyroid radioactivity, which did not show any trend of increasing over time, suggest that deiodination yielding free radiolabeled iodide was limited. Putative [¹²⁵I]-T₁AM catabolites retaining [¹²⁵I]-I (e.g. [¹²⁵I]-3-iodothyroacetic acid) could not be specifically assayed. However, in a few experiments, urine samples were collected at 30 min and thin layer chromatography showed that most of the radioactivity (70%) was associated with [¹²⁵I]-T₁AM (data not shown).

Total tissue [¹²⁵I]-T₁AM uptake and clearance

Total tissue radioactivity was calculated as the product of the values shown in Fig. 1 and tissue weight, which was either

Figure 1 Distribution of radioactivity in different organs after i.v. injection of 100 µCi [¹²⁵I]-T₁AM corresponding to 0.45 pmol. Radioactivity concentration is expressed as percentage of injected dose per gram of wet weight (% ID/g). Histograms represent mean ± S.E.M. of 16 different tissues obtained from animals killed at different times after injection, namely 30 min (n=6), 60 min (n=5), 120 min (n=5), 240 min (n=2), and 1440 min (n=4). Statistical analysis by one-way ANOVA for repeated measures yielded *P*<0.001 at each time point. **P*<0.05, ***P*<0.01 vs blood concentration, by Dunnett's test after one-way ANOVA.

measured directly or estimated on the basis of literature data (Barnett & Widdowson 1965, Griffin & Goldspink 1973, Brochmann *et al.* 2003). The latter was the case for adipose tissue, blood, bone, intestine, muscle, and skin. Overall radioactivity, i.e. the sum overall tissues, is plotted vs time (Fig. 3). A very close fitting (*r*=0.957) was provided by a single exponential, with a rate constant of 0.0167 ± 0.0034/min, corresponding to a half-life of 41 min. The progressive clearance of [¹²⁵I]-T₁AM represented urinary and fecal excretion. In a few experiments, urinary bladder radioactivity was measured at 30 min, and calculations suggested that urinary excretion accounted for about 70% of [¹²⁵I]-T₁AM loss at this time point.

If specific organs were considered individually, the decay was still fitted by a single exponential (*r*>0.830 for blood, brain, and thyroid; *r*>0.900 in all other cases), but the parameters describing the single curves were significantly different, as summarized in Table 2. In particular, the longest half-life was observed in thyroid (558 min), followed by liver (149 min), and gallbladder (138 min), while the shortest half-life was detected in stomach (18 min), skin (24 min), and spleen (45 min). As a consequence of these differences, the relative distribution of radioactivity among organs changed remarkably over time. At 30 min, about 80% of the residual radioactivity was located in intestine (19%), skin (15%), liver (14%), muscle (11%), adipose tissue (10%), and blood (9%). By contrast, after 1440 min, liver alone accounted for 38% of residual radioactivity, with adipose tissue and muscle accounting for another 18% each.

T₁AM distribution vs TAAR gene expression

The observation of displaceable [¹²⁵I]-T₁AM uptake provides evidence for the existence of high-affinity T₁AM binding sites in mouse tissues. As it is well known that T₁AM is the most potent activator of TAAR1 (Scanlan *et al.* 2004), it seemed interesting to compare [¹²⁵I]-T₁AM distribution and

Figure 2 Distribution of radioactivity in different organs 60 min after i.v. injection of 100 µCi [¹²⁵I]-T₁AM corresponding to 0.45 pmol. Filled histograms represent total [¹²⁵I]-T₁AM uptake determined when only [¹²⁵I]-T₁AM was injected (mean of five experiments); empty histograms represent nonspecific uptake, determined when [¹²⁵I]-T₁AM injection was preceded by the injection of 25 µg/kg (i.e. about 1200 pmol) of unlabeled T₁AM (mean of two experiments). Please note that total gallbladder radioactivity actually averaged 21.5% and the corresponding histogram was cut.

Figure 3 Time course of total body radioactivity, determined as described in *In vivo* biodistribution studies. Data are expressed as percentage of injected radioactivity. Interpolation by a single exponential (solid line) yielded a rate constant of $0.0167 \pm 0.0073/\text{min}$ corresponding to a half-life of 41 min ($r=0.971$). If zero time was excluded, then analysis interpolation (dotted line) yielded a rate constant of $0.0077 \pm 0.023/\text{min}$, with a half-life of 89 min ($r=0.957$).

TAAR distribution. In view of the fact that reliable antibodies for TAAR western blot analyses are not yet available, we decided to investigate the expression of each TAAR gene at the mRNA level by absolute quantitative PCR. The results obtained after screening several mouse tissues are summarized in Table 3. In general, expression levels were very low, at the limit of the linearity range of the system (namely ten cDNA copies/ μg of total RNA), and a significant expression was observed only for TAAR1 and TAAR8 in stomach, intestine, spleen, and testis. Notably, TAAR expression was not observed in liver and kidney, in spite of the high [¹²⁵I]-T₁AM uptake.

To further investigate the TAAR distribution in rodents, quantitative gene expression analyses were also performed in rat tissues. As summarized in Table 4, TAAR expression was higher in rat than in mouse, particularly for TAAR8a. However, the number of copies was still quite low, except possibly for testis. These results confirmed the absence of a correlation between endogenous T₁AM tissue concentrations and TAAR gene expressions, as we have previously reported in the T₁AM quantitative analysis studies using LC-MS/MS (Saba *et al.* 2010).

Discussion

T₁AM fulfills the criteria that define chemical messengers, namely it is an endogenous compound able to interact with specific receptors and to produce functional effects. It is thought to derive from TH through decarboxylation and deiodination, although the precise pathway and site of T₁AM production remain to be established (Ianculescu & Scanlan 2010, Zucchi *et al.* 2010, Piehl *et al.* 2011). The wide distribution of endogenous T₁AM and the high ratio of tissue concentration to blood concentration (Saba *et al.* 2010) suggest that T₁AM may act like a true hormone, that is a chemical messenger with a systemic distribution able to

produce specific functional effects by binding to specific molecular targets located in different organs. As a first step in the evaluation of this hypothesis, we determined the distribution of exogenous radiolabeled T₁AM after injecting about 0.45 pmol of [¹²⁵I]-T₁AM i.v. in mouse. Assuming a total blood volume of about 1.5 ml (Barnett & Widdowson 1965), this corresponds to an initial concentration of about 0.3 nM, which is similar to the concentration of endogenous T₁AM that we have measured in rat and human blood by HPLC coupled to mass spectrometry (Saba *et al.* 2010, Galli *et al.* 2012) and significantly lower than the concentration assayed by immunological methods (Hoefig *et al.* 2011).

At this physiological concentration, [¹²⁵I]-T₁AM reached virtually every organ. The high levels detected in gallbladder and intestine up to 30–60 min after injection may reflect biliary excretion and enteric reabsorption, while the high kidney concentration over the same time frame is consistent with urinary excretion, which apparently accounted for the largest fraction of whole-body radioactivity washout. In liver, [¹²⁵I]-T₁AM concentration was significantly higher than blood concentration at all time points, and after 24 h over two-thirds of the residual radioactivity were detected either in liver or muscle or in adipose tissue, suggesting that these tissues should be regarded as T₁AM storage sites. Interestingly, T₁AM has been reported to stimulate lipid catabolism over glucose metabolism (Bräulke *et al.* 2008). As hepatocytes, muscle cells, and adipocytes are the major players in lipid metabolism, our results are consistent with a physiological role of T₁AM in metabolic control. Stomach is the other

Table 2 Decay of tissue radioactivity. Tissue radioactivity was calculated as the product of tissue weight and radioactivity concentration. The average values at different time points were interpolated by a single exponential (see Statistical analysis), whose parameters are shown in the table. Values are expressed as mean $\pm$ S.E.M.

Tissue	K (per min)	Span (%)	Half-life (min)	r
Adipose tissue	0.0077 ± 0.0024	6.31 ± 1.03	89	0.951
Blood	0.0056 ± 0.0039	4.43 ± 1.40	123	0.834
Bone	0.0097 ± 0.0036	2.28 ± 0.49	71	0.947
Brain	0.0090 ± 0.0077	0.29 ± 0.13	77	0.868
Gallbladder	0.0050 ± 0.0007	2.39 ± 0.15	138	0.993
Heart	0.0096 ± 0.0027	0.29 ± 0.05	72	0.967
Intestine	0.0143 ± 0.0009	15.55 ± 0.62	48	0.999
Kidney	0.0097 ± 0.0014	3.81 ± 0.32	72	0.992
Liver	0.0046 ± 0.0022	8.99 ± 1.75	149	0.901
Lung	0.0073 ± 0.0016	1.16 ± 0.13	94	0.981
Muscle	0.0096 ± 0.0028	8.16 ± 1.38	72	0.965
Pancreas	0.0107 ± 0.0029	0.73 ± 0.12	64	0.968
Skin	0.0283 ± 0.0084	18.51 ± 5.76	24	0.968
Spleen	0.0153 ± 0.0061	0.23 ± 0.07	45	0.930
Stomach	0.0372 ± 0.0222	7.22 ± 5.43	19	0.909
Thyroid	0.0012 ± 0.0010	0.38 ± 0.06	558	0.892
P	0.003	<0.001		

K, rate constant; P, probability level for significant differences between groups, by ANOVA applied to nonlinear fitting.

Table 3 Trace amine-associated receptor (TAAR) expression in mouse tissues. Gene expression was determined by absolute quantitative real-time PCR and is expressed as number of cDNA copies/μg of total RNA. Data represent mean ± s.e.m. of two preparations

Tissue	Taar1	Taar2	Taar3	Taar4	Taar5	Taar6	Taar7	Taar8	Taar9
Brain (cortex)	–	–	<10	–	–	–	–	–	–
Brain (hemisphere)	<10	–	<10	–	–	–	–	–	–
Heart	–	–	–	–	–	–	–	–	–
Intestine	<10	–	–	–	–	–	–	16 ± 1	<10
Kidney	–	–	–	–	–	–	–	–	–
Liver	–	–	–	–	–	–	–	–	–
Lung	–	–	–	–	–	–	–	–	–
Spleen	–	–	–	–	–	–	–	19 ± 1	–
Stomach	83 ± 11	–	–	–	–	–	–	–	–
Testis	54 ± 8	–	ND	ND	ND	ND	ND	12 ± 3	ND
Thyroid	<10	–	–	–	–	–	–	<10	–

‘–’, not detected (below the sensibility threshold of the system); ‘ND’, not determined; the value ‘<10’ indicates signals detected under the linearity range of the assay (ten cDNA copies/μg total RNA).

organ in which [¹²⁵I]-T₁AM concentration significantly exceeded blood concentration at 30 min, although the difference vs blood was lost at later time points. A possible explanation is that gastric secretion may represent another pathway of T₁AM excretion. Alternatively, the stomach may be regarded as another short-term storage site.

A limitation of this study is that only five time points were measured. A more extensive investigation with more points collected at shorter times might provide a deeper insight into T₁AM distribution through the use of refined mathematical modeling (Orsi *et al.* 2011). Another limitation comes from the fact that tissue radioactivity does not necessarily represent tissue [¹²⁵I]-T₁AM. [¹²⁵I]-T₁AM can be deiodinated by type 1 or type 3 deiodinases, which would produce free ¹²⁵I[–] (Piehl *et al.* 2008). Free iodide then undergoes urinary excretion, gastric secretion, and is largely accumulated in the thyroid, skin, and to a minor extent in other organs, such as salivary glands, mammary glands, and ovary (Brown-Grant 1961). Therefore, ¹²⁵I[–] may have contributed to the measured radioactivity from thyroid, skin, and possibly stomach. However, as no progressive increase in thyroid

radioactivity was observed over time, and even at the later time points total thyroid radioactivity did not exceed 1–3% of the residual radioactivity, we can safely assume that free ¹²⁵I[–] did not significantly contribute to the total radioactivity measured.

Finally, T₁AM can also undergo oxidative deamination, and production of 3-iodothyroacetic from T₁AM has been demonstrated in isolated organs and in cell cultures as well (Wood *et al.* 2009, Saba *et al.* 2010). However, in rat blood and tissues, the endogenous concentration of 3-iodothyroacetic acid was lower than the T₁AM concentration (Saba *et al.* 2010), and in the present experimental model we could confirm that most of the radioactivity detected in urine after 30 min was still associated with [¹²⁵I]-T₁AM.

In blood and in most of the organs, over 90% of [¹²⁵I]-T₁AM was displaced by an excess of unlabeled T₁AM, suggesting the existence of specific high-affinity binding sites. The most obvious candidate is TAAR1, as nanomolar T₁AM has been reported to interact with TAAR1 in heterologous expression models (Scanlan *et al.* 2004). For this reason, we compared [¹²⁵I]-T₁AM distribution and

Table 4 Trace amine-associated receptor (TAAR) expression in rat tissues. Gene expression was determined by absolute quantitative real-time PCR and is expressed as number of cDNA copies/μg of total RNA. Data represent mean ± s.e.m. of two preparations

Tissue	Taar1	Taar2	Taar3	Taar4	Taar5	Taar6	Taar7a	Taar8a	Taar9
Brain (cortex)	<10	–	<10	–	–	–	–	65 ± 20	<10
Brain (white matter)	<10	–	<10	–	–	–	–	81 ± 12	<10
Cerebellum	–	–	–	–	–	–	–	121 ± 3	<10
Heart	<10	<10	<10	<10	–	–	–	192 ± 23	–
Intestine	11 ± 1	–	–	<10	–	–	–	<10	<10
Kidney	<10	–	–	–	–	–	–	26 ± 17	–
Liver	<10	–	–	–	–	–	–	0	–
Lung	<10	–	–	<10	<10	–	–	21 ± 1	–
Muscle	24 ± 33	–	–	13 ± 2	–	–	–	14 ± 20	–
Spleen	<10	–	–	93 ± 4	–	–	–	12 ± 2	–
Stomach	297 ± 44	–	–	–	–	–	–	13 ± 1	–
Testis	1802 ± 874	89 ± 19	324 ± 63	230 ± 2	19 ± 1	18 ± 2	<10	763 ± 142	12 ± 1

‘–’, not detected (below the sensibility threshold of the system); the value ‘<10’ indicates signals detected under the linearity range of the assay (ten cDNA copies/μg total RNA).

TAAR expression. Among the tissues tested, *TAAR1* expression was limited to stomach, where it was quite low, namely 83 copies/ μg of total RNA. The expression of other TAAR subtypes was also low, with a significant amplification obtained only for *TAAR8* in intestine and spleen. Most notably, no significant TAAR expression was found in liver. Therefore, binding to TAAR cannot be the only factor accounting for tissue $[^{125}\text{I}]\text{-T}_1\text{AM}$ distribution in mouse.

As we have previously shown that TAARs are significantly expressed in rat heart (Chiellini *et al.* 2007), we also investigated T_1AM distribution and TAAR expression in various rat tissues. Although higher expression levels were observed, especially for *TAAR8a*, the dissociation between T_1AM distribution and TAAR expression was also found in rat, with very low TAAR expression found in organs that show high endogenous T_1AM concentration (Saba *et al.* 2010), such as stomach and skeletal muscle, and was virtually absent in liver.

The issue of TAAR distribution has raised some controversy. In mouse, Liberles & Buck concluded that *TAAR1* had a widespread distribution, while all other TAAR subtypes were expressed only in the olfactory epithelium (Liberles & Buck 2006, Liberles 2009). The specific location of *TAAR5* in olfactory vs nonolfactory epithelium was also confirmed in humans (Carnicelli *et al.* 2010). Furthermore, several investigators have reported different TAAR subtypes to be expressed in many tissues, such as whole brain, amygdala, pituitary, stomach, kidney, lung, heart, small intestine, and leukocytes (reviewed by Zucchi *et al.* (2006)). In the present investigation, we used a quantitative approach and observed that TAAR expression was very low mostly in rat and mouse tissues, although higher levels were detected in testis. Obviously, our studies cannot exclude that TAAR may be expressed at relatively high levels only in specific cell types and that their expression level is therefore underestimated when whole organs are assayed. Protein expression studies may be able to shed further light on this issue, but so far, they have been limited by the lack of reliable antibodies. Thus, given the data available at present, our preliminary conclusion is that TAAR may play a physiological role in the olfactory epithelium, in testis, and possibly in other sites, including gastrointestinal tract and central nervous system. However, it should be noted that, as our method could not properly characterize TAAR expression at the protein level, we could not prove conclusively that $[^{125}\text{I}]\text{-T}_1\text{AM}$ is actually bound to TAAR. In any case, the tissue distribution of T_1AM measured cannot be accounted for by TAAR expression alone.

Cellular specific uptake of T_1AM into a variety of cell types has been previously reported (Ianculescu *et al.* 2010), and this process appears to involve specific, saturable, and inhibitable transport mechanisms. Recent investigations demonstrate that T_1AM , like the THs T_4 and T_3 , is present in circulation mostly in a protein-bound state (Roy *et al.* 2012). In particular, T_1AM specifically binds with high affinity ($K_d = 17 \text{ nM}$) to apolipoprotein B-100, the protein component of low-density lipoprotein particles, which may then

serve as the carrier for circulating T_1AM . T_1AM association with apo-B100 may provide a mechanism for transportation and entry of T_1AM into target cells, thus indicating the existence of intracellular biological targets of T_1AM 's action. The presence of T_1AM intracellular binding sites would be consistent with the results from our previous studies in isolated cardiomyocytes, where T_1AM was found to be concentrated intracellularly, possibly by a sodium-dependent mechanism (Saba *et al.* 2010).

While binding to apo-B100 may account for the presence of saturable high-affinity T_1AM binding sites in blood, it cannot possibly be responsible for extravascular T_1AM binding. Additional carriers for T_1AM may be represented by membrane transporters, such as vesicle monoamine transporter 2, dopamine transporter (Snead *et al.* 2007), and organic ion transporters like OATP1A2 (SLCO1A4), OAT1C1, and MCT8 (SLC16A2; Ianculescu *et al.* 2010). Functional evidence for mitochondrial binding sites has also been reported (Venditti *et al.* 2011). Additional investigations are required to clarify whether these targets are responsible for tissue T_1AM binding.

In summary, this study shows that exogenous T_1AM was taken up by virtually every mouse tissue. The highest concentrations were detected in liver, kidney, and gastrointestinal tract, suggesting biliary and urinary excretion associated with long-term liver storage. Late accumulation in adipose tissue and muscle was also apparent, consistent with a role for T_1AM in metabolic regulation. T_1AM in most of the tissue was associated with saturable high-affinity binding sites, but TAAR expression did not correlate with T_1AM distribution, suggesting the existence of additional intracellular targets.

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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