Exogenous 3-Iodothyronamine (T$_{1}$AM) Can Affect Phosphorylation of Proteins Involved on Signal Transduction Pathways in In Vitro Models of Brain Cell Lines, but These Effects Are Not Strengthened by Its Catabolite, 3-Iodothyroacetic Acid (TA$_{1}$)

Lavinia Bandini †, Ginevra Sacripanti †, Marco Borsò ○, Maria Tartaria, Maria Pia Fogliaro, Giulia Giannini, Vittoria Carnicelli, Matteo Emanuele Figuccia, Sara Verlotta, Fiammetta De Antoni, Riccardo Zucchi and Sandra Ghelardoni *

Department of Pathology, University of Pisa, 56126 Pisa, Italy
* Correspondence: sandra.ghelardoni@unipi.it; Tel.: +39-050-221-8665
† These authors contributed equally to this work.

Abstract: T$_{1}$AM, a derivative of thyroid hormones, and its major catabolite, TA$_{1}$, produce effects on memory acquisition in rodents. In the present study, we compared the effects of exogenous T$_{1}$AM and TA$_{1}$ on protein belonging to signal transduction pathways, assuming that TA$_{1}$ may strengthen T$_{1}$AM’s effects in brain tissue. A hybrid line of cancer cells of mouse neuroblastoma and rat glioma (NG 108-15), as well as a human glioblastoma cell line (U-87 MG) were used. We first characterized the in vitro model by analyzing gene expression of proteins involved in the glutamatergic cascade and cellular uptake of T$_{1}$AM and TA$_{1}$. Then, cell viability, glucose consumption, and protein expression were assessed. Both cell lines expressed receptors implicated in glutamatergic pathway, namely Nmdar1, Glur2, and EphB2, but only U-87 MG cells expressed TAAR1. At pharmacological concentrations, T$_{1}$AM was taken up and catabolized to TA$_{1}$ and resulted in more cytotoxicity compared to TA$_{1}$. The major effect, highlighted in both cell lines, albeit on different proteins involved in the glutamatergic signaling, was an increase in phosphorylation, exerted by T$_{1}$AM but not reproduced by TA$_{1}$. These findings indicate that, in our in vitro models, T$_{1}$AM can affect proteins involved in the glutamatergic and other signaling pathways, but these effects are not strengthened by TA$_{1}$.

Keywords: 3-iodothyronamine; 3-iodothyroacetic acid; brain; glutamatergic system; protein phosphorylation

1. Introduction

Thyronamines represent a new class of endogenous signaling compounds detected in blood and tissues of humans and of several animals, which have been postulated to act as neurotransmitters [1–3]. Among thyronamines, 3-iodothyronamine (T$_{1}$AM) has been the compound with more physiological effects [1,2]. Probably derived from decarboxylation and deiodination of thyroid hormone thyroxine (T4) [4], it is catabolized to 3-iodothyroacetic acid (TA$_{1}$) by oxidative deamination [5].

T$_{1}$AM can activate G protein coupled receptors, such as Trace Amine Associated Receptor (TAAR)1 [1], even though interactions with other targets, such as plasma membrane transporters or vesicular biogenic amine transporters, cannot be excluded [6,7]. Significant functional effects have also been observed in the central nervous system: regarding its similarity with monoaminergic neurotransmitters, T$_{1}$AM can inhibit the transport of catecholamines, either vesicular or through the plasma membrane, discovering a novel role for T$_{1}$AM as a neuromodulator [6]. Several findings have led to consider T$_{1}$AM as an endogenous adrenergic-blocking neuromodulator at level of the central noradrenergic system [8].
In 2013, Manni et al. [9] observed an enhancement of learning and memory after intracerebral ventricle injections of T1AM in mice. The behavioral effects of T1AM may be achieved through the modulation of intracellular pathways, counteracting cell stress signaling and leading to the increase in ERK 1/2 phosphorylation and cFos expression [9,10], which have been demonstrated to play a fundamental role in plasticity mechanisms and in memory processes [11,12].

Interestingly, many of the effects attributed to T1AM or to its major metabolite TA1 appear to oppose those of the classical thyromimetic effects exerted by T3 [13] as a potent hypothermia, a decrease in heart rate and cardiac output [1], and the induction of a shift from carbohydrates to fatty acids as preferential metabolic source [14]. Therefore, some investigators consider T1AM as a multitarget ligand, but the patho-physiological role of the individual receptors and binding sites is presently under debate. In general, both T1AM and TA1 targets seem to overlap. TA1 can be virtually produced in every tissue, considering the wide distribution of monoamine oxidases [15]. At doses close to the endogenous levels, TA1 modified behavior, favored memory acquisition and hyperglycaemia, and reduced nociceptive thresholds via histamine H1 and H2 receptors [16]. These results led to the speculation that effects of T1AM may be due, at least in part, to TA1 production.

Glutamatergic neurotransmission, the major excitatory system in the brain, plays a key role in regulating neuroplasticity, neural development, learning, and memory, and it is often compromised in neurological disorders [17]. These processes involve the recruitment of multiple signaling pathways and gene expression, activating downstream signaling effectors, i.e., calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC), extracellular signal-regulated kinases (ERK), and cAMP response element-binding protein (CREB), which are shared by other different neurotransmission processes [18].

Due to the emerging interest on the effect of thyronamines in brain, the present study sought to explore the impact of T1AM and its main catabolite, TA1, on signal transduction pathways in brain cell lines. As an in vitro model, we used NG108-15 cells, a hybrid cell line of mouse neuroblastoma and rat glioma, and U-87 MG cells, derived from a human malignant glioma. First, we characterized the experimental models, by assessing uptake and metabolism of T1AM and TA1; then, we evaluated and compared the effects on proteins involved in signaling cascades, hypothesizing that TA1 may strengthen T1AM’s actions, especially in brain tissue. We analyzed the effects on cell viability, glucose uptake, second messenger production and expression, and post-translational modifications of proteins involved in signaling cascades after chronic treatment.

2. Materials and Methods
2.1. Chemicals

T1AM was purchased from Cayman Chemical (Ann Arbor, MI, USA). TA1 was kindly provided by Dr. Thomas S Scanlan (Oregon Health and Science University, Portland, OR, USA). Unless otherwise specified, all other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Solvents for HPLC-MS/MS measurements were HPLC grade, and the other chemicals were reagent grade.

The vehicle for T1AM and TA1 in the cell treatment was dimethyl sulfoxide (DMSO).

A graphical abstract of methods was included in the Supplementary Material (Figure S1).

2.2. Cell Treatments

NG108-15 cell line, a hybrid cell line of mouse neuroblastoma and rat glioma, and U-87 MG cell line, from human malignant glioma, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% (v/v) of fetal bovine serum (FBS), 1 mM pyruvate, 4.5 g/L glucose, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO2, and subcultured before confluence.
To assess protein expression, cells were exposed, for 24 h, to exogenous T1AM (in a range from 0.1 µM to 10 µM) Control cells were incubated with supplemented DMEM containing DMSO.

Western blotting was performed on the supernatant fraction of cell lysate [19]. The protein concentration was determined by the Bradford method.

2.3. Gene Expression Analysis

The expression of eight genes (Glur2, Nmdar1, Nmdar2b, Ephb2, Pkca, Pkcy, Sirt1, Erk1) (Tables S1 and S2) was evaluated in NG108-15 and U-87 MG cell lines by real-time PCR, which was performed according to the manufacturer’s instruction (Euroclone, Milan, Italy) in three independent samples.

RNA isolation was performed using chloroform extraction provided as TRIzol Kit (Themo Fisher Scientific, Milan, Italy), and the procedure followed as described in the manufacturer’s protocol.

After resuspended RNA in RNAase free water, all samples were purified performing digestion with DNase by RNA Clean & Concentrator (Zymo Research, Irvine, CA, USA). RNA concentration and purity were then analyzed using a Qubit RNA HS Assay kit (Life Technologies, Carlsbad, CA, USA) with a Qubit 1.0 fluorometer from Invitrogen (Waltham, MA, USA).

Total RNA was then retrotranscribed (5 min at 25 °C, 20 min at 46 °C, and 1 min at 95 °C) using iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA).

Relative quantity of gene transcripts was measured by real-time PCR, on samples’ cDNA, using a SYBRGreen chemistry and iQ5 instrument (Bio-Rad). Additionally, 4 µL of 2 µM primer solution were added to 10 µL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) in a 20 µL total volume reaction. The PCR cycle program consisted of an initial 30 s denaturation at 95 °C, followed by 40 cycles of 10 s denaturation at 95 °C and 15 s annealing/extension at 60 °C. For primers with low Ta, we used a lower temperature for the annealing/extension step. Primers were designed with Beacon Designer Software v.8.20 (Premier Biosoft International, Palo Alto, CA, USA) with a junction primer strategy (Tables S1 and S2 in supplementary material). For the hybrid cell line, we used ClustalW [20] to find the homology region where we designed the primer. In any case, negative control of retro-transcription was performed to exclude any interference from residual genomic DNA contamination.

2.4. Uptake of T1AM and TA1

To evaluate T1AM or TA1 uptake, experiments were performed, as previously described, with minor modifications [21], and extracts were analyzed by HPLC-coupled to tandem mass spectrometry (HPLC-MS/MS) [21] upon 24 h treatment with T1AM or TA1, at concentration ranging from 0.1 to 10 µM.

To assess distribution in cellular fractions, cells were treated in flasks with 10 µM T1AM for 1 h. The nuclear pellet was extracted by using a nuclear extraction kit (Abcam, Cambridge, UK); the resulting cytoplasmic extract was centrifuged at 10,000 x g, for 30 min at 4 °C, to separate the mitochondrial and the cytosolic fractions. Fractions were extracted following the same protocol mentioned above.

2.5. Cell Viability

To assess cell viability by using the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) test, cells were treated for 24 h with T1AM or TA1 at different concentrations (0.1–10 µM), and then, MTT staining was performed [22]. The absorbance of the solution was read at 570 nm in a microplate reader (BioRad Laboratories, Milan, Italy). Results were expressed as a percentage of control.
2.6. Glucose Consumption

To assess glucose uptake, cells were exposed, for 4 h, to exogenous T$_1$AM or TA$_1$ (0.1–10 µM) in DMEM base (phenol red free) supplemented with 0.5 mg/mL glucose [23]. Control group was incubated with DMEM containing the same volume of vehicle. Cell culture medium was then collected, and glucose concentration was evaluated in the medium with a spectrophotometric assay kit (Sigma-Aldrich) at 340 nm. Metabolite concentration referred to the total protein content of whole-cell lysates.

2.7. cAMP Concentration Assay

cAMP concentration was assessed in cell lysate with an ELISA assay kit (BioVision Incorporated, Milpitas, CA, USA) according to manufacturer’s instruction. Briefly, cells were treated for 24 h with T$_1$AM or TA$_1$ (0.1–10 µM). At the end of treatment, 0.1 M HCl was added to stop enzyme activity (phosphodiesterases) The cAMP concentration in supernatant was spectrophotometrically evaluated, and results were normalized to total protein concentration in supernatant.

2.8. Protein Expression Analysis

Protein expression analysis was performed according to manufacturer’s instructions (Bio-Rad). In brief, proteins were subjected to SDS-PAGE (4–20% acrylamide separating gel, Criterion stain-free TGX Biorad). The separated proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA, USA), which was incubated overnight with the diluted antibody. Primary antibodies against CREB, pCREB, pERK, Sirt1, and c-Fos, and secondary antibodies were purchased from Cell Signaling (Danvers, MA, USA); CAMKII, pCAMKII, ERK, and PKC were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Immunoblots were visualized by means of a chemiluminescence reaction (Millipore) by Image LabTM Software (Biorad) under a luminescent image analyzer (Chemidoc XSR+ Bio-Rad). Only bands below the saturation limit were analyzed. The protein level was normalized to the optical density of total proteins in each lane.

2.9. Statistical Analysis

Results are expressed as the mean ± SEM. Differences between groups were analyzed by one-way or two-way ANOVA, as detailed for each figure. In the experiments aimed at determining differences vs. a single control group, Dunnett’s post-hoc test was applied. The threshold of statistical significance was set at $p < 0.05$. GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for data processing and statistical analysis.

3. Results
3.1. Expression of the Receptors of the Glutamatergic Pathway

Both cell lines, NG108-15 and U87MG, were characterized by real time PCR to evaluate the expression of receptors implicated in the glutamatergic postsynaptic pathway. The expression of Nmdar1, Nmdar2b, Glur2, Ephb2, and Taar1 genes was evaluated using a mouse TATA box binding protein (Tbp), as a housekeeping gene for NG108-15 cells and Hypoxanthine phosphoribosyltransferase 1 (Hprt1), for U87 MG cells.

The receptor expression was evaluated using real-time PCR. As shown in Figure 1, both cell lines expressed receptors, albeit to different extents, if compared to the corresponding housekeeping gene implicated in glutamatergic postsynaptic pathway: Nmdar1, Glur2, and Ephb2. Taar1, the putative T$_1$AM receptor, was only expressed in the U87 MG. Other genes, namely Pkca, Sirt1, and Erk1, whose protein expression was also evaluated, were also expressed; by contrast, neither Pkcy nor Nmdar2B gene expression was detected in our in vitro models.
3. Results

3.1. Expression of the Receptors of the Glutamatergic Pathway

Expression of receptors in NG108-15 and U-87 MG cell lines was assessed by using real time PCR. Values are expressed as mean ± SD of 2−ΔΔCq of three independent samples. Ct of receptors was compared to the housekeeping gene, TATA box binding protein (Tbp), for NG108-15 cells and Hypoxanthine phosphoribosyltransferase 1 (Hprt1) for U87 MG cells.

3.2. Cellular Uptake of T1AM and TA1

We measured HPLC MS/MS T1AM uptake and TA1 production in NG108-15 and U-87 MG cell lines, in the presence of FBS and T1AM (0.1, 1 or 10 µM), and in cell medium and lysate at the end of treatment (Instrument detection limits: T1AM > 0.3 nM; TA1 > 5 nM).

As summarized in Table 1, in the medium used to treat NG108-15 cells, after 24 h, T1AM was detectable only at the highest concentration of infusion (10 µM) and averaged 0.66 ± 0.14 nM, while its catabolite, TA1, was detected starting from the lowest concentration. Similar results were obtained in the U-87 MG cell line, where, in the medium, T1AM was present in trace amounts (0.36 ± 0.01 nM, at 10 µM T1AM), and TA1 was detected at every infused concentration. In cell lysates, T1AM was still measurable only at 10 µM T1AM and present in trace amounts at the other tested concentrations. Differently, TA1 was clearly detectable at either 1 or 10 µM T1AM, and it was present in trace amounts at 0.1 µM T1AM in both cell lines.

Table 1. Concentrations of T1AM and TA1 in medium and cell lysate after 24 h of infusion. Data represent mean ± SEM, n = 3–4 per group, and are expressed as nM. T1AM or TA1 contents were measured in medium and lysate of NG108-15 or U-87 MG cells, which were incubated for 24 h with T1AM (1–10 µM). [*p < 0.0001 for TA1 in medium and lysate in both cell lines (ANOVA)]. N.D., Not Detectable.

| T1AM (µM) | NG 108-15 Cells | U-87 MG Cells |
|-----------|-----------------|---------------|
|           | Medium | Cell Lysate | Medium | Cell Lysate |
| 0.1       | T1AM (nM)  | TA1 (nM)  | T1AM (nM)  | TA1 (nM)  | T1AM (nM)  | TA1 (nM)  | T1AM (nM)  | TA1 (nM)  |
| 1         | N.D.      | 135 ± 12    | N.D.      | N.D.      | 95 ± 6     | N.D.      | N.D.      | N.D.      |
| 10        | 0.66 ± 0.14| 4996 ± 97   | 10 ± 6    | 91 ± 19   | 0.36 ± 0.01| 13,214 ± 302| 6 ± 0.3   | 144 ± 80  |

The values of the concentrations of T1AM and of its catabolite, TA1, in the different cell fractions are summarized in Table 2 and expressed as µM and nM, respectively. The results showed a similar T1AM distribution in the two cell lines: T1AM was detected in all
fractions, albeit at a higher concentration in cytosol and nuclear fractions. Differently, TA$_1$ was measurable in all fractions of NG108-15 cells, while in U-87 MG cells, TA$_1$ was detected only in cytosol. Due to pellet resuspension, the concentrations of T$_1$AM and TA$_1$ in the mitochondrial and nuclear fractions might be underestimated. These results indicated a wide distribution of T$_1$AM in cells and confirmed that different experimental models may produce diverse behaviors.

Table 2. Concentrations of T$_1$AM and TA$_1$ in cellular fractions after 1 h of infusion. Data represent mean ± SEM, n = 3 per group, and are expressed as μM (T$_1$AM) or nM (TA$_1$). T1AM or TA1 contents were measured in mitochondrial, nuclear, and cytosolic fractions of NG108-15 or U-87 MG cells that were incubated for 1 h with 10 μM T$_1$AM. [Within each row, p < 0.0001 for T$_1$AM, and p < 0.001 for TA$_1$ for differences among cellular fractions (ANOVA)]. N.D., Not Detectable.

| Cell Lines | Cytosolic Fraction | Mitochondrial Fraction | Nuclear Fraction |
|------------|--------------------|------------------------|------------------|
|            | T$_1$AM (μM)      | TA$_1$ (nM)            | T$_1$AM (μM)    | TA$_1$ (nM) |
| NG108-15   | 2.89 ± 0.13       | 205.3 ± 27.6           | 1.66 ± 0.1      | 18.6 ± 2.9  | 2.77 ± 0.07 | 46.5 ± 9.3 |
| U-87 MG    | 2.62 ± 0.24       | 20.5 ± 0.9             | 0.54 ± 0.17     | N.D.        | 1.63 ± 0.34 | N.D.       |

To exclude any endogenous production of thyronamines or catabolites, the same experimental procedure was repeated with supplemented DMEM in the absence of exogenous T$_1$AM, incubated alone or in the presence of cells: neither T$_1$AM nor TA$_1$ was revealed.

To complete the uptake, the treatment was repeated with only TA$_1$, which was detectable in medium and cell lysate after 24 h of infusion (Table 3). Even though the concentration in the medium was reduced only at 10 μM, TA$_1$ was measurable in cell lysates at any tested concentration, indicating that cells could take it up. At 0.1 or 1 μM, we observed values that were higher than the infused concentrations, probably due to a matrix effect, since similar results were obtained with the only medium, after 24 h of infusion, and averaged as follows (nM): 128 ± 13, TA$_1$ 0.1 μM; 1102 ± 42, TA$_1$ 1 μM; 10,392 ± 200, TA$_1$ 10 μM. No further metabolism of TA$_1$ was recorded during infusion. T$_1$AM was present in trace, maybe as an impurity of TA$_1$, since neither T$_1$AM nor TA$_1$ were revealed in a previous assessment of the experimental model.

Table 3. Concentrations of TA$_1$ in medium and cell lysate after 24 h of infusion. Data represent mean ± SEM, n = 3–4 per group and are expressed as nM. TA$_1$ content was measured in medium and lysate of NG108-15 or U-87 MG cells, which were incubated for 24 h with TA$_1$ (1–10 μM). [p < 0.0001 for TA$_1$ in medium and lysate in both cell lines (ANOVA)].

| Cell Lines/TA$_1$ | Medium (nM) | Lysate (nM) |
|-------------------|-------------|-------------|
|                   | 0.1 μM      | 1 μM        | 10 μM       | 0.1 μM      | 1 μM        | 10 μM       |
| NG 108-15         | 233 ± 18    | 1795 ± 55   | 5355 ± 175  | 6.8 ± 1.9   | 72.4 ± 6.9  | 995 ± 95    |
| U-87 MG           | 135 ± 26    | 1193 ± 140  | 1520 ± 327  | 1.6 ± 0.1   | 15.4 ± 3.9  | 120 ± 22    |

3.3. Cell Viability

Cell viability was evaluated by MTT test in NG108-15 and U87 MG cells (Figure 2) treated with different concentrations of T$_1$AM or TA$_1$. 
In both cell lines, T1AM showed a slight—but significant—cytotoxic action starting from 0.1 μM (Figure 2a, −10–15%, \( p < 0.001 \) vs. control), implying a decrease oxidative metabolism. Below this concentration, T1AM was not cytotoxic (0.01 μM vs. control: NG108-15 cells, 101 ± 1.7 vs. 100.0 ± 1.7; U-87 MG cells, 96.1 ± 1.7 vs. 100.0 ± 1.5; \( p = \text{NS} \)).

TA1 was not cytotoxic, and a significant increase, by 50%, was measured after treatment with TA1 at 10 μM (\( ** p < 0.01 \)) in the U-87 MG cell line, while no change was observed in the NG 108-15 cell line (Figure 2b).

### 3.4. Glucose Consumption

To assess glucose consumption, NG 108-15 and U-87 MG cells were incubated for 4 h in phenol red-free DMEM containing 0.5 g/L glucose. At the end of treatment, glucose concentration was assayed in the medium, and the results were expressed as the difference between the initial and final concentrations normalized to the total protein content in cell lysates.

As indicated in Figure 3a, a 20% decrease in glucose consumption was observed upon 4 h treatment at 1–10 μM T1AM in the NG108-15 cell line (\( p < 0.05 \) vs. control by one-way ANOVA test and Dunnett’s test), while no significant change was observed after treatment in the U-87 MG cell line (Figure 3b \( p = \text{NS} \) vs. control). By comparison, glucose consumption was not affected in NG 108-15 cells exposed to TA1 (Figure 3c).

### 3.5. cAMP Assay

The concentration of cAMP was evaluated using a colorimetric assay kit, as described above, after 24 h of treatment with T1AM or TA1 at concentration, ranging from 0.1 μM to 10 μM.

As shown in Figure 4, only 0.1 μM T1AM increased cAMP production in the U87-MG cell line (Figure 4b, \( p < 0.01 \) vs. Con). No further change was observed in the other treatments with T1AM or TA1.
Figure 3. Glucose consumption was evaluated using a spectrophotometric assay kit, after 4 h of treatment: (a) with T1AM in NG108-15 cells, (b) with T1AM in U87-MG cells, and (c) with TA1 in NG108-15 cells. Results are the difference between the initial glucose concentration in medium and the final concentration, normalized to the total content of proteins in cell lysate. Control cells (Con) were incubated with a medium containing the same volume of vehicle. Values are mean ± SEM and are expressed as µg glucose/mg of total protein in lysate. [(a) T1AM, one-way ANOVA, p < 0.05, Dunnett’s post-hoc test for multiple comparison, * p < 0.05, vs. control (Con); n = 4 per groups].

Figure 4. cAMP production was evaluated using a spectrophotometric assay kit, after 24 h of treatment: (a) with T1AM in NG108-15 cells, (b) with T1AM in U-87 MG cells, (c) with TA1 in NG108-15 cells. Results are the difference between the initial glucose concentration in medium and the final concentration, normalized to the total content of proteins in cell lysate. Control cells (Con) were incubated with a medium containing the same amount of vehicle. Values are mean ± SEM and are expressed as µg glucose/mg of total protein in lysate. [(a) T1AM, one-way ANOVA, p < 0.05, Dunnett’s post-hoc test for multiple comparison, * p < 0.05, vs. control (Con); n = 4 per groups].
3.6. Effects of T\textsubscript{1}AM and TA\textsubscript{1} on the Expression of Signaling Cascade

We investigated changes in expression and post-translational modification of some proteins involved in the glutamatergic postsynaptic signaling, in NG 108-15 and U-87 MG cells, upon infusion of T\textsubscript{1}AM or TA\textsubscript{1}. Protein expression analysis was performed after 24 h of treatment with T\textsubscript{1}AM or TA\textsubscript{1}, ranging from 0.1 to 10 M, resulting in different effects according to the cell line.

The major effect highlighted in both cell lines was an increase in the phosphorylation of members of the signaling cascade, as shown in Figure 4 (and in Supplementary Material, Figure S2), albeit on different target proteins.

In the NG 108-15, an increase in phosphorylation of ERK and CaMKII was observed at the highest T\textsubscript{1}AM concentrations (Figure 5a, 1 \textmu M T\textsubscript{1}AM, pERK/ERK + 60%, * \textit{p} < 0.05 vs. Control; Figure 5a, 10 \textmu M T1AM, pCAMKII/CAMKII + 50%, * \textit{p} < 0.05 vs. control), while in U87 MG cells, T\textsubscript{1}AM induced the phosphorylation of the transcriptional factor CREB at 1 \textmu M (Figure 5b, pCREB/CREB, + 70%, * \textit{p} < 0.01 vs. control). Neither expression nor post-translational modifications of other proteins were affected. We also investigated changes by TA\textsubscript{1} in expression and post-translational modifications in proteins, which were significantly affected by T\textsubscript{1}AM (Figure 5c): neither in U-87 MG, nor in NG 108-15 cells, the phosphorylation of the above-mentioned proteins was altered upon infusion of TA\textsubscript{1}, indicating that effects may be attributed to T\textsubscript{1}AM, rather than to the production of its catabolite TA\textsubscript{1}.

![Figure 5](image-url)
Figure 5. Western blot analysis of T1AM or TA1 effects on protein expression, belonging to the glutamatergic system, after 24 h treatment. (a) T1AM in NG108-15 cells, (b) T1AM in U-87 MG cell lines, and (c) TA1 in NG108-15 cells. Histograms represent mean ± SEM. Representative immunoblots of proteins, which reached significance, are shown. All results are normalized against total proteins, measured using stain-free gels, and are expressed as relative protein levels. All treatments received the same amount of vehicle, and control group (Con) was incubated with a medium containing only vehicle (DMSO). Two way ANOVA, $p < 0.001$, and Dunnett’s post-hoc test are used for multiple comparisons, * $p < 0.05$, *** $p < 0.001$ vs. control, Con (a, b)].
4. Discussion

In this project, we firstly characterized the experimental model, and then, we evaluated the effects of T₁AM on proteins involved in signal transduction pathways. Since T₁AM was metabolized to TA₁, experiments with significant results were extended to TA₁ with the aim of evaluating if its catabolite might reproduce T₁AM’s effects.

Our results indicated that NG108-15 and U-87 MG cell lines expressed, although to a different extent, the main receptors of the glutamatergic system: namely, Nmdar1, Glur2, Ephb2, but not Nmdar2b. Taar1, the putative T₁AM receptor, was expressed only by U87 MG cells, making the NG108-15 cell line a valid negative control for effects that are assumed to be mediated by this receptor.

NMDA receptors consist in four subunits: two obligatory NR1 subunits and two regulatory subunits that can be NR2A→D, or NR3A→B. The precise combination of NMDAR subunits determines the functional properties of the NMDAR channels [24]. NR2A and NR2B subunits, which are the predominant NMDAR subtypes in the forebrain, undergo a particularly well-characterized developmental shift in the cortex. NR2B subunits are abundant in the early postnatal brain, and NR2A levels increase progressively with development [25–27]. The absence of Nmdar2b may be justified by the fact that the NR2B subunit is usually expressed only in some regions of brain and only in the postnatal stage, while subunit NR1 is widely expressed in adult human and mouse brain but only in the embryonic brain [28]. EphB-ephrinB, particularly B2, interaction has recently emerged as major role player in synaptic plasticity and neuronal process development, including long term potentiation [29]. EphrinB2 signaling is critical for the stabilization of AMPA receptors at the cellular membrane, and its expression is elevated in cancer cell lines, such as neuroblastoma and glioma, compared to primary cell lines [30]. Both cell lines expressed Pkcx, Erk1, and Sirt1 proteins involved in several post synaptic signaling cascades, while Pkcg was not detected. Protein kinases PKCa and PKCy belong to the family of PKC activated by cytosolic Ca²⁺ ions and diacylglycerol; while the alpha isoform is expressed in several brain regions and other tissues, only brain, spinal cord, cerebellum, hippocampus, and cerebral cortex have been found to express the Pkcy isoform [31]. The activity of the Pkcx contributes to the enhancement of plasticity in the hippocampal CA1 region. Indeed, the inhibition of this kinase can eliminate long term potentiation [32].

To complete the validation of our models, we assessed the uptake of exogenous T₁AM and TA₁ in the cell medium and lysate at the end of treatment (24 h) and their metabolism. We observed that T₁AM, incubated in standard cell culture medium supplemented with FBS, was absorbed by U87 MG and NG108-15 cell lines, diffused into the cell fractions, and rapidly catabolized to TA₁, which was measurable in all fractions of NG 108-15 cells, while in U-87 MG cells, it was detected only in cytosol. These results indicated a wide distribution of T₁AM into the cell and confirmed that different experimental models may produce diverse experimental response. No further metabolism was observed with exogenous TA₁.

The last step of cell characterization was to test T₁AM cytotoxicity. T₁AM showed a slight cytotoxic effect in both NG108-15 and U87 MG cells, as revealed from the MTT test, also implying a reduced oxidative metabolism. Differently, TA₁ did not affect cell viability: this could be explained by considering that no further oxidative deamination occurred in presence of TA₁, and consequently, there was no production of hydrogen peroxide, ammonia, or other compounds, which are usually produced by monooamino oxidase catalysis and can alter the redox state of cells and induce cytotoxicity [33].

Thus, both NG108-15 and U87 MG cell lines expressed proteins associated with the glutamatergic and other systems of signal transduction pathways, and they may be considered an in vitro model to evaluate effects of T₁AM or TA₁ on different intracellular cascades.

We then investigated changes in expression and post-translational modifications of some of these proteins upon infusion of T₁AM. The major effect we observed was an increase in phosphorylation, exerted by T₁AM, but not reproduced by TA₁ alone. We showed that T₁AM was able to induce phosphorylation of nuclear factor CREB in U-87 MG cells, while it was able to induce phosphorylation of CaMKII and ERK in NG108-15 cells.
These findings indicated that infusion with exogenous T₁AM might alter the glutamatergic signaling cascades and, in general, other signal pathways which contain this kind of protein kinases, albeit with different effects, depending on the in vitro model used.

Both cell lines shared a similar receptor pattern, except for TAAR1, which was not expressed in NG108-15 line: this might account for differences in sensitivity towards exogenous T₁AM. Notably, most significant effects were recorded at T₁AM 10 µM, the only treatment which allowed the detection of T₁AM in the medium after 24 h, indicating that effects could be attributable to T₁AM rather than to its catabolite, as confirmed by treatment with TA₁.

CaMKII is a kinase activated by calcium ions, a key mediator in connecting transient calcium influx to neuronal plasticity, and its autophosphorylation at Thr-286 induces a persistent activation [34]. This kinase phosphorylates and activates different substrates, including the extracellular regulated kinase (ERK), which, in turn, leads to the activation of transcription factors, CREB and c-fos among them, and then, the gene transcription that triggers the synthesis of new proteins. The transcriptional factor CREB is the heart of the glutamatergic signaling cascade, and its activation at the end of the cascade leads to the transcription of factors fundamental for memory consolidation and LTP [35]. We have to take into account that ERK enzymes are points of convergence for several pathways, and the wide range of effects exerted by ERK activation in downstream cascades of different signaling system has been well established [36].

The observed increase in phosphorylation may underlie the potential mechanism of T₁AM of its prolearning effects. Even though our hypothesis is not fully sustained by the increase in the second messenger cAMP, which was almost unchanged, we can hypothesize that a cross talk between pathways might have occurred. Moreover, to confirm this hypothesis, it could be of interest to evaluate changes in protein phosphorylation at different time course points.

Glucose is the main energetic substrate for the brain. Differences between cell lines were encountered in glucose consumption as well, which was slightly decreased after 4 h by 1–10 µM T₁AM, only in the NG108-15 cells, in contrast with the need of ATP to support protein phosphorylation. Assadi-Porter et al. [37] demonstrate that T₁AM can act as a regulator of both glucose and lipid metabolism in mice through sirtuin-mediated pathways, but in our model, no change in sirtuins was observed.

Our biochemical observations are partially consistent with previous results [10,37]; differences may be attributed to different circumstances, such as environmental conditions, cell stages/passages, subcellular localization, experimental models, and procedures.

However, our study suffers from limitations in experimental models. These cell lines are an unlimited auto-replicative source, relatively easy to culture, but they suffer from the limitations induced by a cancer cell, which expresses a particular gene profile. For this reason, the assessment of the overall effects of T₁AM and TA₁ should be extended to primary cell lines (astrocytes or primary neuronal/hippocampal cells) and, then, to in vivo experimental models, which are more relevant and reflective of the original environment.

5. Conclusions

The cell lines investigated express receptors and signaling molecules implicated in several signaling pathways, and they can uptake and metabolize T₁AM.

Most of the experiment results are statistically significant only at the highest concentration of T₁AM. The observed effects are mediated, mainly, by T₁AM rather than its metabolite, TA₁; as a matter of fact, all the significant changes by T₁AM were not reproduced by the infusion of TA₁ alone. This may indicate that the effects induced by T₁AM were not influenced or strengthened by the production of TA₁, as described differently in the literature on animal models [33].

T₁AM can affect biological processes mediated by phosphorylation of proteins in several pathways, including the glutamatergic system. It demonstrates a very complex pharmacology, and an alteration in these signaling molecules or phosphorylation could not
be its only primary effect, but other mechanisms may also be implicated. These findings should be considered during the development of potential T1AM-based therapies.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/life12091352/s1, Figure S1: Graphical abstract showing all methods used in our study, Figure S2: Western blotting: Representative immunoblots of proteins after 24 h of treatment with T1AM in NG108-15(A) and U-87 MG (B) cell lines, or with TA1 (C), Table S1: Primer sequences of target genes used in hybrid cell line NG108-15. Homology gene sequences of rat and mouse were found using ClustalW [20] and primers were designed with Beacon Designer Software v8.20 on these regions, Table S2: Primer sequences of target genes used in the human cell line U-87 MG. Primers were designed with Beacon Designer Software v8.20.

Author Contributions: Conceptualization, G.S., L.B. and S.G.; supervision, S.G.; formal analysis, G.S., L.B. and S.G.; funding acquisition, S.G.; investigation, L.B., G.S., G.G., F.D.A., M.E.F., M.P.F., M.T. and S.V.; methodology, V.C., S.V. and M.B.; resources, V.C.; visualization and writing—original draft preparation, L.B. and G.S.; writing—review and editing, R.Z. and S.G. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by University of Pisa, Pisa, Italy [549901_2018_GHELARDONI_ATENEO; 549901_2020_Ghelardoni_Ateneo; 549901_2021_Ghelardoni_Ateneo; 549901_2022_Ghelardoni_Ateneo].

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Raw data of this research study are available by contacting the authors.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References
1. Scanlan, T.S.; Suchland, K.L.; Hart, M.E.; Chiellini, G.; Huang, Y.; Kruzich, P.J.; Frascarelli, S.; Crossley, D.A.; Bunzow, J.R.; Ronca-Testoni, S.; et al. 3-Iodothyronamine is an e-endogenous and rapid-acting derivative of thyroid hormone. Nat. Med. 2004, 10, 638–642. [CrossRef] [PubMed]
2. Köhrle, J. The Colorful Diversity of Thyroid Hormone Metabolites. Eur. Thyroid J. 2019, 8, 115–129. [CrossRef] [PubMed]
3. Saba, A.; Chiellini, G.; Frascarelli, S.; Marchini, M.; Ghelardoni, S.; Raffaelli, A.; Tonacchera, M.; Vitti, P.; Scanlan, T.S.; Zucchi, R. Tissue distribution and cardiac metabolism of 3-iodothyronamine. Endocrinology 2010, 151, 5063–5073. [CrossRef]
4. Hoefig, C.S.; Wuensch, T.; Rijntjes, E.; Lehmphul, I.; Daniel, H.; Schweizer, U.; Mittag, J.; Köhrle, J. Biosynthesis of 3-iodothyronamine From T4 in Murine Intestinal Tissue. Endocrinology 2015, 156, 4356–4364. [CrossRef]
5. Wood, W.J.; Geraci, T.; Nilsen, A.; DeBarber, A.E.; Scanlan, T.S. Thyronamines are oxidatively deaminated to iodothyroacetic acids in vivo. Chembiochem 2009, 10, 361–365. [CrossRef] [PubMed]
6. Snead, A.N.; Santos, M.S.; Seal, R.P.; Miyakawa, M.; Edwards, R.H.; Scanlan, T.S. Thyronamines inhibit plasma membrane and vesicular monoamine transport. ACS Chem. Biol. 2007, 2, 390–398. [CrossRef] [PubMed]
7. Regard, J.B.; Kataoka, H.; Cano, D.A.; Camerer, E.; Yin, L.; Zheng, Y.W.; Scanlan, T.S.; Hebrok, M.; Coughlin, S.R. Probing cell type-specific functions of Gi in vivo identifies GPCR regulators of insulin secretion. J. Clin. Investig. 2007, 117, 4034–4043. [CrossRef]
8. Gompf, H.S.; Greenberg, J.H.; Aston-Jones, G.; Ianculescu, A.G.; Scanlan, T.S.; Dratman, M.B. 3-Monoiodothyronamine: The rationale for its action as an endogenous adrenergic-blocking neuromodulator. Brain Res. 2010, 10, 130–140. [CrossRef]
9. Manni, M.E.; De Siena, G.; Saba, A.; Marchini, M.; Landucci, E.; Gerace, E.; Zazzeri, M.; Musilli, C.; Pellegrini-Giampietro, D.; Matucci, R.; et al. Pharmacological effects of 3-iodothyronamine (T1AM) in mice include facilitation of memory acquisition and retention and reduction of pain threshold. Br. J. Pharmacol. 2013, 168, 354–362. [CrossRef]
10. Bellusci, L.; Laurino, A.; Sabatini, M.; Sestito, S.; Lenzi, P.; Raimondi, L.; Rapposelli, S.; Biagioni, F.; Fornai, F.; Salvetti, A.; et al. New Insights into the Potential Roles of 3-Iodothyronamine (T1AM) and Newly Developed Thyronamine-Like TAAR1 Agonists in Neuroprotection. Front. Pharmacol. 2017, 8, 905. [CrossRef]
11. Minato, A.; Akiyoshi, M.; Okuno, H. Role of Immediate-Early Genes in Synaptic Plasticity and Neuronal Ensembles Underlying the Memory Trace. Front. Mol. Neurosci. 2016, 7, 58. [CrossRef] [PubMed]
12. Giese, K.P.; Mizuno, K. The roles of protein kinases in learning and memory. Learn Mem. 2013, 20, 540–552. [CrossRef] [PubMed]
13. Piehl, S.; Hoefig, C.S.; Scanlan, T.S.; Köhrle, J. Thyronamines—past, present, and future. Endocr. Rev. 2011, 32, 64–80. [CrossRef] [PubMed]
14. Braulke, L.J.; Klingenspor, M.; DeBarber, A.; Tobias, S.C.; Grandy, D.K.; Scanlan, T.S.; Heldmaier, G. 3-Iodothyronamine: A novel hormone controlling the balance between glucose and lipid utilisation. J. Comp. Physiol. B 2008, 178, 167–177. [CrossRef]
15. Laurino, A.; De Siena, G.; Resta, F.; Masi, A.; Musilli, C.; Zucchi, R.; Raimondi, L. 3-Iodothyroacetic acid, a metabolite of thyroid hormone, induces itch and reduces threshold to noxious and to painful heat stimuli in mice. Br. J. Pharmacol. 2015, 172, 1859–1868. [CrossRef][PubMed]
16. Musilli, C.; De Siena, G.; Manni, M.E.; Logli, A.; Landucci, E.; Zucchi, R.; Saba, A.; Donzelli, R.; Passani, M.B.; Provensi, G.; et al. Histamine mediates behavioural and metabolic effects of 3-iodothyroacetic acid, an endogenous end product of thyroid hormone metabolism. Br. J. Pharmacol. 2014, 171, 3476–3484. [CrossRef]
17. Ge, Y.; Wang, Y.T. Postsynaptic signaling at glutamatergic synapses as therapeutic targets. Curr. Opin. Neurobiol. 2022, 75, 102585. [CrossRef]
18. Ménard, C.; Gaudreau, P.; Quirion, R. Signaling pathways relevant to cognition-enhancing drug targets. Handb. Exp. Pharmacol. 2015, 228, 59–98. [CrossRef]
19. Sacripanti, G.; Lorenzini, L.; Bandini, L.; Frascarelli, S.; Zucchi, R.; Ghelardoni, S. 3-Iodothyronamine and 3,5,3′-triiodo-L-thyronine reduce SIRT1 protein expression in the HepG2 cell line. Horm. Mol. Biol. Clin. Investig. 2020, 2, 41. [CrossRef]
20. Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; McGettigan, P.A.; McWilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; et al. Clustal W and Clustal X version 2.0. Bioinformatics 2007, 23, 2947–2948. [CrossRef]
21. Lorenzini, L.; Ghelardoni, S.; Saba, A.; Sacripanti, G.; ChIELlinI, G.; Zucchi, R. Recovery of 3-Iodothyronamine and Derivatives in Biological Matrixes: Problems and Pitfalls. Thyroid 2017, 27, 1323–1331. [CrossRef][PubMed]
22. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods 1983, 65, 55–63. [CrossRef]
23. Sacripanti, G.; Nguyen, N.M.; Lorenzini, L.; Frascarelli, S.; Saba, A.; Zucchi, R.; Ghelardoni, S. 3,5-Diiodo-L-Thyronine Increases Glucose Consumption in Cardiomyoblasts Without Affecting the Contractile Performance in Rat Heart. Front Endocrinol. 2018, 9, 282. [CrossRef][PubMed]
24. Cull-Candy, S.G.; Leszkiewicz, D.N. Role of distinct NMDA receptor subtypes at central synapses. Sci. STKE 2004, 255, 16. [CrossRef][PubMed]
25. Flint, A.C.; Maisch, U.S.; Weishaupt, J.H.; Kriegstein, A.R.; Monyer, H. NR2A subunit expression shortens NMDA receptor enhancement of long-term potentiation by nefiracetam in the rat hippocampal CA1 region. J. Neurochem. 2002, 81, 1387–1397. [CrossRef][PubMed]
26. Sheng, M.; Cummings, J.; Roldan, L.A.; Jan, Y.N.; Jan, L.Y. Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. Nature 1994, 68, 144–147. [CrossRef]
27. Böckers, T.M.; Zimmer, M.; Müller, A.; Bergmann, M.; Brose, N.; Kreutz, M.R. Expression of the NMDA R1 receptor in selected human brain regions. Neuroreport 1994, 5, 965–969. [CrossRef]
28. Henderson, J.T.; Georgiou, J.; Jia, Z.; Robertson, J.; Elowe, S.; Roder, J.C.; Pawson, T. The Receptor Tyrosine Kinase EphB2 Regulates NMDA-Dependent Synaptic Function. Neuron 2001, 32, 1041–1056. [CrossRef]
29. Pozniak, P.D.; White, M.K.; Khalili, K. TNF-α/NF-kB Signaling in the CNS: Possible Connection to EPHB2. J. Neuroimmun Pharmacol. 2014, 9, 133–141. [CrossRef][PubMed]
30. Saito, N.; Shirai, Y. Protein kinase C gamma (PKC gamma): Function of neuron specific isotype. J. Biochem. 2002, 132, 683–687. [CrossRef][PubMed]
31. Kida, S. A Functional Role for CREB as a Positive Regulator of Memory Formation and LTP. Exp. Neurobiol. 2012, 21, 136–140. [CrossRef][PubMed]
32. Lynch, M.A. Long-term potentiation and memory. Physiol. Rev. 2004, 84, 87–136. [CrossRef]
33. Laurino, A.; De Siena, G.; Resta, F.; Masi, A.; Manni, M.E.; Logli, A.; Landucci, E.; Zucchi, R.; Saba, A.; et al. Metabolic Reprogramming by 3-Iodothyronamine (T1AM): A New Perspective to Reverse Obesity through Co-Regulation of Sirtuin 4 and 6 Expression. Int. J. Mol. Sci. 2018, 19, 1535. [CrossRef][PubMed]