ACS chemical biology

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Thyronamines Inhibit Plasma Membrane and Vesicular Monoamine Transport

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ABSTRACT Thyroid hormone has long been known to have important transcriptional regulatory activities. Recently, however, the presence of endogenous derivatives of thyroid hormone, thyronamines, has been reported in various mammalian tissues. These derivatives have potent *in vitro* activity with a class of orphan G-protein-coupled receptors, the trace amine-associated receptors, and profound *in vivo* effects when administered to mice. We report here a novel neuromodulatory role for thyronamines. In synaptosomal preparations and heterologous expression systems, thyronamines act as specific dopamine and norepinephrine reuptake inhibitors. Thyronamines also inhibit the transport of monoamines into synaptic vesicles. These observations expand the nontranscriptional role of thyroid hormone derivatives and may help to explain the pharmacological effects of thyronamines *in vivo*.

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Received for review March 14, 2007 and accepted May 4, 2007.

Published online May 25, 2007

10.1021/cb700057b CCC: $37.00

A previous report described the synthesis and pharmacology of a class of compounds, thyronamines (*1*) (Supplementary Figure 1), which are presumably decarboxylated metabolites of thyroid hormone, a classic endocrine hormone known to exert its actions by means of transcriptional regulation (*2, 3*). This work demonstrated that at least two of these compounds, thyronamine (T_0AM) and 3-iodothyronamine (T_1AM), are present in various vertebrate tissues and are potent and rapid activators of the rat and mouse trace amine associated receptors 1 ($TAAR_1$), members of the G-protein-coupled receptor (GPCR) superfamily. T_1AM, when administered by intraperitoneal injection in mice, induces profound hypothermia and bradycardia within minutes, too rapid for a transcriptional mechanism.

Notably, thyronamines contain the arylethylamine scaffold and exhibit activity kinetics that are common to the catecholamine and serotonin neuromodulators and neurotransmitters. Trace amines, another well-described class of molecules with arylethylamine functionality, are endogenous derivatives of monoamine neurotransmitters. Despite their presence in the brain, trace amines were originally presumed to be transient byproducts of neurotransmitter synthesis and metabolism, causing striking pharmacological effects, but only at high concentrations. Additionally, trace amines have been shown to influence neurotransmission by regulating the active transport of monoamine transmitters, thus acting as neuromodulators (*4*). However, recent evidence suggests a physiological role for these trace amines. Like thyronamines, they have been shown to activate the TAAR family of GPCRs (*5–9*).

Neurotransmitters are transported across membranes at two distinct cellular locations. The first is at

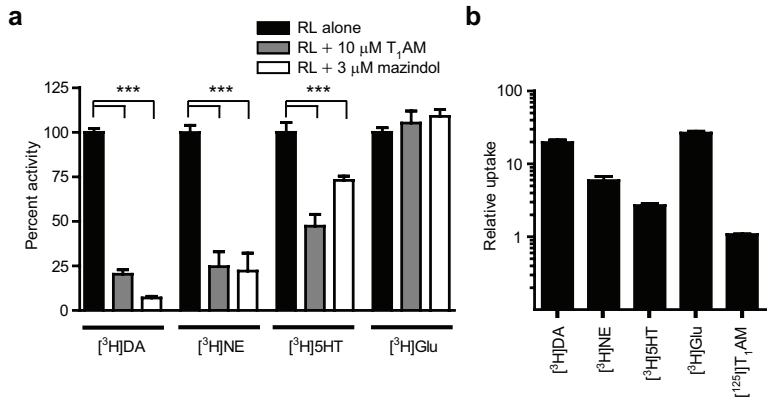

Figure 1. T_1AM inhibition of synaptosomal transport of monoamines. a) Uptake is shown as percent activity normalized for each of the tritiated radioligands [^{3}H]5HT, [^{3}H]DA, [^{3}H]NE, and [^{3}H]Glu. Statistical significance was determined using the paired two-tailed *t* test. * designates statistical significance, $p < 0.001$. b) Alternately, uptake of these radioligands or [^{125}I]T_1AM is shown relative to uptake at time 0 min, in which membranes were held on ice and the reaction mixture was immediately quenched with cold KRH buffer. Membranes treated with radioligand and 1% DMSO showed no significant difference in uptake compared with cells incubated with radioligand alone (data not shown). The mean values reported are from three separate experiments performed in triplicate.**

the plasma membrane, where specific transporters for each of the classical neurotransmitters act to terminate signaling and to recycle transmitters for future rounds of release. The second location is at the membrane of the secretory vesicle, where transmitters are accumulated from the cytoplasm in preparation for regulated exocytotic release (*10*–*12*). The plasma membrane transporters responsible for cellular uptake of the monoamine neurotransmitters are the dopamine transporter (DAT), the norepinephrine transporter (NET), and the serotonin transporter (SERT), and the sole transporter responsible for vesicular transport of all monoamines in the central nervous system (CNS) is the vesicular monoamine transporter 2 (VMAT2) (*13, 14*).

Neurotransmitter transport has an important role in normal neurotransmission and in a wide variety of disease states (*11*). However, the specific molecular mechanisms that regulate these activities remain poorly understood. Given the presence of thyronamines in the brain and their structural similarity to the monoamine transmitters and trace amines, we hypothesized that thyronamines might interact with monoamine transporters. Herein we demonstrate that several thyronamines, including T_1AM, act as specific dopamine and norepinephrine reuptake inhibitors, as well as inhibitors of VMAT2. These data shed light on the molecular mechanisms that contribute to the physiological and pharmacological effects of monoamines.

RESULTS AND DISCUSSION

The current study tested the ability of a panel of synthetically derived iodothyronamines (*1*) to perturb monoamine neurotransmitter transport, with the goal of expanding the potential physiological roles of thyronamines and understanding the molecular mechanisms of thyronamine pharmacology. This analysis was motivated by the significant structural similarity of thyronamines to the trace amines and monoamine neurotransmitters, as well as the demonstrated GPCR activity with TAARs. T_1AM became the primary focus of this work because of its profound pharmacological activity *in vivo* and its potent ability to activate rat and mouse $TAAR_1$ relative to other trace amines *in vitro* (*1, 5, 6*). Herein, we demonstrate that T_1AM is a DAT, NET, and VMAT2 inhibitor with low micromolar potencies. This novel neuromodulatory role for T_1AM indicates a potentially expanded physiological role for thyroid hormone derivatives, distinct from transcriptional regulation, and adds to the complexity of thyronamine pharmacology.

T_1AM Inhibition of Synaptosomal Monoamine Transport. To study the effect of T_1AM on plasma membrane monoamine transport, we prepared rat brain synaptosomes. Synaptosomal preparations have been used previously to study plasma membrane neurotransmitter transport (*4, 15*). Membranes were assayed with or without either T_1AM (10 μM) or the reuptake inhibitor mazindol (3 μM). At this concentration, T_1AM significantly inhibited dopamine, norepinephrine, and serotonin transport (77.6%, 72.1%, and 42.2% inhibition, respectively, as compared with no T_1AM controls) (Figure 1, panel a). This mirrors the activity of mazindol, a potent inhibitor of dopamine, norepinephrine and

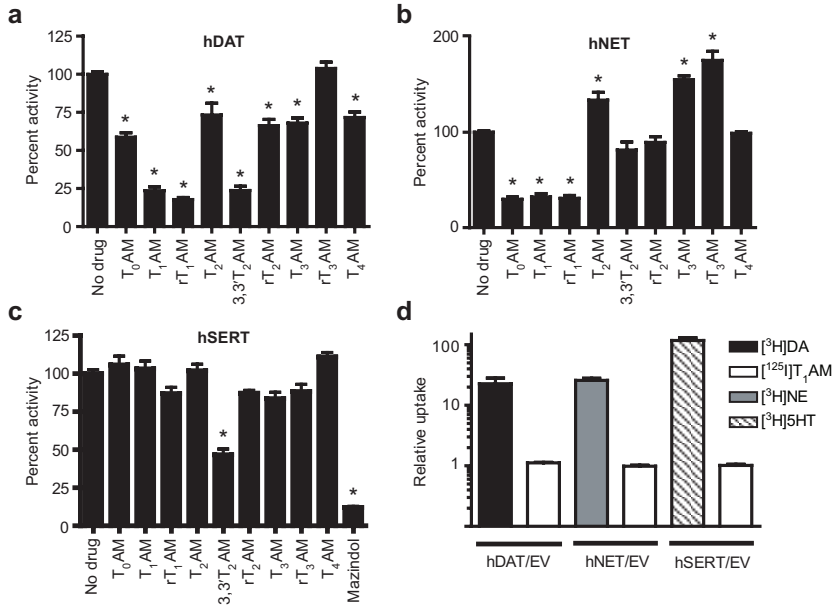

Figure 2. DAT, NET, and SERT inhibition by thyronamines. a–c) Results are shown as percent uptake normalized to 100% for hDAT, hNET, and rSERT with radioligand alone. d) Alternatively, uptake is shown relative to empty pBSK or empty pcDNA3 transfected cells. Statistical significance was determined using the paired two-tailed *t* test. * designates statistical significance, $p < 0.001$. Cells treated with radioligand and 1% DMSO showed no significant difference in uptake compared with cells incubated with radioligand alone (data not shown). The mean values reported are from three separate experiments performed in triplicate.

serotonin transporters (*12*), which at 3 μM inhibited transport of the respective radioligands in this assay by 92.5%, 76.4%, and 15.5%. Both T_1AM and mazindol demonstrated specificity for inhibition of monoamine transport, as neither had any significant effect on glutamate transport.

Several of the classical trace amines and pharmacological ligands that are structurally similar to T_1AM, are known both to inhibit neurotransmitter transport and also to be recognized as substrates (*16, 17*). To distinguish between a role as an inhibitor or a substrate, [^{125}I]T_1AM was synthesized and used to directly measure T_1AM transport (*18*). Membranes incubated with [^{125}I]T_1AM demonstrated no significant uptake above background binding (Figure 1, panel b). Additionally, increasing concentrations of unlabeled T_1AM had no effect on the relative uptake (data not shown), indicating that T_1AM is not a substrate.

DAT, NET, and SERT Inhibition by Thyronamines. The dopamine transporter (hDAT), norepinephrine transporter (hNET), and serotonin transporter (hSERT) were expressed in HeLa cells to identify the specificity of T_1AM for each of the plasma membrane monoamine transporters and to screen for activity of the other thyronamines. Heterologous expression of the various plasma membrane transporters has been demonstrated (*19*) and provides an excellent means of selectively analyzing transport by each of the known transporters outside the nervous system. To determine the effects of various thyronamines on each of the transporters, transfected cells were assayed for uptake of their preferred substrates ([^{3}H]5 hydroxytryptamine (5HT) for SERT, [^{3}H]DA for DAT, and [^{3}H]NE for NET) in the presence of each of the nine thyronamines at a single concentration (10 μM). Consistent with the rat synaptosomal uptake, hDAT and hNET were sensitive to T_1AM, with transport inhibited by 76.5% and 67.7%, respectively, as compared with non-T_1AM-treated controls (Figure 2, panels a and b). Additionally, both hDAT and hNET had varying sensitivities to the other thyronamines at 10 μM. In the case of hNET transport, we found that several thyronamines, T_2AM, T_3AM, and rT_3AM, increased NE transport (33–75%). In contrast to the synaptosomal results, however, heterologously expressed hSERT was insensitive to T_1AM and most of the thyronamines at 10 μM (Figure 2, panel c). Only $3,3'T_2AM$ demonstrated any significant inhibition of hSERT (~50% inhibition at 10 μM $3,3'T_2AM$). Controls with mazindol showed near complete inhibition, indicating that hSERT was fully sensitive to a known inhibitor. Furthermore, identical experiments performed with rDAT and rSERT showed similar overall activity and thyronamine sensitivity (data not shown). Additionally, neither DAT, NET, nor SERT demonstrated detectable uptake of [^{125}I]T_1AM under a variety of conditions (Figure 2, panel d).

To characterize more thoroughly the potency of T_1AM inhibition, a dose–response study was performed with hDAT and hNET, varying the T_1AM concentrations from 10 nM to 100 μM. The analysis yielded IC_{50}'s for T_1AM of 3.6 μM in the case of hDAT and 4.4 μM in the case of hNET (Figure 3, panels a and b; Table 1). The mode of T_1AM inhibition was then assessed by measuring the effects of T_1AM on the K_m and V_{max} of DA transport by DAT and NE transport by NET. hDAT-transfected HeLa cells were incubated with 20 nM to 100 μM [^{3}H]DA with or without 5 μM T_1AM. It was found that 5 μM T_1AM increased the K_m (from ~5 to ~16 μM) and decreased (by ~25%) the V_{max} (Figure 3, panel c; Table 1), suggesting that T_1AM acts by mixed inhibition, with competitive and noncompetitive components. The kinetic analysis was repeated for hNET-transfected cells incubated with 10 nM to 100 μM [^{3}H]NE with or without 5 μM T_1AM. T_1AM addition resulted in a nearly 3-fold increase (1.8 to ~5.0 μM) in K_m (Figure 3, panel d; Table 1) but

had no significant effect on V_{max}, indicating that T_1AM behaves as a competitive inhibitor of hNET.

Synaptosomal preparations and heterologous expression systems allowed us to assess the pharmacological effects of thyronamines on the plasma membrane transport of monoamines and generated a detailed analysis of thyronamine sensitivities and T_1AM inhibition kinetics. In synaptosomal uptake assays, T_1AM demonstrated near complete inhibition of DA and NE uptake at 10 μM, with limited effects on 5HT uptake and no effects on glutamate transport. Coupling this with data from heterologous expression systems for DAT, NET, and SERT, T_1AM selectively inhibits DAT and NET at concentrations similar to those demonstrated for several of the trace amines (*4*). Although DAT and NET are both inhibited by T_1AM, there are subtle differences in their overall thyronamine structure–activity relationships (SARs), with DAT showing additional sensitivity to rT_1AM and 3,3′T_2AM, and NET to T_0AM and rT_1AM. Surprisingly, we found that T_2AM, T_3AM, and rT_3AM hyperactivate NET (33–75%) by an unknown mechanism. Given the similarity of DAT and NET in sequence (65% identity) and substrate preference, it is noteworthy that structural changes as small as the presence and location of a single iodide atom can lead to differences in recognition by these two transporters.

In contrast to the consistent inhibition of DAT and NET in synaptosomes and heterologous expression systems, T_1AM inhibits uptake of [^{3}H]5HT into rat synaptosomal preparations but has no effect on transport of [^{3}H]5HT by hSERT. Although these observations seem contradictory, there are possible SERT-independent sources of 5HT uptake in the synaptosome assay. Thus, considering the relatively low level of synaptosomal uptake seen for 5HT (2.7-fold over background) compared to that for DA (19.6-fold), NE (5.9-fold), and glutamic acid (26.5-fold), the poor ability of mazindol to block 5HT uptake in this assay, as well as the inability of T_1AM to inhibit recombinant hSERT, we conclude that T_1AM is not a SERT inhibitor.

Effects of T_1AM on Synaptic Vesicle Transport. Because we found that T_1AM may modulate plasma membrane monoamine transport, we sought to determine its effect on vesicular monoamine transport, as this is often a coupled process. To assess the effects of T_1AM on synaptic vesicle amine transport, synaptic vesicles were purified from crude synaptosomal preparations by means of differential centrifugation. Using vesicle preparations, the uptake of [^{3}H]5HT was measured with or without T_1AM or tetrabenazine (TBZ), a known VMAT2 inhibitor (IC_{50} of 460 nM) (*20*). TBZ is an appropriate positive control for inhibition because TBZ-sensitive VMAT2 is the only known vesicular transporter for monoamines in the CNS (*13, 14*). [^{3}H]5HT was used as the test substrate because it is recognized with high apparent affinity by VMAT2 (K_m ~0.29 μM). We observed that T_1AM (10 μM) inhibited vesicular transport of 5HT by 62% (Figure 4, panel a). In the same assay, saturating levels of TBZ inhibited monoamine transport by 87%. However, no transport of [^{125}I]T_1AM could be detected under a variety of conditions (Figure 4, panel b).

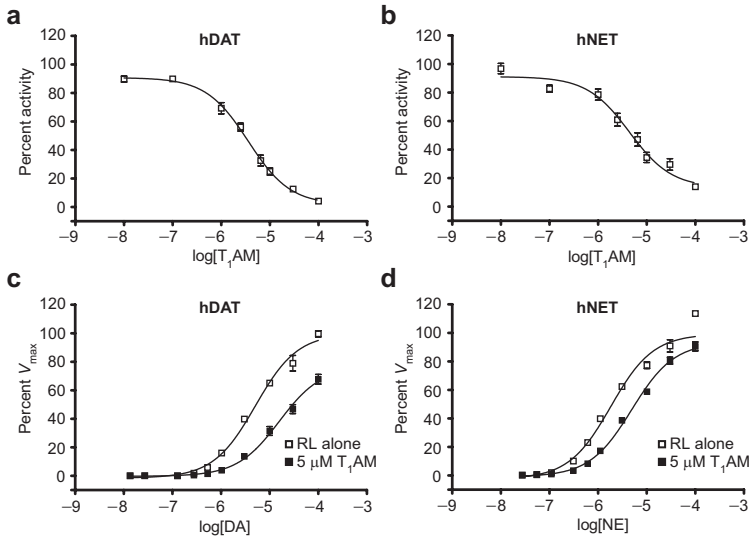

Figure 3. DAT and NET dose responses to T_1AM treatment. a, b) Data are shown as percent uptake of cells and incubated with radioligand alone. c, d) Data are shown as percent V_{max} of non-T_1AM-treated cells as calculated by Prism 4.0. The mean values reported are from three separate experiments performed in triplicate.

TABLE 1. T_1AM effects on neurotransmitter transport[a]

	T_1AM	0 μM T_1AM		5 μM T_1AM	
	IC_{50} (μM)	K_m (μM)	% V_{max}	K_m (μM)	% V_{max}
hDAT	3.59	5.11	100 ± 2.1	15.79	76.8 ± 2.9
hNET	4.41	1.83	100 ± 1.9	4.95	94.1 ± 1.4
rVMAT2	3.20	0.45	100 ± 3.1	1.10	74.0 ± 3.5

[a]The mean values reported are from three separate experiments performed in triplicate. The standard error of the log of the IC_{50} or K_m values was <1.5% for all values. % V_{max} values are presented ± SE.

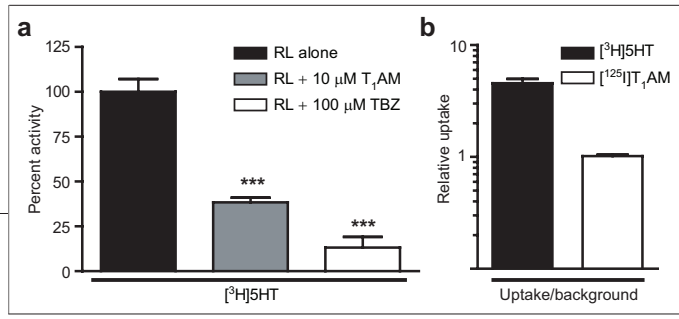

Figure 4. Effects of T_1AM on synaptic vesicle transport. a) Uptake is shown as percent activity normalized for the uptake of 20–30 nM [^{3}H]5HT alone. Statistical significance was determined using the paired two-tailed *t* test. * designates statistical significance, $p < 0.001$. b) Alternately, uptake of [^{3}H]5HT or [^{125}I]T_1AM is shown relative to uptake at time 0 min, in which membranes were held on ice and the reaction mixture was immediately quenched with cold SH buffer. Membranes treated with radioligand and 1% DMSO showed no significant difference in uptake compared with cells incubated with radioligand alone (data not shown). The mean values reported are from three separate experiments performed in duplicate.**

VMAT2 Inhibition by Thyronamines. To understand the interaction of T_1AM with vesicular monoamine transport, we assayed 5HT uptake in the presence of thyronamines (10 μM final concentration) into endosomal membranes prepared from rVMAT2-transfected human embryonic kidney (Hek) 293 cells. Consistent with the effects seen in synaptic vesicles, T_1AM significantly inhibited VMAT2 (by 80%) (Figure 5, panel a). In addition to T_1AM, all thyronamines except T_4AM inhibited VMAT2-mediated transport of [^{3}H]5HT to varying degrees. The potency we observed for T_1AM (IC_{50} of 3.2 μM) is comparable to several well-characterized VMAT2 inhibitors (*21, 22*) (Figure 6, panel a; Table 1). To address the possible recognition of T_1AM as a VMAT2 substrate, we also assayed the uptake of [^{125}I]T_1AM. No [^{125}I]T_1AM transport could be detected in membranes prepared from cells transfected with VMAT2 (Figure 5, panel b).

To determine the mode of T_1AM inhibition, kinetic analyses were performed using 20 nM to 100 μM [^{3}H]5HT with or without 5 μM T_1AM. It was found that 5 μM T_1AM resulted in a >2-fold increase in K_m (450 nM to 1.1 μM) and a 26% reduction in V_{max} (Figure 6, panel b; Table 1), suggesting that T_1AM inhibits VMAT2 by mixed inhibition, with both competitive and noncompetitive components.

Unlike the plasma membrane monoamine transporters, VMAT2 tolerates a variety of monoamine substrates (*21, 23*), and this tolerance translated into a broader sensitivity to thyronamines. All thyronamines except T_4AM demonstrate significant inhibition of VMAT2, and the potency of T_1AM (3.2 μM) is comparable to that of the trace amines and other VMAT2 inhibitors (*21, 22*). Notably, compounds with <2 iodines in their phenolic ring showed greater VMAT2 inhibition. Most significantly, our data revealed inhibition of neurotransmitter transport by T_1AM but no detectable uptake of radiolabeled T_1AM under a variety of conditions, by either the synaptic vesicle preparations or by membranes heterologously expressing VMAT2. This is surprising given the wide variety of substrates transported by VMAT2. Thus, T_1AM is the only known endogenous phenethylamine examined to date that inhibits VMAT2 but fails to be recognized as a substrate.

Implications of T_1AM Modulation of Monoamine Transport. Given T_1AM's ability to inhibit DAT-, NET-, and VMAT2-mediated transport, one might expect T_1AM inhibition of plasma membrane monoamine traffic to cause a significant accumulation of extracellular monoamines and other downstream effects (*24, 25*). Additionally, inhibition of vesicular monoamine transport would likely deplete neurotransmitter stores, reducing further monoamine transmission. This would produce effects in the CNS similar to but perhaps distinct from supraphysiological levels of trace amines and other neuromodulators such as cocaine and amphetamines. Although the observed potencies of thyronamine inhibition (low μM IC_{50}'s) are lower than that of the pharmacological reuptake inhibitors cocaine and mazindol (low–mid nM IC_{50}'s, K_i's) (*16, 17*), they are comparable to the potencies of the biogenic trace amines (low μM) and known VMAT2 inhibitor TBZ (<1 μM) (*20*).

Despite the differences in potencies, aminergic agents that regulate monoamine neurotransmission have been associated with thermoregulatory and cardiac effects (*26–28*). Although not as rapidly acting as T_1AM, the hypothermic induction by reserpine administration highlights such an effect (*29, 30*). Thus T_1AM effects on monoamine transport may contribute to the observed hypothermia and reduction in cardiac performance *in vivo*. There is evidence, however, that activation of $TAAR_1$ correlates with the hypothermic induction by T_1AM and related derivatives (*31*). This led to the supposition that TAAR-mediated activity may be the underlying molecular mechanism of the observed T_1AM pharmacology.

Evidence for three possibilities now exists. The first is that thyronamine activity with TAAR GPCRs alone leads to the observed pharmacological effects, and the neuromodulatory effects on monoamine transport have an as yet undiscovered effect on mammalian physiology. Second, the neuromodulatory activities of thyronamines are responsible for the hypothermic and cardiac effects. This could be consistent with the TAAR studies if the synthetic thyronamine derivatives tested for GPCR activity also inhibit mono-

amine transport with the same SAR. Lastly, it is possible that the thyronamine effects on monoamine transport and activity with $TAAR_1$ synergistically lead to the observed pharmacology. This would be possible if thyronamines simultaneously block monoamine transport, leading to higher extracellular levels of monoamines, and activate TAAR GPCRs to regulate neurotransmission through intracellular mechanisms (*32*). Such dual activity with transporters and GPCRs is common for neuromodulators and other pharmacological agents such as 3,4 methylenedioxymethamphetamine (*7, 33*). This synergistic mechanism is the most feasible given the complex nature of the effects seen coupled with the recent demonstration of the feedback between $TAAR_1$ and DAT activity (*34, 35*).

Beyond their pharmacological properties, thyronamines may also have unique physiological roles. T_0AM and T_1AM are present in a variety of mammalian tissues including the brain, as well as in circulation (*1*). The hypothesis of a physiological role for thyroid hormone derivatives is not new. Specifically, Dratman (*36*) hypothesized that thyroid hormone metabolites may be active neurotransmitters or neuromodulators. Dratman and colleagues later demonstrated that intravenous administration of radiolabeled thyroid hormone is efficiently localized to, concentrated, retained, and metabolized in synaptosomal components of rat brains, specifically regions associated with noradrenergic signaling (*37–40*). T_1AM was not specifically identified in these studies, but metabolites with complete phenolic ring deiodination, as in T_1AM, were not observed because of location of the [^{125}I] tag in the radioligand used. Despite the discovery of thyroid hormone localization and subsequent metabolism in the brain, determination of local concentrations of the hormone or its metabolites has, to our knowledge, not been quantified.

Evidence for specific active transport into synaptosomes of physiological levels of thyroid hormone, the presumed parent compound for thyronamines, raises the physiological potential of thyronamine effects on neurotransmitter transport above that of the other trace amines, for which no active transport mechanism at physiological levels has been described (*8*). Despite the unknown synaptosomal concentrations of thyroid hormone or its derivatives, it is widely appreciated that, given active localization mechanisms local concentrations can far exceed circulating levels (*41*). As an extension of the demonstrated trafficking and subsequent

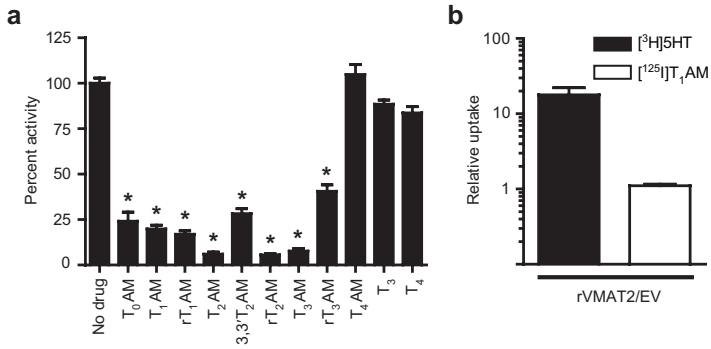

Figure 5. VMAT2 activity with thyronamines. a) Uptake is shown as percent activity normalized for 20 nM [^{3}H]5HT alone. Statistical significance was determined using the paired two-tailed *t* test. * designates statistical significance, $p < 0.001$. b) Additionally for [^{3}H]5HT and [^{125}I]T_1AM, uptake is shown as an uptake relative to wild type HEK293 cell membrane preparation. Membranes treated with radioligand and 1% DMSO showed no significant difference in uptake compared with cells incubated with radioligand alone (data not shown). The mean values reported are from three separate experiments performed in triplicate.

metabolism of thyroid hormone (*37–40*), it is feasible that local concentrations of thyronamines in specific brain regions may reach levels determined here to be sufficient to affect catecholamine transport. This supports the hypothesis of a specific physiological mechanism for thyroid hormone transport, metabolism, and neuromodulation by means of thyronamine activity in the mammalian CNS.

In summary, several thyronamines, including T_1AM, inhibit neuronal transport of DA and NE, as well as vesicular transport of biogenic amines. The vesicular activity of T_1AM is particularly interesting, because it is the only demonstrated instance of an endogenous monoamine inhibitor of VMAT2 that is itself not a substrate.

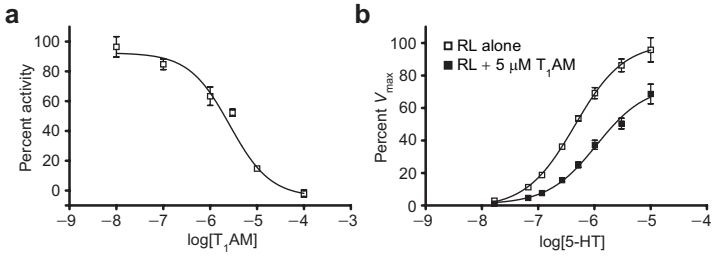

Figure 6. Mode of T_1AM inhibition. a) Data are shown as percent uptake of non-T_1AM-treated cells. b) Data are shown as percent V_{max} of untreated VMAT2 membranes, as calculated by Prism 4.0. The mean values reported are from three separate experiments performed in triplicate.

The now expanded role of T_1AM highlights its potentially novel mechanism of action as a physiologically relevant neuromodulator, consistent with the definition supplied by Berry (*8*) and raises the possibility of a unique pharmacology in the CNS distinct from the known activity of the monoamine neurotransmitters, any of the other endogenous trace amines, or known pharmacological agent, including cocaine and amphetamines. Though this demonstrated neuromodulatory activity of T_1AM can explain some of the previously observed pharmacology, these effects are highly complex and may have multiple underlying causes.

METHODS

Synaptosome Purification. Crude synaptosomes were prepared as previously described (*42*). Briefly, brains, avoiding myelin-rich areas, were removed from freshly decapitated Sprague-Dawley rats >10 weeks of age, placed into ice-cold homogenization buffer (320 mM sucrose, 4 mM HEPES-NaOH, pH 7.3 (10 mL per brain)) with protease inhibitors (Complete protease inhibitor tablet, Roche), 200 μM phenylmethanesulfonyl fluoride (PMSF), and 1 μg mL^{-1} Pepstatin A), homogenized in a glass-Teflon homogenizer at 900 rpm, and centrifuged for 10 min at 1000*g*. The supernatant was then centrifuged for 15 min at 12,000*g*. The pellet was resuspended in homogenization buffer (10 mL per brain) (avoiding the dark brown bottom of the pellet) and recentrifuged for 15 min at 13,000*g*. The resulting pellet was carefully resuspended in homogenization buffer to yield the crude synaptosomal preparation.

Synaptosomal Transport Assay. For each experiment, 75 μg of protein of the synaptosome preparation was added to 200 μL of KRH buffer (125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM HEPES, 5.6 mM glucose, pH 7.3) containing 100 μM sodium ascorbate (Sigma), 100 μM pargyline (Sigma), and either 40 nM 5-[1,2-^{3}H] HT ([^{3}H]serotonin, [^{3}H]5HT), 20 nM 3,4-[Ring-2,5,6-^{3}H]-dihydroxyphenethylamine ([^{3}H]dopamine, [^{3}H]DA) (Perkin-Elmer), 180 nM DL-[7-^{3}H(N)]-norepinephrine ([^{3}H]norepinephrine, [^{3}H]NE) (Perkin-Elmer), 40 nM L-[3,4,^{3}H]-glutamic acid ([^{3}H]Glu) (Perkin-Elmer), or several nonradioactive T_1AM concentrations (from 100 nM to 10 μM) doped with [^{125}I]T_1AM (*18*). To determine the effect of compounds on uptake of radioligands, either nonradioactive T_1AM (10 μM final concentration) (*31*) or mazindol (3 μM final concentration, Research Biochemicals International-Sigma) was added to the reaction solution, and the solution was incubated for 5 min at 37 °C. The transport reaction was terminated by rapid dilution with two 1.5-mL aliquots of cold KRH buffer and filtration through 0.2 μm HT 200 Tuffryn membranes (Pall Life Sciences). The filters were then dried, and bound radioactivity was measured by scintillation counting in CytoScint (ICN) scintillation fluid. Transport activity for each condition was measured in triplicate on at least three separate occasions. The protein concentration of each synaptosome preparation was measured using a Coomassie protein assay (Pierce) (*20*). In order to determine background measurements for each radioligand, membranes in the reaction mixture were held on ice, and the mixture was immediately quenched with cold KRH buffer and filtered as described above.

Cell Transfection. For transfection of hNET, hDAT (Susan G. Amara Lab, University of Pittsburgh, Pittsburgh, PA), hSERT (Randy D. Blakely Lab, Vanderbilt University, Nashville, TN), empty pBluescript SKII(−) (pBSK), and empty pcDNA3, HeLa cells were grown to confluency in 15-cm plates in DMEH21 containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Hyclone Laboratories) and 1% (v/v) pen/strep. Cells were seeded into 24-well plates at ~100,000 cells per well 16 h prior to transfection and were transfected using the vaccinia/T7 transient expression system, described previously (*19*).

For rVMAT2 expressing stable HEK293 cell lines, HEK293 cells were transfected with rVMAT2 pcDNA3 plasmid. HEK293 cells were grown to confluency in 15-cm plates in DMEH21 media containing 10% (v/v) heat-inactivated FBS and 1% (v/v) pen/strep; 16 h prior to transfection cells were split 1:2. Cells were transfected with lipofectamine (Invitrogen) using standard protocol, placed in G418 selective media 48 h after transfection, and propagated until harvest.

Membrane Preparation. HEK293 cells expressing VMAT2 or untransfected HEK293 cells from 15-cm plates were washed in prewarmed (37 °C) PBS, manually detached from the plate with a cell scraper (Fisherbrand, Fisher Scientific), collected by centrifugation (5 min at 1000*g*), and resuspended in cold SH buffer (0.32 M sucrose, 10 mM HEPES, pH 7.4) containing protease inhibitors (Complete protease inhibitor tablet, Roche). The cell suspension was then sonicated in an ice-cooled bath sonicator (Branson) at medium intensity twice for 20 s, and the cell debris was removed by sedimentation at 1300*g* for 5 min at 4 °C. All membranes were frozen and used within 60 d with no significant reduction in transport activity. The protein concentration of each membrane preparation was measured using a Coomassie protein assay.

DAT/NET/SERT Transport Assay. For each experiment, 24-well plates with HeLa cells transiently expressing either hNET, hDAT, hSERT, empty pBSK, or empty pcDNA3 (described above) were assayed as previously described (*19*). Briefly, cells were washed and preincubated with prewarmed KRTH (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM Tris, 10 mM HEPES, pH 7.4) containing 100 μM ascorbate (500 μL per well) for 30 min at 37 °C. Uptake was initiated by the addition of 55 nM [^{3}H]NE, 20 nM [^{3}H]DA, 30 nM [^{3}H]5HT, or several nonradioactive T_1AM concentrations (from 100 nM to 10 μM) doped with [^{125}I]T_1AM. To determine the effect of each of the thyronamines on transport of radioligands, thyronamines (*31*) dissolved in DMSO were added to the reaction to a final concentration of 10 μM, or mazindol to a final concentration of 3 μM, and transport was measured after incubation for 20 min at 37 °C. To determine the effects of T_1AM on K_m for dopamine, nonradioactive DA (Sigma) dissolved in 100 μM ascorbate was added to the reaction solution at concentrations ranging from 10 nM to 100 μM, with or without 5 μM nonradioactive T_1AM. Transport was measured after incubation for 20 min at 37 °C. To determine effects of T_1AM on the K_m for norepinephrine, nonradioactive NE (Sigma) dissolved in 100 μM ascorbate was added to the reaction solution at concentrations ranging from 100 nM to 100 μM, with or without 5 μM nonradioactive T_1AM. Transport was measured after incubation for 20 min at 37 °C. Following incubation, the transport reaction was terminated (cells were washed three times with 0.5 mL cold (4 °C) KRTH and solubilized in 1% (w/v) SDS), and radioactivity was measured by scintillation counting in CytoScint scintillation fluid (ICN). Transport activity for each condition was measured in triplicate on at least three separate occasions. Data given for relative uptake of hDAT and hNET show representative uptake for a single experiment done in triplicate. In order to determine back-

ground measurements for each ligand, membranes from cells transfected with empty pBSK vector were added to the reaction mixture and treated as described above.

Synaptic Vesicle Purification. Synaptic vesicles were prepared as previously described (*42*). Briefly, synaptic vesicles were released from synaptosomes in a crude synaptosome preparation. Synaptosomes were resuspended with 9 vol of cold water with protease inhibitors (Complete protease inhibitor tablet (Roche), 200 μM PMSF, and 1 μg mL^{-1} Pepstatin A) and lysed with a glass-Teflon homogenizer at 2000 rpm. The homogenate suspension was centrifuged for 20 min at 33,000*g*, and the resulting supernatant was removed and centrifuged for 2 h at 260,000*g*. The remaining supernatant was discarded, and the pellet was carefully resuspended to yield the crude synaptic vesicle preparation.

Synaptic Vesicle and VMAT2 Transport Assay. Membrane preparations were assayed as described previously (*20*). For each experiment, an aliquot of membranes (50–100 μg of protein), either frozen rVMAT2 membranes thawed on ice or fresh synaptic vesicle preparation, was added to 200 μL of SH buffer containing 4 mM KCl, 2 mM $MgSO_4$, 2.5 mM ATP, and either 20–30 nM [^{3}H]5HT or [^{125}I]T_1AM (from 100 nM to 10 μM). To determine the effect of each of the thyronamines on transport of radioligands, 10 μM final concentration of either thyronamines, 3,5,3′-triiodothyronine (thyroid hormone, T_3), thyroxine (T_4) (all dissolved in DMSO), or 100 μM TBZ was added to the reaction. To determine the concentration of compounds needed to inhibit serotonin transport by 50% (IC_{50}), T_1AM dissolved in DMSO was added to the reaction solution to final concentrations ranging from 10 nM to 100 μM. To determine effects of T_1AM on the K_m for 5HT, nonradioactive 5HT dissolved in SH buffer was added to the reaction solution at concentrations ranging from 10 nM to 10 μM, with or without 5 μM nonradioactive T_1AM. After incubation for 5 min at 37 °C, the transport reaction was terminated by rapid dilution with two 1.5-mL aliquots of cold SH buffer and filtration through 0.2 μm HT 200 Tuffryn membranes (Pall Life Sciences). The filters were then dried, and bound radioactivity was measured by scintillation counting in CytoScint scintillation fluid. Transport activity for each condition was measured in triplicate (duplicate for the synaptic vesicle assay) on at least three separate occasions (*20*). To determine background measurements for synaptic vesicle transport, purified vesicles were added to the reaction mixture, held on ice, and the mixture was immediately quenched with cold SH buffer and filtered as described above. To determine background measurements for recombinant VMAT2 transport, membranes from cells transfected with empty pcDNA3 vector were added to the reaction mixture, held on ice, and the mixture was immediately quenched with cold KRH buffer and treated as described above.

Statistical Analyses. Statistical analyses were performed with the GraphPad Prism version 4.00 software, with values expressed as means ± SEM. Statistical significance was determined using the paired two-tailed *t* test.

Acknowledgment: We thank B. Lin, E. Tan, D. Kadnikov, A. Ianculescu, and J. Paulson for critical review of the manuscript, the S. Amara Lab for the donation of the hNET and hDAT DNA constructs, and the R. Blakely Lab for the donation of the hSERT DNA construct. This work was supported by a fellowship from the Ford Foundation (A.S.), the PEW Latin America Fellowship (M.S.), a National Institute of Mental Health (NIMH) Postdoctoral Fellowship (R.S.), a grant from the NIMH (R.H.E.), and a grant from the National Institutes of Health (DK-52798, T.S.S.).

Supporting Information Available: This material is available free of charge *via* the Internet.

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