HORMONAL AND NEUROENDOCRINE REGULATION OF ENERGY BALANCE

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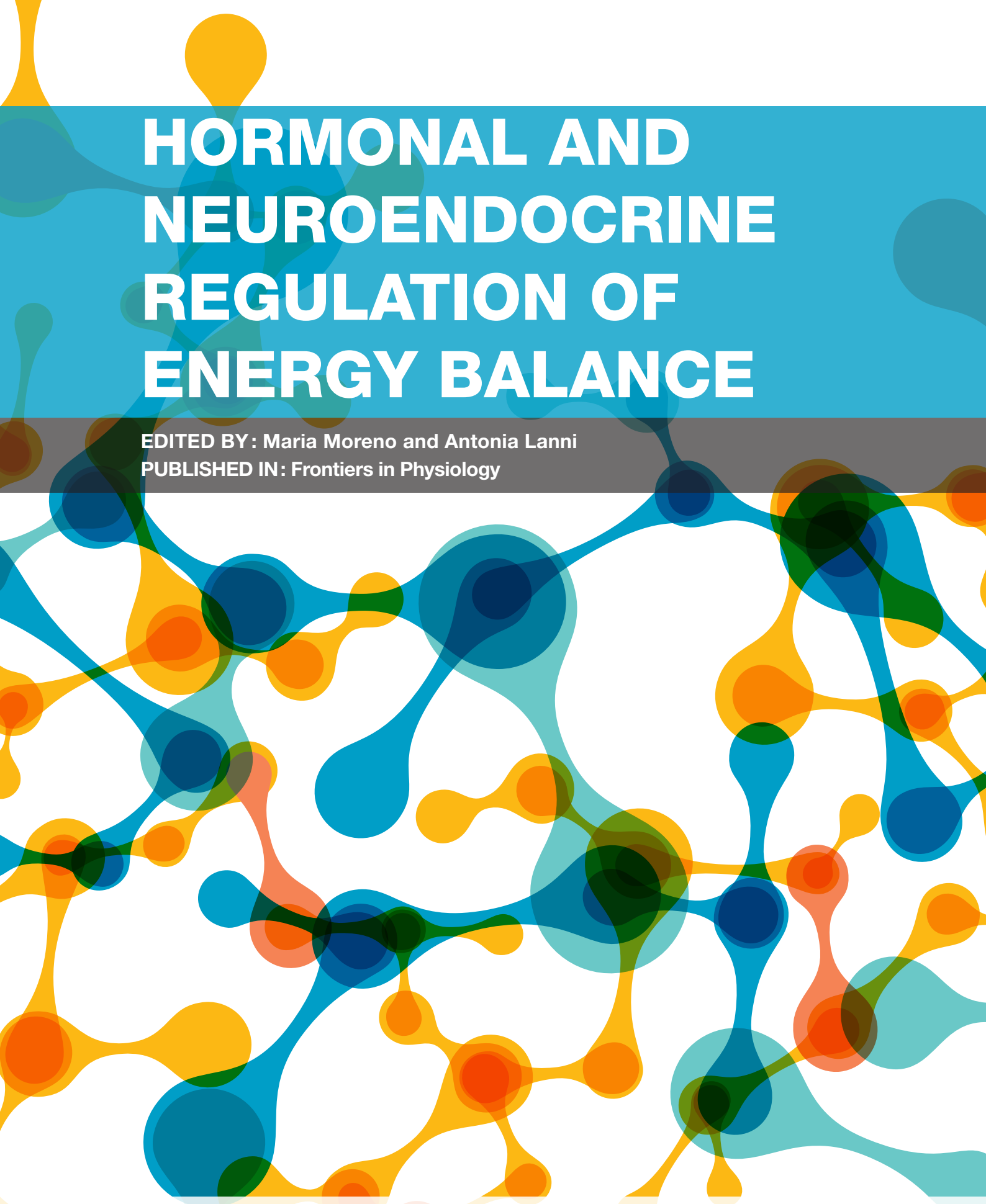

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HORMONAL AND NEUROENDOCRINE REGULATION OF ENERGY BALANCE

Topic Editors:
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Alteration in adequate energy balance maintenance results in serious disturbances such as obesity and its related metabolic disorders. In Mammals, energy balance is homeostatically controlled through hormonal and neuroendocrine systems which cooperation is based on cross-talk between central and peripheral signals. The hypothalamus as well as peripheral hormones among which adipokines from adipose tissue and thyroid hormones play a crucial role in energy homeostasis. Unraveling the physiological, cellular and molecular mechanisms through which hormonal and neuroendocrine systems regulate energy balance has been a long-standing challenge in biology and is now more necessary when considering the world-wide increasing prevalence of obesity. Indeed, recognizing and understanding the biochemical and nutrient signaling pathways contributing to the nervous and endocrine integration of physiological mechanisms involved in the normal and/or abnormal regulation of energy balance is fundamental also to the development of new, effective, and targeted treatments for obesity.

Recent studies have highlighted the role of hypothalamic pro-opiomelanocortin-expressing neurons in the regulation of energy homeostasis by controlling energy expenditure and food intake. This is accomplished through a precise balance of production and degradation of α-melanocyte-stimulating hormone, an anorexigenic neuropeptide which is degraded to an inactive form unable to inhibit food intake by the key enzyme prolyl carboxypeptidase (PRCP), thus suggesting that pharmacologic approaches targeting PRCP may provide a novel and effective option for the management of obesity and its associated metabolic disorders. Indeed, efforts have been made to generate potent, brain-penetrant PRCP inhibitors. Weight loss due to negative energy balance is a goal for obese subjects not always reachable by dietary caloric restriction or increased physical activity. Lipid-lowering therapies have been suggested to have potential benefits, however, the establishment of comprehensive therapeutic strategies is still awaited. Recently, it has been reported that thyroid hormone (TH)- derivatives such as 3,5-diiodothyronine and 3-iodothyronamine possess interesting biological activities, opening new perspectives in thyroid physiology and TH derivatives therapeutic usage.

Moreover, several studies, focusing on the interaction between thyroid hormone (TH), the autonomic nervous system and the liver, revealed an important role for the hypothalamus in the differential effects of TH on autonomic outflow to peripheral organs controlling energy balance.

This research topic aims to give a comprehensive and integrate view of the factors involved in the endocrine and neuroendocrine signaling in energy balance regulation to highlight their involvement into physiological processes and regulatory systems as well as their perturbation during pathological processes.

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EDITORIAL
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Editorial: Hormonal and Neuroendocrine Regulation of Energy Balance

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Keywords: energy balance, thyroid hormones, mitochondria, apelin, brown adipose tissue, melanocortin, catch up fat, uncoupling

The Editorial on the Research Topic

Hormonal and Neuroendocrine Regulation of Energy Balance

Energy balance is the relationship between energy intake and energy expenditure plus body energy storage. In mammals, the regulation of energy balance and body weight is a complex process wherein hormonal and neuroendocrine systems cooperate via cross-talk between central and peripheral signals. When energy intake exceeds energy expenditure, an increase in body mass, of which about 70% is body fat, occurs. The maintenance of this situation, over a given period of time, results in serious disturbances such as obesity and its related metabolic disorders. Quite recently, it is emerged that also the adaptation to a deficit in energy intake promotes hyperinsulemic states and the associated risks for later development of the metabolic syndrome by increasing the efficiency of energy utilization and storage as body's adipose tissue, a phenomenon known as catch-up fat. Thus, changing the food environment can push adiposity of a population upwards and result in increased prevalence of metabolic disorders. At cellular level, mitochondria have a fundamental role in regulating metabolic pathways, and tight control of mitochondrial functions and dynamics is critical to maintaining adequate energy balance. Consequently, it is no wonder that mitochondrial dysfunctions are implicated in metabolic disorders. Recent studies have highlighted the role of: (i) hypothalamic melanocortin system as an integrative center in the regulation of energy balance and (ii) adipokines, thyroid hormones (THs) and thyroid hormone-analogs as modulators of metabolic efficiency. A better understanding of physiological processes involved in the regulation of energy balance may well offer clues for developing strategies to target metabolic disorders. For this Research Topic, our initial aim was to give a comprehensive and integrate view of the factors involved in the endocrine and neuroendocrine signaling of energy balance regulation. We expected to highlight their involvement into physiological processes and regulatory systems as well as their perturbation during pathological processes. In this endeavor, 14 papers, including 1 Opinion Article, 11 review articles, and 2 original articles reveal an appealing, broad scope of subjects involving the aim of the Topic that exploit a variety of novel informations and potential applications. Insights into highly investigated areas, concerning new avenues for regulation of energy balance by TH-derivatives such as 3,5-diiodothyronine (3,5-T2) and 3-iodothyronamine (T1AM) and analogs like GC-1, are presented in a series of review articles which describe their involvement in the regulation of lipid and energy metabolism (Senese et al.), the role of 3,5-T2 on energy balance (Goglia) and the metabolic and neurological actions of T1AM (Zucchi et al.). These contributions highlight the huge progress made in identifying "novel" mechanisms of action of TH and their derivatives, opening new perspectives in the analysis of hormonal regulation and providing new targets for potential therapeutic interventions in metabolic and endocrine diseases.

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A review contribution by Silvestri et al. highlights the important role of proteomic approaches in the understanding of complex mechanisms such as those involved in thyroid control of metabolism. The authors of this contribution discuss new leads (i.e., target proteins and metabolic pathways) emerging in applying proteomics to the actions of 3,5-T2 and Triiodothyronine (T3) in conditions of altered energy metabolism in animal tissues having a central role in the control of energy balance. A relatively new area of research is covered by Kim et al. who review the role of the melanocortin system in the control of energy homeostasis. Many peripheral signals including hormones, nutrients such as glucose, lipids, and amino acids which regulate the melanocortin system by affecting the activity levels of different components of the system are discussed. Among the wide range of adipokines identified over the past years and implied into physiological and metabolic control of energy balance, Bertrand et al. review the various systemic effects of apelin on energy metabolism by addressing its mechanism of action. Due to its beneficial effects on both energy metabolism and insulin sensitivity, apelin is gaining an important physiological role and is being suggested as an interesting therapeutic anti-diabetic target. Newly, in a context where no safe and effective drugs are available to treat obesity, a recent research is focused on considering the possibility that stimulating Brown Adipose Tissue (BAT) might be effective in treating obesity and its associated metabolic disorders. In a review article, Poher et al. discuss these new emerging issues and underline the perspective that new alternatives, focusing on adipose tissue function, could potentially be of therapeutic relevance in the future. A review article by Obregon examines the difference between Brown, White and Brite/Beige adipocytes underlying their contribution to energy balance supporting the reactivation of BAT and the induction of Beige/Brite adipocytes in humans as a therapeutic option to fight obesity. Moreover, this contribution reviews the role played by THs on proliferation and differentiation of adipocytes mainly via regulation of gene expression. Lecoutre and Breton review the link between nutrient supply perturbations in the fetus or neonate and increased risk of adult-onset metabolic disorders. They discuss how maternal nutritional manipulations reprogram offspring's adipose tissue predisposing offspring to fat accumulation and, thus, to metabolic disorders. The impact of adiposity on energy balance is reported in an original research article by De Andrade et al. who, by using a rat model of semistarvation-refeeding, in which fat recover is driven by suppressed thermogenesis, conclude that the putative mechanisms leading to catch up-fat involve alterations in skeletal muscle contraction, fiber composition and local TH metabolism. Another original article reports the study performed by Wang et al. who detect metabolic alterations in pre-manifest and manifest Huntington's disease patients and suggest that in addition, and prior, to overt neuronal damage, Huntington's disease affects hormone secretion and energy regulation. These findings may shed light on Huntington's pathogenesis and provide new opportunities for biomarker development. Bioenergetic aspects related to mitochondrial activity also gave a prominent contribution to this Research Topic. The physiological role of mitochondrial uncoupling proteins (UCPs) in energy metabolism is reviewed by Busiello et al. who discuss the relevance of UCPs in determining mild uncoupling, in protecting from oxidative stress and in the amelioration of fatty acids oxidation. The alterations in mitochondrial efficiency as well as the impact of this parameter on metabolic homeostasis of skeletal muscle are reviewed in the contribution by Crescenzo et al. The reported evidences led to the conclusion that an increased mitochondrial efficiency precedes and may contribute to the development of high-fat induced insulin resistance. Emerging findings reveal an important role for mitochondrial dynamics in the regulation of energy balance. Putti et al. in a review article contribution highlight the impact of diet on mitochondrial dynamic behavior and function in liver and skeletal muscle as well as the involvement of mitochondrial fusion and fission processes in body energy balance regulation in response to the nutritional status. Overall, all the original articles and the review articles covering this topic, in all their diversity, contribute to clarify unanswered questions on the physiological processes involved in the regulation of energy balance and offer clues for developing strategies to target metabolic disorders. In each of the articles contributing to this topic, there is much more than it can be commented in this short introduction. We hope that the readers will go through the articles where they will find a lot of updated information, shedding light on hormone's actions, and metabolic pathways related to energy balance which are opening new promising perspectives in the field focalized in the Research Topic.

AUTHOR CONTRIBUTIONS

AL: contributions to the conception of the work; MM: contributions in drafting the work and to final approval of the version to be published.

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Caloric restriction induces energy-sparing alterations in skeletal muscle contraction, fiber composition and local thyroid hormone metabolism that persist during catch-up fat upon refeeding

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Weight regain after caloric restriction results in accelerated fat storage in adipose tissue. This catch-up fat phenomenon is postulated to result partly from suppressed skeletal muscle thermogenesis, but the underlying mechanisms are elusive. We investigated whether the reduced rate of skeletal muscle contraction-relaxation cycle that occurs after caloric restriction persists during weight recovery and could contribute to catch-up fat. Using a rat model of semistarvation-refeeding, in which fat recovery is driven by suppressed thermogenesis, we show that contraction and relaxation of leg muscles are slower after both semistarvation and refeeding. These effects are associated with (i) higher expression of muscle deiodinase type 3 (DIO3), which inactivates tri-iodothyronine (T_3), and lower expression of T_3-activating enzyme, deiodinase type 2 (DIO2), (ii) slower net formation of T_3 from its T_4 precursor in muscles, and (iii) accumulation of slow fibers at the expense of fast fibers. These semistarvation-induced changes persisted during recovery and correlated with impaired expression of transcription factors involved in slow-twitch muscle development. We conclude that diminished muscle thermogenesis following caloric restriction results from reduced muscle T_3 levels, alteration in muscle-specific transcription factors, and fast-to-slow fiber shift causing slower contractility. These energy-sparing effects persist during weight recovery and contribute to catch-up fat.

Keywords: thermogenesis, catch-up growth, weight regain, obesity, deiodinase, skeletal muscle, contraction-relaxation, rat

Abbreviations: CSA, cross-sectional area; CSQ, calsequestrin; DIO, iodothyronine deiodinase; FoxO1, forkhead box protein O1; MyHC, myosin heavy chain; PGC1-α, peroxisome proliferator-activated receptor gamma coactivator 1-α; $RT_{1/2}$, time for half-relaxation from the peak; SERCA, sarco-endoplasmic reticulum calcium ATPase; TTP, time to peak.

Introduction

Mammals adapt to food scarcity by increasing the efficiency of energy utilization. This has been documented in longitudinal studies of starvation and caloric restriction in humans as well as animals (Keys et al., 1950; Keesey and Powley, 2008) and is regarded as an outcome of adaptive processes to a deficit in energy intake. There is evidence that this ability persists, at least in part, during weight recovery upon refeeding (Boyle et al., 1978; Harris and Martin, 1984; Hill et al., 1984) and that the energy thus conserved is directed at accelerating specifically the recovery of the body's adipose tissue rather than that of other tissues in animals (Dulloo and Girardier, 1990; MacLean et al., 2004; Evans et al., 2005) and in humans (Dulloo and Jacquet, 1998; Weyer et al., 2000). This preference for "catch-up fat" is viewed as a result of a feedback loop between adipose tissue and thermogenesis (Dulloo and Jacquet, 2001); it probably evolved to optimize survival capacity during an ancestral life characterized by periodic food shortage. Nowadays, it is the key factor causing higher body fat gain relative to lean tissue and is commonly observed in adults after malnutrition, weight loss cures, anorexia nervosa and cancer-cachexia (Dulloo and Jacquet, 2001). This preferential catch-up fat phenomenon has also been linked to the hyperinsulinemic state of catch-up growth and the associated risks for later development of the metabolic syndrome (Crescenzo et al., 2003; Dulloo et al., 2006).

While the underlying mechanisms of the thrifty metabolism and catch-up fat are still poorly understood, studies using a rat model of semistarvation-refeeding in which catch-up fat is driven solely by suppressed thermogenesis (Dulloo and Girardier, 1990; Crescenzo et al., 2003) have implicated a role of impaired skeletal muscle metabolism for the following reasons: First, *in vivo* assessment of insulin-stimulated tissue glucose utilization during weight recovery indicates that glucose uptake is increased in white adipose tissue but reduced in skeletal muscle, suggesting a preference for storage via *de novo* lipogenesis and, perhaps, muscular insulin resistance (Cettour-Rose et al., 2005). Second, assessments of the mitochondrial subpopulations and oxidative capacities in hindlimb muscle suggest that the subsarcolemmal mitochondrial mass and its oxidative capacity, known to be diminished during caloric restriction, remained lower after refeeding (Crescenzo et al., 2006). Third, the circulating level of the main active thyroid hormone, tri-iodothyronine (T_3)—for which skeletal muscle is a major target—is diminished during caloric restriction and tends to remain low in refed animals during the energy-sparing phase of driving catch-up fat (Mainieri et al., 2006; Summermatter et al., 2008).

Given the evidence that this hypothyroid state is associated with diminished ATP turnover but increased mechanical efficiency (Lambert et al., 1951; Wiles et al., 1979; Johansson et al., 2003), we addressed the question of whether the slowed kinetics of skeletal muscle contraction-relaxation, known to occur during caloric restriction (Russell et al., 1983a,b, 1984a; Chan et al., 1986; Lewis et al., 1986; Pichard and Jeejeebhoy, 1988; Sieck et al., 1989; Nishio and Jeejeebhoy, 1991; Mijan de la Torre et al., 1993; Bissonnette et al., 1997), persist during weight recovery and might hence be linked to the thrifty metabolism driving catch-up fat.

Using our previously validated rat model of semistarvation-refeeding (Dulloo and Girardier, 1990; Crescenzo et al., 2003, 2006; Cettour-Rose et al., 2005; Mainieri et al., 2006; Summermatter et al., 2008), we found altered *in vivo* contractile properties of the hindlimb muscle not only at the end of a 2 week period of caloric restriction but also after 1 week of refeeding. These functional changes correlated with a fast-to-slow fiber transition, impaired expression of transcription factors involved in the development of slow-twitch muscles, namely calcineurin, peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1-α), and forkhead box protein O1 (FoxO1), and with a decreased rate of muscular T_3 synthesis linked to changes in the levels of the deiodinases DIO1, DIO2, and DIO3. The reduced T_3 synthesis, the slower rate of contraction-relaxation and the alterations in muscle size and shape persisted after refeeding.

Materials and Methods

Animals and Diet

A total of 40 male Sprague Dawley rats (Janvier Labs, le Genest-Saint-Isle, France) were used in this study. Rats were housed single in a temperature-controlled room (22 ± 1°C) with a 12-h light/12-h dark cycle. They were maintained on chow diet (Provimi-Kliba, Kaiseraugst, Switzerland) consisting, by energy, of 24% protein, 66% carbohydrates, and 10% fat, and had free access to water. Animals were maintained in accordance with the regulations and guidelines of the Department of Medicine (University of Fribourg) for the care and use of laboratory animals. All the experimental procedures involving animals were approved by ethical committees and veterinary offices of the States of Fribourg and Geneva.

Design of the Study

The experimental design was similar to that previously described in establishing a rat model for studying changes in energy expenditure that occur during accelerated fat recovery (i.e., catch-up fat) upon refeeding after growth arrest (Dulloo and Girardier, 1990; Crescenzo et al., 2003; Cettour-Rose et al., 2005; Mainieri et al., 2006). This approach allows investigating changes in thermogenesis specific for catch-up fat in the absence of confounding variables, such as food intake and differential rates of protein gain. In brief, 7-week-old rats were food-restricted at 50% of their spontaneous food intake for 2 weeks (semistarved rats, SS rats), after which they were refed (RF rats) the amount of chow corresponding to the spontaneous chow intake of control rats matched for weight at the onset of refeeding (control rats). Under these conditions, the refed animals showed a similar gain in lean mass, but an about 2-fold increase in body fat gain as compared to controls over a period of 2 weeks, due to 10–13% lower energy expenditure resulting from suppressed thermogenesis (Dulloo and Girardier, 1990; Crescenzo et al., 2003). The present study was conducted on SS rats and their fed controls (C_{SS}) at the end of 2 weeks of semistarvation, as well as on RF rats and their weight-matched controls (C_{RF}) after 1 week of refeeding.

Isometric Force Recordings

Isometric force recordings of the triceps surae muscle were performed *in situ* as previously described in mice (Dorchies et al., 2006, 2013; Reutenauer et al., 2008; Hibaoui et al., 2011; Nakae et al., 2012; Reutenauer-Patte et al., 2012) but were adapted to rats: a larger platform for accommodating larger animals on the force equipment setup was constructed, and the stimulus controller was modified to deliver stimulations up to 80 V instead of 40 V. In brief, electrical stimulations were delivered to the triceps surae of deeply anesthetized rats. Time to peak (TTP), time for half-relaxation from the peak (RT1/2), absolute and specific phasic twitch peak tension (*Pt*) and tetanic tension (*Po*), force-frequency relationship, and fatigability of muscle exposed to repetitive tetanic contractions were determined. Experimental details are provided in Supplementary Material.

Tissue Sampling

Rats were euthanized immediately after the force parameters were recorded. The triceps surae muscle from the stimulated leg was carefully dissected and weighed for the calculation of total muscle cross-sectional area (CSA) (see Supplementary Material). The gastrocnemius muscle was chosen for further analyses because it accounts for about 80% of the triceps mass, and therefore, it is the muscle that contributes the most to the analyzed force parameters. Gastrocnemius muscles are made up of fast-twitch fibers located in the external, glycolytic regions, and slow-twitch fibers that define an oxidative area in the deep region of the muscle. Gastrocnemius muscles from stimulated legs were embedded in Tissue-Tek® OCT™ compound, frozen in liquid nitrogen, and stored at −80°C until processed for histology. Gastrocnemius muscles from non-stimulated legs were also dissected, frozen in liquid nitrogen, and stored at −80°C for biochemical and molecular assays.

Histology and Immunofluorescent Labeling

Muscle Sections

Serial transverse sections (10 μm thick) were prepared from the largest aspect of the gastrocnemius muscle belly (approximately one-third of muscle length from proximal end) using an HM 560 M cryostat (Microm, Volketswil, Switzerland) and collected on SuperFrost Plus slides. Every slide held sections of either SS or RF samples together with their respective controls. Slides were stored at −80°C until processed.

Hematoxylin-eosin Staining

The muscle sections were stained with hematoxylin-eosin according to classical procedures (Dorchies et al., 2006, 2013; Nakae et al., 2012; Reutenauer-Patte et al., 2012) to assess overall muscle morphology. Activities of NADH-reductase, succinate dehydrogenase and cytochrome C oxidase were evaluated by enzyme histochemistry.

Immuno-labeling of Myosin Heavy Chains

The muscle sections were exposed to antibodies specific to myosin heavy chain isoforms 1, 2A, and 2B, which are markers of muscle fibers of type I, IIA and IIB, respectively, essentially as described (Dorchies et al., 2013). Technical details about the immuno-labeling procedures are provided in Supplementary Material.

Pictures

For each gastrocnemius, high-resolution pictures (final magnification 50X) were taken from the very same area of the deep, oxidative region with an AxioCam MRc digital camera (Zeiss, Feldbach, Switzerland) mounted on an inverted microscope (Zeiss Axiovert 200 M) equipped for epifluorescence. Visualization of green (AF_{488}), red (AF_{594}), and blue (Hoechst) fluorescence was achieved with specific filter sets (Zeiss). Black and white pictures were pseudo-colored and overlayed with the MetaMorph software (Visitron Systems, Puchheim, Germany) (Dorchies et al., 2013). Pictures were coded so that the investigators were blind to the group details.

Analysis of MyHC-positive Fibers

Fiber analysis was performed as described in detail elsewhere (Dorchies et al., 2013). The fibers labeled positively for MyHC 1-, 2A-, or 2B were assigned as fibers of type I, IIA, or IIB, respectively. The remaining fibers (negative for MyHC 1, 2A, and 2B) were designated as type IIX fibers. Fibers were counted using built-in tools of the ImageJ software (NIH, MD, USA) and expressed as the percentage of the total number of fibers (Dorchies et al., 2013). For each MyHC isoform, one picture (approximately 1900 fibers) taken from the deep area of each cross-section was analyzed. In order to minimize counting errors, the full analysis was performed by 2 investigators (PBMA and OPV) and the results were averaged.

Net T_3 Neogenesis Assay

The kinetics of thyroid hormone metabolism in skeletal muscle were assessed *in vitro* using the method of Kaplan and Utiger (Kaplan and Utiger, 1978) by incubating muscle homogenates in Tris buffer at 37°C. The T_3 neogenesis reaction was started by adding T_4 (1.3 μM) dissolved in PBS containing 0.25% BSA. Aliquots of the homogenate were removed after 0, 5, 10, 15, and 30 min, the reaction was stopped by adding 95% ethanol and samples were stored at 4°C until assayed for thyroid hormone content using a T_3/T_4 enzyme immunoassay kits (Diagnostic Systems Laboratories, Webster, TX, USA).

Semi-quantitative Determination of Muscle Proteins by Western Blot

The expression levels of gastrocnemius muscle proteins were determined by Western blots according to standard procedures described in detail elsewhere (Reutenauer-Patte et al., 2012; Dorchies et al., 2013). In brief, gastrocnemius muscles were pulverized in liquid nitrogen-cooled mortars and extracts were prepared in Guba-Straub buffer containing 1% Triton X-100 and 0.1% 2-mercaptoethanol. Protein content in the extracts was assayed and adjusted to 3 μg/μL with 2x reducing Laemmli buffer. Thirty μg of the extracts were resolved by SDS-PAGE before being transferred to nitrocellulose membranes. Equal loading and transfer efficiency were verified by staining with Ponceau Red. The membranes were then exposed to primary antibodies specific for selected muscle proteins, to appropriate

HRP-conjugated secondary antibodies, and finally to an ECL reagent. Signals were captured on X-ray films and analyzed by densitometry using the ImageJ software. Technical tips used for ensuring intra-gel and inter-gel comparison as well as semi-quantitative analysis of the signals are described in Supplementary Material.

The primary antibodies used were directed against calcineurin (catalytic subunit), calsequestrin type 1 (CSQ1) and type 2 (CSQ2), DIO1, DIO2, DIO3, FoxO1, parvalbumin, PGC-1α, and the sarco-endoplasmic reticulum Ca^{2+}-ATPases SERCA1 and SERCA2. Technical details about these antibodies are given in **Table 1**. Validation of the antibodies used for detecting and quantifying DIO1, DIO2, and DIO3 is shown in Supplementary Material.

SERCA Activity

The enzymatic activity of the SERCA pumps was measured by an NADH-coupled assay (Warren et al., 1974; Strosova et al., 2011; Viskupicova et al., 2015). Muscle microsomes were prepared from the gastrocnemius muscle of semistarved, refed and controls rats according to Warren et al. (1974), modified by Karlovska et al. (2006). Because of their skeletal muscle origin, microsomes comprised mostly sarcoplasmic reticulum (SR) vesicles, and 70–80% of total protein content was made up of SERCA protein (Lenoir et al., 2002). The sarcoplasmic reticulum-rich vesicles (final concentration 12.5 μg protein/cuvette) were added to the assay mixture (40 mM Hepes pH 7.2, 0.1 M KCl, 5.1 mM $MgSO_4$, 2.1 mM ATP, 0.52 mM phosphoenolpyruvate, 1 mM EGTA, 0.15 mM NADH, 7.5 IU of pyruvate kinase, 18 IU of lactate dehydrogenase, pre-incubated at 37°C for 10 min). The reaction was started by addition of $CaCl_2$ (final concentration 1 mM). The reaction rate was determined by measuring the decrease in NADH absorbance at 340 nm, at 37°C. Specific SERCA activity (IU/mg; i.e., μmol substrate/min/mg of protein) was calculated using the following equation:

$$\frac{IU}{mg} = \frac{\Delta A_{340nm} \cdot V}{6.22 \cdot m},$$

where ΔA_{340nm} represents a change in absorbance at 340 nm per min, V is the volume of the reaction mixture (mL), $6.22.10^3$ $L.mol^{-1}.cm^{-1}$ is the absorption coefficient for NADH, and m represents the total amount of protein in the reaction mixture (mg).

Data Analysis and Statistics

All data are presented as means ± SEM (n = 8–10 rats per group). Direct comparisons between the two experimental groups and their respective control groups, i.e., SS vs. C_{SS} or RF vs. C_{RF}, were performed using Mann-Whitney tests, considering that the sample size was too small for assuming Gaussian distribution of the values. The level of statistical significance was set at $P \leq 0.05$. Statistical analyses were performed and graphs were constructed using GraphPad Prism version 6.01 (GraphPad software Inc., La Jolla, CA, USA).

Results

Effects of Semistarvation-refeeding on Rat Body Weight and Triceps Size

In accordance with our earlier observations with this model (Crescenzo et al., 2003; Cettour-Rose et al., 2005; Mainieri et al., 2006), caloric restriction led to growth arrest, whereas isocaloric refeeding led to weight gain at a slightly higher rate than in the control rats. As shown in **Table 2**, at the end of the 2-week period of caloric restriction, body weights of the semistarved rats and their controls were similar (SS vs. Css: 222 vs. 224 g), and after 1 week of isocaloric refeeding, body weights tended to be higher in the refed than in the control animals (RF vs. CRF: 296 vs. 281; non-significant). The data on the triceps surae muscles are also shown in **Table 2**. After semistarvation, the triceps were 20.4% bigger than the control ones. As the rats' body weight remained

TABLE 1 | Primary antibodies used for analysis of protein levels.

Target protein	Host (clonality[a])	Dilution (competitor[b])	Source	Cat. number
Calcineurin	Rabbit (P)	1:1000 (BSA)	CST[c]	#2614
Calsequestrin 1	Mouse (M)	1:3000 (BSA)	Thermo scientific	MA3-913
Calsequestrin 2	Rabbit (P)	1:2000 (BSA)	Thermo scientific	PA1-913
DIO1	Rabbit (P)	1:1000 (BSA)	Proteintech Europe	11790-1-AP
DIO2	Rabbit (P)	1:200 (milk)	SCB[d]	sc-98716
DIO3	Rabbit (P)	1:1000 (milk)	Novus biologicals	NBP1-05767
FoxO1	Rabbit (M)	1:1000 (BSA)	CST	#2880
Parvalbumin	Mouse (M)	1:2000 (BSA)	Merck-Millipore	MAB1572
PGC-1α	Rabbit (P)	1:1000 (BSA)	Novus biologicals	NBP1-04676
SERCA1	Mouse (M)	1:2000 (BSA)	Thermo scientific	MA3-911
SERCA2	Mouse (M)	1:1000 (BSA)	Abcam	ab3625

[a] *M, monoclonal; P, polyclonal.*
[b] *Refers to the blocking reagent used (5%) during the incubation step with the primary antibodies.*
[c] *Cell Signaling Technology.*
[d] *Santa Cruz Biotechnology.*

TABLE 2 | Effects of the dietary interventions on body weight and triceps size.

	C_{SS}	SS	C_{RF}	RF
RAT				
Body weight (g)	224 ± 4	222 ± 2^{ns}	281 ± 4	296 ± 4^{ns}
TRICEPS				
Absolute mass (mg)	1467 ± 32	1765 ± 33***	1968 ± 36	2217 ± 75**
Specific mass (mg/g)	6.65 ± 0.17	7.97 ± 0.11***	7.02 ± 0.09	7.50 ± 0.23^{ns}
Optimal length (mm)	30.7 ± 0.4	30.5 ± 0.6^{ns}	33.8 ± 0.6	36.0 ± 0.7*
CSA (mm^2)	45.2 ± 0.9	54.7 ± 1.3***	55.1 ± 1.2	60.5 ± 1.4*

$^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; ^{ns}, not significant, comparing SS or RF to their related control. Mann-Whitney test, n = 10.

unchanged, this translated into a +19.9% higher triceps specific mass. This increase was due do an enlargement of the muscles, perpendicular to the long axis, as demonstrated by the +21.2% increase in the triceps CSA.

The alterations in triceps mass and shape partly persisted after refeeding for 1 week: the absolute mass, and CSA in the refed RF group remained significantly higher than in the C_{RF} control group. Of note, the optimal length required for the triceps to generate the maximum force was increased (+6.7%) after refeeding, underscoring an effect of semistarvation-refeeding on muscle plasticity and visco-elastic properties.

Effects of Semistarvation-refeeding on Contractile Properties of the Triceps Surae

Phasic twitches were recorded after electrical stimulation of the triceps surae from rats after semistarvation for 2 weeks and after refeeding for 1 week. As illustrated in **Figure 1A** for the C_{RF} and RF groups, the electrical stimulation of the triceps triggered its contraction (rising phase), followed by its complete relaxation (descending phase), allowing to determine phasic tensions (**Figures 1B,C**), and kinetics of contraction and relaxation (**Figure 1D**). Then, the stimulation frequency was gradually increased from 10 to 100 Hz and the strongest contraction was used for determining the tetanic tension (**Figures 1B,C**).

Phasic and Tetanic Tensions

Semistarvation caused the muscle to generate higher phasic (*Po*) and tetanic (*Pt*) absolute tensions (**Figure 1B**). These effects on force development persisted after refeeding. Importantly, the augmentation of the forces generated by the muscles was not seen any longer after the absolute tensions were transformed into specific tensions (**Figure 1C**), i.e., after taking into account muscle cross sectional area CSA (**Table 2**). This revealed that the increase in force was solely due to muscle enlargement and not to a higher performance per surface unit.

Kinetics of Contraction and Relaxation

The dietary interventions caused a marked slowing of the kinetics of contraction and relaxation. This is demonstrated by the increased time required for reaching maximal contraction from baseline (time to peak; TTP) and by the increased time for achieving 50% relaxation from the peak values (time for half-relaxation from the peak; $RT_{1/2}$) (**Figures 1A,D**). This slowing, which was evident after semistarvation, became even more pronounced after refeeding. Also, the effects on relaxation were much more pronounced ($RT_{1/2}$: SS vs. C_{SS}: +4.12 ms, $P \leq 0.001$; RF vs. C_{RF}: +4.75 ms, $P \leq 0.01$) than those on contraction (TTP: SS vs. C_{SS}: +1.45 ms, $P = 0.251$; RF vs. C_{RF}: +2.01 ms, $P \leq 0.05$).

Force-frequency Relationship

Force-frequency relationships were determined. The average curves for the C_{SS} and SS groups are shown in **Figure 1E**. The muscle of the SS rats developed a markedly augmented relative force up to 50 Hz when compared to controls, resulting in a clear leftward shift of the force-frequency curve. The control rats required a stimulation frequency of ∼38 Hz to develop 50% of the maximum force, whereas the SS rats required only ∼30 Hz to reach the same relative force. This is in accordance with the decreased kinetics of contraction and relaxation (**Figure 1D**). After 1 week of refeeding, the leftward shift of the force-frequency relationship became non-significant (not shown).

Fatigability of Muscle Exposed to Repetitive Tetanic Contractions

We assessed muscle fatigue via a protocol in which muscles are challenged by repetitive tetanizations (Dorchies et al., 2006, 2013; Reutenauer et al., 2008; Reutenauer-Patte et al., 2012). Muscle fatigue was determined at the end of the semistarvation period and after 1 week of refeeding. No difference was found between the groups (**Figure 1F**; SS vs. C_{SS} or RF vs. C_{RF}).

Effects of Semistarvation-refeeding on Muscle Structure and Fiber Type Composition

No alterations of muscle fiber morphology were found as assessed by hematoxylin-eosin staining of gastrocnemius muscle cross-sections. In particular, no changes in fiber size and in cytochrome C oxidase, lactate dehydrogenase, and succinate dehydrogenase activities were found (data not shown).

In order to elucidate the reasons for the slower kinetics of contraction and relaxation, we performed exhaustive fiber typing by labeling the MyHC with isoform-specific antibodies. **Figure 2A** illustrates the labeling of MyHC type 1. We found that the fibers positive for MyHC 1 were more abundant in the oxidative regions of gastrocnemius muscles after semistarvation, compared to the C_{SS} control rats (+31.5%; $P \leq 0.01$). MyHC 1 is characteristic for the type I, slow-twitch fibers (**Figure 2B**). An augmented proportion of type I fibers partly persisted in the gastrocnemius of RF rats (+13.4%), without reaching statistical significance. The accumulation of the type I fibers occurred not only at the expense of the type IIA fibers but also of type IIB fibers and to a lesser extent, of type IIX fibers, demonstrating an overall conversion of fast fibers into slower fibers. As a result, the ratio of type I to IIA fibers was moderately increased (+ ∼51%) but the ratio of type I to IIB fibers was more than doubled (+127%) in the gastrocnemius from SS rats and remained ∼78% higher in the RF rats, compared to their respective controls (**Figure 2C**). The fiber typing data supported our findings on the slower contraction and relaxation kinetics.

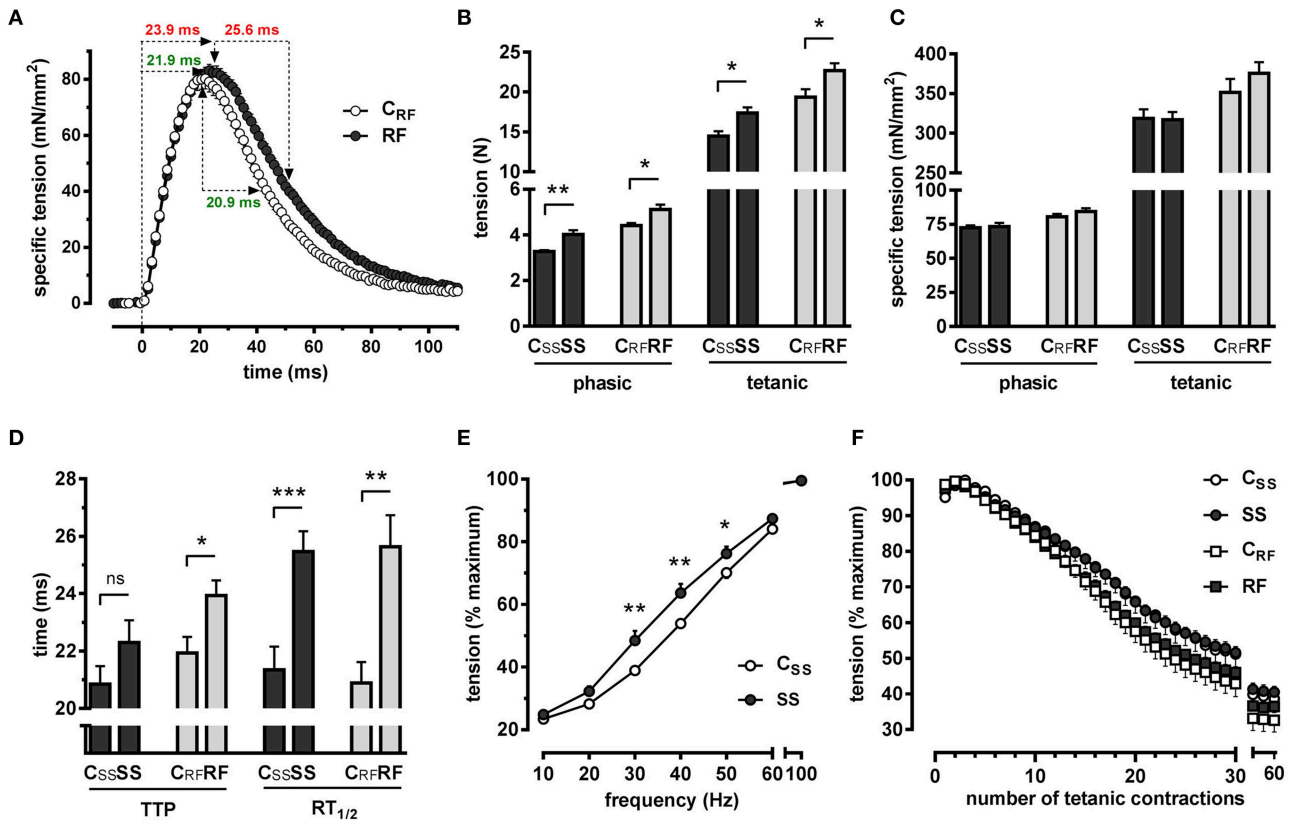

FIGURE 1 | Electrically-evoked isometric contractions of the triceps surae were recorded *in situ* in semistarved and refed rats and in their respective controls. Force characteristics and kinetics of contraction and relaxation were determined. **(A)** The average phasic traces of refed rats and their controls are depicted, illustrating muscle contraction followed by complete relaxation. The kinetics values (time to peak and time for half-relaxation from the peak) are highlighted in green (control) and in red (refed). **(B)** Absolute phasic and tetanic tensions, **(C)** specific phasic and tetanic tensions, **(D)** kinetics of contraction and relaxation, **(E)** force-frequency relationships, and **(F)** muscle fatigue were determined as described in the text. SS, semistarved rats; C_{SS}, control of semistarved rats; RF, refed rats; C_{RF}, control of refed rats. $n = 8$–10; Mann-Whitney test; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; ^{ns}, not significant, comparing SS or RF to their respective control.

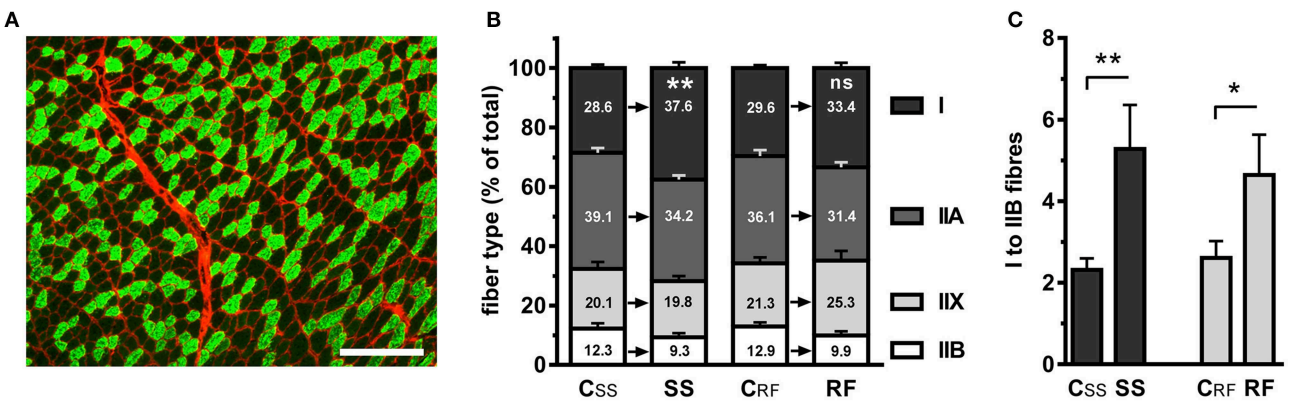

FIGURE 2 | The distribution of fiber types in gastrocnemius muscles of semistarved and refed rats and their respective controls was determined based on the expression of myosin heavy chain (MyHC) isoforms. Muscle sections were incubated with monoclonal antibodies to specific MyHC isoforms and fluorescent secondary antibodies. The extracellular matrix was stained with a fluorescent lectin in order to help demarcating the muscle fibers. **(A)** Example of MyHC 1 labeling in the gastrocnemius of a control rat, revealing the slow-twitch, type I fibers (green) and the counterstained extracellular matrix (red). A similar procedure was employed for staining type IIA and type IIB fibers using anti-MyHC 2A and anti-MyHC 2B, respectively. The positive fibers were expressed as the percentage of the total number of fibers. Approximately 1900 fibers were counted on every section. Bar: 100 μm. **(B)** Distribution of fiber types in the experimental groups. The fibers that were not of type I, IIA, or IIB were identified as type IIX. **(C)** Ratio of type I to type IIB fibers. SS, semistarved rats; C_{SS}, control of semistarved rats; RF, refed rats; C_{RF}, control of refed rats. $n = 8$–10; Mann-Whitney test; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; ^{ns}, not significant, comparing SS or RF to their respective control.

TABLE 3 | Effects of the dietary interventions on SERCA activity in rat gastrocnemius.

	C_{SS}	SS	C_{RF}	RF
Activity (IU/mg prot.)	3.39 ± 0.45	3.80 ± 0.34^{ns}	3.62 ± 0.34	4.29 ± 0.62^{ns}

^{ns}, not significant, comparing SS or RF to their related control. Mann-Whitney test, n = 10.

Effects of Semistarvation-refeeding on Expression Levels of Slow-twitch Muscle Markers

The kinetics of contraction and relaxation are partly controlled by the nature and activity of the SERCA pumps. SERCA1 is expressed in fast-twitch fibers (IIB, IIX, IIA), whereas SERCA2 is specific for slow-twitch fibers (type I). SERCA activity was measured in microsomes prepared from the gastrocnemius of the rats. No difference in SERCA activity was found after semistarvation (SS vs. C_{SS}) or refeeding (RF vs. C_{RF}), although there was a trend toward enhanced activity for both (**Table 3**).

We investigated whether the slower kinetics of contraction and relaxation were accompanied by alterations in the expression of selected markers of slow *vs.* fast fibers in rat gastrocnemius (**Figure 3**). The expression levels of SERCA pumps and of the calcium-buffering proteins, CSQ1, CSQ2, and parvalbumin were normalized to that of total MyHC (**Figure 3A**). The level of SERCA1 (specific for fast muscle fibers) tended to increase after semistarvation and refeeding, although the inter group differences were not significant (**Figure 3B**). SERCA2, which is specific for type I fibers, found predominantly in slow muscles, was significantly decreased after semistarvation; an effect that was completely reversed by refeeding (**Figure 3C**). As shown in **Figures 3D–F**, semistarvation and refeeding had no impact on the abundance of the fast muscle-specific markers CSQ1 and parvalbumin or on the slow muscle-specific marker CSQ2.

The characterization of muscle markers was extended to several transcription factors known to control the acquisition of slow-twitch vs. fast-twitch phenotype, namely calcineurin, PGC1-α, and FoxO1. We found that after semistarvation, the levels of calcineurin were significantly increased (+26%) and those of FoxO1 were more than doubled (+141%), whereas PGC1-α amounts were decreased (−16%) (**Figures 3G–I**). After refeeding, only the levels of FoxO1 remained elevated (+60%) compared to the control group.

Effects of Semistarvation-refeeding on Muscular Net T_3 Neogenesis and Deiodinase Levels

The rate of net T_3 neogenesis was assessed *ex vivo* in extracts of gastrocnemius muscles from rats after semistarvation and after refeeding. The *de novo* net T_3 synthesis was found to be significantly lower in muscles of SS rats (−18%), as well as in muscles of RF rats (−14%) relative to their controls (**Table 4**).

Because net T_3 neogenesis is the result of T_3 synthesis from T_4 (through deiodination catalyzed by DIO1 and/or DIO2) and T_3 degradation by DIO3, we analyzed the expression of the deiodinases DIO1, DIO2 and DIO3 in gastrocnemius muscles (**Figure 4**). As shown in Supplementary Material, we perform an extensive validation of the antibodies used for Western-blot analysis of DIO expression in rat skeletal muscle. We found that DIO1 and DIO3 were more abundant (+38% and +71%, respectively), whereas DIO2 was less abundant (−16%) in gastrocnemius muscle after semistarvation (**Figures 4A–C**). These differences were all highly significant and partly persisted after refeeding (+28%, −13%, and +25% for DIO1, DIO2, and DIO3, respectively). As a consequence of these differential changes in DIO levels, the relative DIO1-to-DIO3 ratio was not significantly affected by semistarvation (**Figure 4D**), whereas the DIO2-to-DIO3 ratio was strongly diminished to ∼48% of the control value and was only partly corrected to normal values after refeeding (**Figure 4E**). These alterations in DIO levels likely accounted for the diminished local net T_3 neogenesis in gastrocnemius muscles.

Discussion

It has long been known that patients clinically diagnosed as being malnourished have a reduced rate of skeletal muscle contraction-relaxation (Lopes et al., 1982; Russell et al., 1983b; Chan et al., 1986; Pichard and Jeejeebhoy, 1988; Nishio and Jeejeebhoy, 1991) and that these changes in contractile properties can be induced by caloric restriction or prolonged starvation in humans (Russell et al., 1983a) and rats (Russell et al., 1984a). The focus of these past studies has been in relation to the mechanisms by which malnutrition leads to impaired skeletal muscle mechanical functions and physical disability (Lopes et al., 1982; Pichard and Jeejeebhoy, 1988). However, a lower speed of the contraction–relaxation cycle, by virtue of the associated reductions in ATP turnover (Pichard et al., 1988; Mijan de la Torre et al., 1993), and hence increased muscle mechanical efficiency, can also contribute to the adaptive reduction in thermogenesis that occurs in response to caloric restriction. We report here, that during caloric restriction and refeeding, hindlimb muscles showed delayed contraction-relaxation kinetics, supported by an increased proportion of slow at the expense of fast muscle fibers. From a molecular point of view, we demonstrated that semistarvation-refeeding caused major changes in the muscular expression of transcription factors that control slow *vs.* fast phenotype, and of the deiodinases DIO1, DIO2, and DIO3, which is in agreement with decreased availability of muscular T_3, the main active thyroid hormone. Collectively, altered thyroid hormone metabolism, fiber type composition and contractile properties constitute mechanisms by which diminished skeletal muscle thermogenesis could contribute to energy conservation during weight loss and weight recovery, and hence contribute to the thrifty metabolism that accelerates fat recovery or catch-up fat during refeeding.

Consequences of Semistarvation-refeeding on Muscle Contraction, Fiber Type switch, and Calcium Handling

Slower Contraction-relaxation Kinetics: Roles of Muscle Structure and Fiber Type

The prolonged contraction-relaxation times, assessed as time to peak and time for half-relaxation from the peak in response to

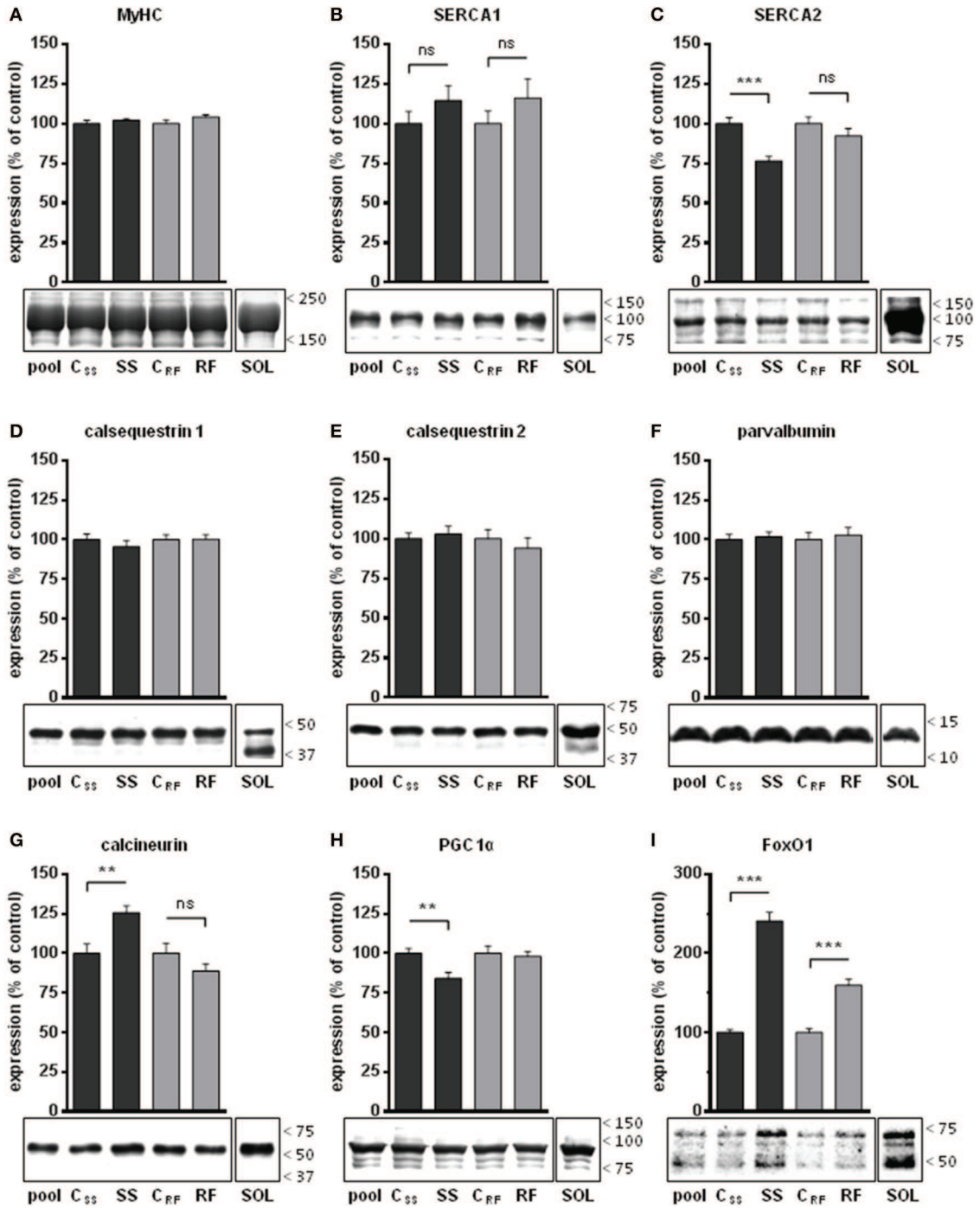

FIGURE 3 | Expression levels of selected proteins in gastrocnemius extracts from semistarved and refed rats and their respective controls were determined by Western blots. (A) Amount of myosin heavy chains (MyHC) were determined by Coomassie staining in 3 independents experiments. MyHC were similarly abundant in all 4 groups and were thus validated as internal controls for the quantification of the other muscle markers under study. Selected proteins that are well known markers of Ca^{2+} handling in slow-twitch or fast-twitch fibers were analyzed: **(B)** SERCA1, **(C)** SERCA2, **(D)** calsequestrin 1, **(E)** calsequestrin 2, and **(F)** parvalbumin. The abundance of transcription factors that control the development of slow-twitch muscles either positively or negatively were also determined: **(G)** calcineurin, **(H)** PGC1-α (the most intense band at the expected size for the full-length protein was analyzed; the smaller molecular weight bands likely to be degradation products were excluded), and **(I)** FoxO1 (all bands, likely representing native, methylated, and acetylated forms, were quantified). The signals **(B–I)** were corrected for their MyHC content and normalized to the signal of a pool sample (a mixture of aliquots of all extracts) loaded on the gels for the purpose of intra-gel and inter-gel comparison. The signals from an extract of soleus muscle (a slow-twitch muscle), referred to as SOL, are shown for comparison. They were acquired under the same exposure condition as the experimental groups. The position of the molecular weight markers (kDa) is shown on the right side of the blots. SS, semistarved rats; C_{SS}, control of semistarved rats; RF, refed rats; C_{RF}, control of refed rats. $n = 10$; Mann-Whitney test; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; ^{ns}, not significant, comparing SS or RF to their respective control.

electrical stimulation, during caloric restriction and refeeding, were accompanied by changes in muscle fiber type. Specifically, the semistarved and refed groups displayed a slower phenotype than their respective controls. In terms of fuel economy, this would be advantageous as there is evidence that slow-twitch muscles use less ATP per unit of isometric tension than fast-twitch muscles (Wendt and Gibbs, 1973; Crow and Kushmerick, 1982; Henriksson, 1990). Whether this shift from fast-to-slow fiber composition contributes to the prolonged contraction-relaxation times is, however, uncertain. Although, a greater atrophy of fast fibers than slow fibers has often been reported to correlate with delayed contraction-relaxation times in muscle from malnourished rats and humans (Essen et al., 1981; Lopes et al., 1982; Russell et al., 1984a,b; Henriksson, 1990), several studies failed to demonstrate a relationship between the magnitude of changes in relaxation rate and the proportion of fiber types in muscle (Kelsen et al., 1985; Lewis et al., 1986; Sieck et al., 1989), or showed that the alterations identified as the cause of the slowing occurred in both, type I and type II fibers (Nishio and Jeejeebhoy, 1991).

TABLE 4 | Effects of the dietary interventions on net T_3 neogenesis in rat gastrocnemius muscle.

	C_{SS}	SS	C_{RF}	RF
T_3 (pmol/mg prot./min)	0.51 ± 0.02	0.42 ± 0.01***	0.43 ± 0.02	0.37 ± 0.01**

$^{**}P \leq 0.01$; $^{***}P \leq 0.001$, comparing SS or RF to their related control. Mann-Whitney test, $n = 10$.

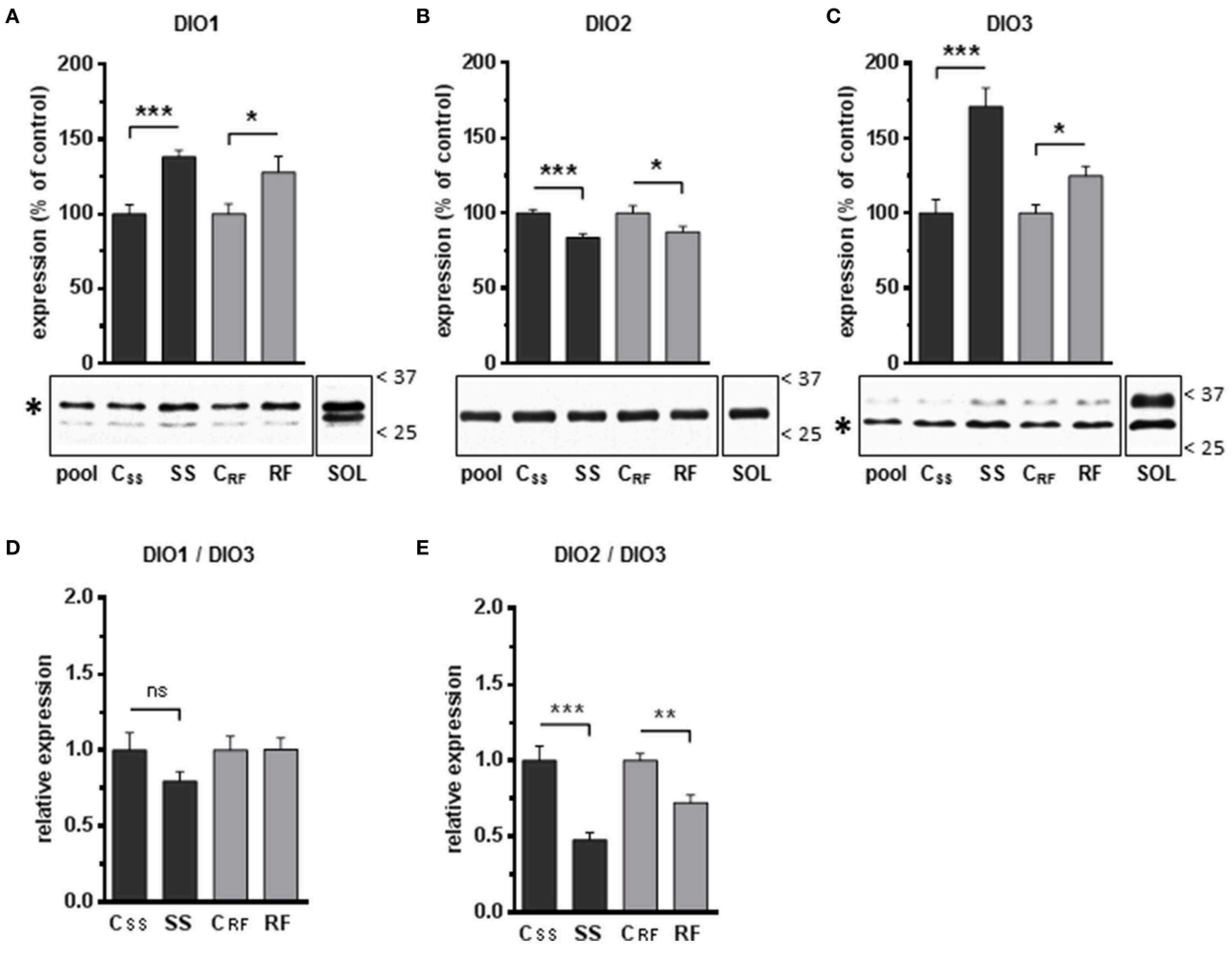

FIGURE 4 | Expression levels of the iodothyronine deiodinases DIO1, DIO2, and DIO3 in gastrocnemius extracts from semistarved and refed rats and their respective controls were determined by Western blots. The graphs show the expression levels of **(A)** DIO1, **(B)** DIO2, and **(C)** DIO3. The procedures for the normalization of the signals as well as the definition of the Pool and SOL extracts are described in the Legend to **Figure 3**. The position of the molecular weight markers (kDa) is shown on the right side of the blots. Asterisks indicate the bands that were identified as DIO1 and DIO3 proteins based on antibody validation experiments (see Supplementary Material for details). The faint extra bands likely represent non-specific labeling of other proteins. The ratios of DIO1-to-DIO3 **(D)**, and DIO2-to-DIO3 **(E)**, were calculated. SS, semistarved rats; C_{SS}, control of semistarved rats; RF, refed rats; C_{RF}, control of refed rats. $n = 10$; Mann-Whitney test; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; ^{ns}, not significant, comparing SS or RF to their respective control.

Our data do not fully clarify these controversies. Although we found a clear increase of type I fibers in the red (slow, oxidative) regions of the gastrocnemius after semistarvation, we did not observe a similar fast-to-slow transition in the white (fast, glycolytic) regions of that muscle (not shown). Therefore, it is not clear whether the slower MyHC signature restricted to the slow region is the only cause for the major slowing in contraction and relaxation kinetics observed in response to electrical stimulation.

Skeletal muscle in both semistarved and refed rats showed no differences in the resistance to fatigue compared to their respective controls. This might be explained by the fact that part of the type IIA fibers were converted into type I fibers, both of which being often referred to as fatigue-resistant fibers. The fatigue assay employed is very challenging to muscle fibers. Based on unpublished observations from our laboratory, we believe that the drop in force in this assay is caused by a combination of metabolic exhaustion, secondary to limited nutriment availability and accumulation of inorganic phosphate, as has been described by Allen and colleagues (Allen et al., 2008; Allen and Trajanovska, 2012) and mechanical damage to the fibers. The similar resistance to fatigue of experimental groups further suggests that semistarvation and refeeding did not induce major disturbances in muscle structure.

Slower Contraction-relaxation Kinetics: Roles of Calcium Handling Proteins

The pattern of calcium handling proteins expressed in a given muscle fiber partly controls contraction intensity and shape. Ca^{2+} ions released from the sarcoplasmic reticulum triggers contraction, whereas reuptake of Ca^{2+} into the sarcoplasmic reticulum allows relaxation to take place (Berchtold et al., 2000; Reggiani and te Kronnie, 2006). Altered Ca^{2+} cycling could *a priori* contribute to the increased time to peak observed: excessive Ca^{2+} release from the sarcoplasmic reticulum and inefficient Ca^{2+} reuptake through SERCA pumps would result in prolonged cytosolic calcium transients and increase the period of time until which relaxation could occur. Moreover, a link between thyroid hormone levels and Ca^{2+} handling in skeletal muscle has been described (Everts, 1990; Minatoya et al., 2007). However, our findings do not support impaired intracellular Ca^{2+} cycling. Indeed, when expressed per cross-sectional area, no differences in skeletal muscle force, neither phasic nor tetanic, were observed between the semistarved rats and their controls or between the refed rats and their controls. This suggests that Ca^{2+} cycling was essentially unaffected (Berchtold et al., 2000). Such a contention is consistent with our findings that SERCA activity remained constant after semistarvation and refeeding. It is likely that the decreased SERCA2 levels after semistarvation were compensated by the trend of SERCA1 to increase, resulting in stable SERCA activity overall. The fact that levels of sarcoplasmic reticulum (CSQ1, CSQ2) as well as cytosolic (parvalbumin) Ca^{2+}-buffering proteins (Berchtold et al., 2000; Reggiani and te Kronnie, 2006) also remained unchanged, further supports the hypothesis that Ca^{2+} cycling was spared in this model of semistarvation-refeeding.

Slower Contraction-relaxation Kinetics: Roles of Myosin Heavy and Light Chains

Muscle relaxation occurs as a consequence of Ca^{2+} removal from the cytosol to concentrations below the myofibrillar activation threshold. In other words, during phasic twitches, Ca^{2+} handling proteins control the time until the onset of relaxation but not the rate at which relaxation occurs (Berchtold et al., 2000; Reggiani and te Kronnie, 2006). The latter is mostly dictated by the nature of the myosins that are expressed in a given muscle. Accordingly, the prolonged time for half-relaxation from the peak could be explained, at least partly, by the increased proportion of type I fibers. Other mechanisms, such as changes in the pattern of myosin light chains and/or their phosphorylation status, may also be involved in the slower relaxation (Edwards et al., 1975; Zhang et al., 2006; Barclay et al., 2007; Westerblad et al., 2010). Indeed, the rate of ATP hydrolysis by the MyHC heads and hence actin-myosin cross-bridge kinetics are mostly controlled by the myosin light chains that are associated to the MyHC heads (Berchtold et al., 2000; Reggiani and te Kronnie, 2006). In support of this, others have shown that the slower relaxation of fatigued mouse fibers was primarily caused by altered actin-myosin cross-bridge properties more than defects in Ca^{2+} handling (Westerblad and Allen, 1993; Westerblad et al., 1997).

Slow-contracting Muscle but Unusual Molecular Signature: Roles of Transcription Factors

As evidenced by the analysis of muscle force in sedated rats and by MyHC typing on gastrocnemius sections, semistarvation caused a remarkable slowing of muscle contraction-relaxation kinetics that persisted after refeeding. The *in vivo* functional findings correlated well with the accumulation of type I fibers that are the molecular motors for slow contraction-relaxation cycling. The greater proportion of slow fibers was also in line with the leftward shift of the force-frequency relationship after semistarvation, as low stimulation frequencies were able to recruit larger fractions of the maximum force.

We hypothesized that the slower fiber phenotype would be substantiated by other differences in the expression of slow muscle markers at the expense of those of fast muscle. For instance, we expected a decrease in SERCA1, CSQ1, and parvalbumin, which are typical of fast-twitch muscles and/or an increase in SERCA2 and CSQ2, which are characteristic of slow-twitch muscles (Berchtold et al., 2000; Reggiani and te Kronnie, 2006). However, this was not the case. On the contrary, we found a trend toward an increase of SERCA1 and a significant decrease in SERCA2 after semistarvation, and the levels of the Ca^{2+} buffering proteins CSQ1, CSQ2, and parvalbumin remained constant, regardless of the dietary status. Therefore, although MyHC expression supports the slower muscle behavior observed, the muscles did not exhibit the typical molecular signature that one may expect from slow-contracting muscles.

We propose that this incomplete slow signature resulted from an aberrant expression of several transcription factors that regulate the acquisition and maintenance of slow phenotype in skeletal muscle. For instance, calcineurin and PGC-1α are positive regulators of the slow phenotype and were reported to stimulate fast-to-slow transition (Lin et al., 2002; Vescovo

et al., 2005; Mallinson et al., 2009; Jiang et al., 2010; Sakuma and Yamaguchi, 2010). We found that calcineurin was increased after semistarvation, which correlated with the observed slower phenotype. PGC-1α levels, on the contrary, were decreased, which may promote the opposite effects. FoxO1 levels were markedly elevated after semistarvation, which is consistent with previous reports demonstrating FoxO1 accumulation after fasting (Gross et al., 2008). FoxO1 is more abundant in the mixed gastrocnemius muscle than in the slow-twitch soleus muscle (Yuan et al., 2011). By contrast to calcineurin and PGC-1α, FoxO1 acts as a negative regulator of slow muscle fibers (Kamei et al., 2004). In this context, it is worth mentioning that persisting elevated levels of FoxO1 may stimulate the reversion of the slow phenotype by inhibiting calcineurin activity directly (Yuan et al., 2011). Accordingly, the shift of MyHC 1-positive fibers and of SERCA2 levels toward control values during refeeding may result from the persistence of elevated FoxO1 together with normalized amounts of calcineurin and PGC-1α.

We propose that the differential expression of calcineurin, PGC-1α, and FoxO1 affects the expression levels of the proteins coded by their target genes, for example MyHC 1, and that may be the cause for the incomplete slow signature in muscle fibers. Such an unusual molecular pattern may also explain that the even slower contraction-relaxation kinetics after refeeding were associated with a normal force-frequency relationship.

Muscular Levels of Deiodinases and Kinetics of Thyroid Hormones during Catch-up Fat

Whatever the specific intracellular events underlying the slowed contraction-relaxation of skeletal muscle and the associated switch from fast-to-slow fibers during caloric restriction and their persistence during refeeding, it is highly likely that they are modulated by alterations in thyroid hormone availability and actions. On the one hand, the hypothyroid status induced by caloric restriction can be expected to lead to a fast-to-slow fiber transition since hypothyrodism in patients, chemically-induced hypothyroidism in rodents, or deficiency of thyroid hormone receptors in mice, have all been shown to markedly affect muscle fiber proportions in this direction (Ianuzzo et al., 1977; Wiles et al., 1979; Johansson et al., 2003; Simonides and van Hardeveld, 2008). On the other hand, such a status can also lead to higher energetic efficiency, affecting the speed of the contraction–relaxation cycle of skeletal muscle (Wiles et al., 1979; Caiozzo and Haddad, 1996; Everts, 1996). In a previous study (Mainieri et al., 2006) using this rat model of semistarvation-refeeding, we observed that plasma levels of both T_4 and T_3 were significantly lower during caloric restriction than in controls, but their restoration kinetics were different. Whereas plasma T_4 was restored to control levels within 5 days of refeeding, plasma T_3 remained lower than controls on day 10 of refeeding. The kinetics of T_3 restoration thus seem to parallel the rate of fat recovery in our rat model. However, thyroid hormone concentrations are known to be affected by local metabolism, such as conversion of T_4 to the active hormone T_3 by type 1 and type 2 iodothyronine deiodinases (DIO1 and DIO2, respectively) and inactivation of T_4 and T_3 by type 3 iodothyronine deiodinase (DIO3) (Salvatore et al., 2014). In fact, DIO3 decreases T_3 availability within the skeletal muscle by two ways: it prevents conversion of T_4 to T_3 by catalyzing the conversion of T_4 to reverse T_3 (rT_3) instead, and it also catalyzes the degradation of T_3 to 3,3′-T_2 (Salvatore et al., 2014).

The regulated expression of DIO2 and DIO3 occurs in many tissues, including skeletal muscle, and allows for a tissue-specific modulation of intracellular thyroid hormone activity that is independent on the circulating thyroid hormone levels, thereby increasing the regulatory potential in gene expression (Huang and Bianco, 2008; Salvatore et al., 2014). Thus, in our rat model, the possibility of a lower T_3 availability in skeletal muscle could be contributed not only from a lower plasma T_3 levels (Mainieri et al., 2006; Summermatter et al., 2008), but also from altered deiodinase activities. This latter contention would be consistent with our findings here that the kinetics of T_3 generation in skeletal muscle homogenates incubated with T_4 were significantly lower after semistarvation and refeeding compared to their respective controls. Additional findings reveal that such reductions in muscle T_3 availability in our rat model likely result from decreased DIO2, together with increased DIO3, during both, caloric restriction and refeeding.

DIO3 is barely detectable in adult tissues and the cellular compartment in which T_3 inactivation occurs remains undetermined (Huang and Bianco, 2008). Thus, a role of DIO3 in local tissue modulation of thyroid hormone metabolism in response to weight loss induced by caloric restriction or disease-cachexia has long been unrecognized, despite earlier reports of robust stimulation of DIO3 in various tissues, including skeletal muscle, in hospitalized critically ill patients (Peeters et al., 2003, 2005; Rodriguez-Perez et al., 2008). Furthermore, results from a study in chicken (Van der Geyten et al., 1999) point to the fact that starvation increases liver DIO3 levels by more than 3-fold within 24 h of caloric restriction, and that this increase is associated with a decrease in plasma T_3 levels. Based on our findings showing marked upregulation of DIO3 expression associated with diminished T_3 availability in muscle during caloric restriction and weight recovery, diminished intracellular T_3 availability could be a key factor underlying the shift in fast-to-slow muscle fiber and the slowed kinetics of contraction-relaxation. These changes would contribute to the suppression of thermogenesis, operating as a function of fat store depletion, and hence in the thrifty metabolism that persists during weight recovery to accelerate the restoration of the fat stores.

Conclusion

The analysis of isometric phasic and tetanic twitches, force-frequency relationship, and fatigue characteristics of leg muscles suggests that the prolonged contraction-relaxation observed during semistarvation persists during the phase of catch-up fat, but is unlikely to be explained by changes in muscle structure or in Ca^{2+} handling. Instead, our data suggest that semistarvation-refeeding causes aberrant expression of transcription factors within skeletal muscle. This stimulates differing signaling pathways, whose integration by the myofibers results in an unusual pattern of expression of slow *vs.* fast muscle proteins. The

altered muscle function and, in particular, the slower kinetics of contraction-relaxation, are likely induced by changes in thyroid hormone levels. This is strongly suggested by diminished T_3 availability within skeletal muscle and supported by a decrease in DIO2 that occurred concomitantly with an increase in DIO3.

Author Contributions

Conceived and designed the experiments: AD, PA, MS, OP, UR, OD, LN. Performed the experiments: PA, OD, MS, OP, DA, LN. Analyzed the data: OD, PA, MS, OP, AD, UR, LN. Contributed reagents/materials/analysis tools: AD, JM, UR, OD, LS. Wrote the paper: AD, OD, PA, LN. Edited the manuscript: UR, JM, MS, DA, OP, LS, LN.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2015.00254

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Maternal nutritional manipulations program adipose tissue dysfunction in offspring

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Based on the concept of Developmental Origin of Health and Disease, both human and animal studies have demonstrated a close link between nutrient supply perturbations in the fetus or neonate (i.e., maternal undernutrition, obesity, gestational diabetes and/or rapid catch-up growth) and increased risk of adult-onset obesity. Indeed, the adipose tissue has been recognized as a key target of developmental programming in a sex-and depot-specific manner. Despite different developmental time windows, similar mechanisms of adipose tissue programming have been described in rodents and in bigger mammals (sheep, primates). Maternal nutritional manipulations reprogram offspring's adipose tissue resulting in series of alterations: enhanced adipogenesis and lipogenesis, impaired sympathetic activity with reduced noradrenergic innervations and thermogenesis as well as low-grade inflammation. These changes affect adipose tissue development, distribution and composition predisposing offspring to fat accumulation. Modifications of hormonal tissue sensitivity (i.e., leptin, insulin, glucocorticoids) and/or epigenetic mechanisms leading to persistent changes in gene expression may account for long-lasting programming across generations.

Keywords: adipocyte, hypertrophy, hyperplasia, fetal programming, epigenetic mechanisms, inflammation, obesity

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Epidemiological and clinical studies demonstrated that nutrient supply perturbations in the fetus or neonate (i.e., maternal undernutrition, obesity, gestational diabetes and/or rapid catch-up growth) are associated with higher adiposity in adulthood (Ravelli et al., 1999). Based on the concept of Developmental Origin of Health and Disease (Barker, 2004), it has been hypothesized that maternal nutritional manipulations during the perinatal period may program the development and cause dysfunction in offspring's adipose tissue later in life. Over the past decade, an increasing number of data from human and animal studies have validated this concept (Lukaszewski et al., 2013; Lecoutre and Breton, 2014).

Several types of adipose tissue coexist in mammals. Although their properties are quite different, they exhibit similar cell composition: mature adipocytes (i.e., lipid storage compartment) and a stromal vascular fraction (a heterogeneous population of cell types including adipose stem cells). The white adipose tissue (WAT) constitutes the main energy reserve, storing triglycerides (TG) during periods of positive energy balance by promoting lipogenesis. Two distinct processes account for WAT growth. First, adipogenesis (i.e., increased adipocyte number) relies on the recruitment and the commitment of adipose stem cells to adipocyte lineage. The differentiation of preadipocytes into adipocytes are regulated by several adipogenic and lipogenic transcription

factors such as peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/enhancer binding protein (C/EBPα, β, γ), the sterol regulatory element-binding protein 1c (SREBP1c) as well as fatty acid synthesis enzymes such as fatty acid synthase (FAS). Second, lipogenesis (i.e., increased adipocyte size) depends on the synthesis and the storage of TG in mature adipocyte (**Figure 1**). Fat cell number and lipolysis are also controlled by the activity of the WAT sympathetic system (Bowers et al., 2004). The brown adipose tissue (BAT) differs from WAT by its cell origin (Seale et al., 2009). Brown adipocytes dissipate energy via thermogenesis mediated via BAT-selective genes such as uncoupling protein 1 (UCP1) and transcription factors [PPARα and PPARγ coactivator 1-α (PGC1-α)]. Despite high similarities to brown adipocytes, brite adipocytes (brown-in-white) derive from a distinct origin and are closer to the white adipocyte cell lineage. Several studies in rodents showed that WAT browning (i.e., enhanced brite adipocytes) can be induced by prolonged cold exposure, treatment with β-adrenergic agonists (via β3-adrenoreceptor activation) and endurance exercise (Vosselman et al., 2013).

Because adipose tissue development occurs at different periods during the perinatal period in rodents (i.e., mainly at the end of gestation, throughout lactation and adolescence) and in bigger mammals (i.e., before birth), the window of vulnerability to environmental factors differs between species (Muhlhausler and Smith, 2009; de Oliveira et al., 2013). However, regardless of the difference in the timing of adipogenesis and adipose tissue development, closely related mechanisms underlying perinatal programming have been reported in altricial and precocial species (Lukaszewski et al., 2013; Lecoutre and Breton, 2014). In this context, we provide a brief overview on the repercussion of perinatal nutritional manipulations on adipose tissue functionality in programmed offspring.

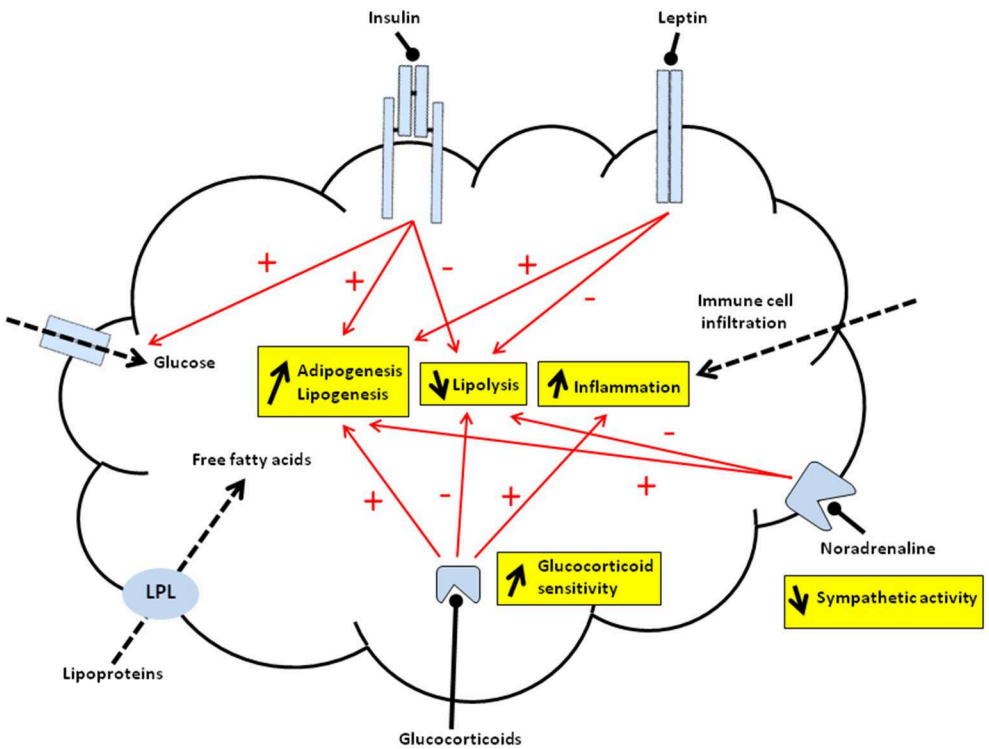

FIGURE 1 | Schematic representation of programmed mechanisms in offspring's adipose tissue of malnourished dams. Overall, offspring from malnourished dams display increased adipogenesis/lipogenesis and inflammation as well as decreased lipolysis within adipose tissue. Red arrows indicate programmed activation (+) or inhibition (–) of major processes controlled by hormones that may predispose offspring to fat accumulation. The accumulation of triglycerides (TG) may be due to increased esterification of either free fatty acids released from lipoproteins, catalyzed by lipoprotein lipase (LPL) (dashed arrow), or free fatty acids synthesized from glucose metabolism mainly driven by lipogenic enzymes. Leptin acting via its receptor (JAK2/STAT3 signaling pathway) may activate preadipocyte proliferation and/or inhibit lipolysis. Insulin after binding to its receptor (IRS/PI3K/Akt signaling pathway) may promote the TG formation via activation of glucose entry (i.e., increased GLUT4 translocation) (dashed arrow) and lipogenic enzymes. Insulin also shows anti-lipolytic effects and may activate adipogenesis. Increased adipogenesis and decreased lipolysis may be due to impaired activity of the sympathetic system (via noradrenaline). Glucocorticoids (GC) that bind intracellular receptor may activate adipogenesis and lipogenesis whereas they inhibit lipolysis. Thus, both increased circulating GC levels and intracellular GC sensivity (i.e., modified GR, MR, 11 β-HSD1, 11 β-HSD2 relative abundance and activities) can directly impact on adipose tissue function. The pro-inflammatory state might be due to either immune cell infiltration (dashed arrow) and/or local expression of pro-inflammatory mediators. The pro-inflammatory state may also originate, at least in part, from increased GC sensitivity within WAT.

Maternal Nutritional Manipulations Alter Adipogenesis and Lipogenesis in Offspring's Adipose Tissue

Low Birth-Weight Offspring

Three principal models of maternal undernutrition leading to low birth weight have been developed: maternal low-protein diet (LP), maternal food restriction (FR) and uterine artery ligation (mimicking uteroplacental insufficiency) in pregnant dams.

Maternal LP during gestation and lactation results in a reduction in adipose cell size of rat offspring (Ferland-McCollough et al., 2012). Analysis of WAT of adult offspring from LP dams revealed an upregulation of C/EBPα and PPARγ gene expression (Guan et al., 2005) with increased rate of cultured preadipocyte proliferation (Bieswal et al., 2004). In addition, adult rats from LP dams displayed elevated miRNA-483-3p expression levels that may reduce the capacity of lipid storage contributing to limit adipocyte hypertrophy (Ferland-McCollough et al., 2012). In contrast, rat offspring from 50% food-restricted (FR50) dams during gestation from day 10 to term (Desai et al., 2008) or from 70% food-restricted (FR70) dams throughout the gestation (Lukaszewski et al., 2011) showed hypertrophic adipocytes. Newborn rats from FR50 dams displayed higher preadipocyte proliferation, C/EBPs and PPARγ expression, along with adipocyte TG accumulation *in vitro* (Yee et al., 2012). Prior to the onset of overt obesity, increased adipocyte size was associated with elevated expression levels of factors promoting lipogenesis (i.e., SREBP1c, FAS, leptin) in weanling pups (Desai et al., 2008) and adult offspring (Lukaszewski et al., 2011) from FR dams. Similarly, uterine artery ligation of pregnant dams causes an upregulation of PPARγ gene expression in WAT of rat offspring before increased adiposity (Joss-Moore et al., 2010).

Several animal model and human studies have highlighted the deleterious effect of rapid postnatal growth, especially when overfeeding paradigms during lactation (i.e., hypercaloric maternal diet, cross-fostering pups, reduced litter size) were applied in low-birth-weight offspring. In particular, the mismatch between pre- and postnatal energy supply was closely related to increased adipogenesis and higher risk of obesity later in life (Druet et al., 2012). Consistent with these findings, postnatal overfeeding of rat pups from LP dams promoted higher proliferation and differentiation rate of cultured preadipocytes (Bol et al., 2008). Overfed adult offspring from FR dams exhibited higher adiposity along with increased lipogenic (Lukaszewski et al., 2011) and clock genes (Sutton et al., 2010) in a depot-specific manner. The latter suggests that circadian rhythm disruptions may participate to the adult-onset obesity. Similar patterns of programming are observed in sheep, which show great similarities with the development of human adipose tissue. Low birth-weight lamb with atrophied fat depots displayed progressive fat accumulation along with increased PPARγ gene expression through accelerated postnatal growth (Muhlhausler and Smith, 2009).

Maternal Overfeeding

Numerous models of maternal overfeeding and obesity [i.e., high-fat (HF) or cafeteria diet] before and/or during gestation and/or lactation were described in the literature. In most cases, despite normal birth weight, offspring of obese dams are sensitized to postnatal adiposity, adipocyte hypertrophy and weight gain.

Several rodent and sheep models of maternal obesity demonstrate that maternal obesity at conception enhances adipogenesis from the fetal period (Muhlhausler and Smith, 2009; Borengasser et al., 2013) to adulthood (Murabayashi et al., 2013) resulting in higher WAT mass and larger adipocytes. Overfeeding during lactation and/or postweaning periods leading to catch-up growth, consistently worsen adipogenesis programming (Desai and Ross, 2011; Guberman et al., 2013). Upregulated PPARγ is one of the characteristic feature of enhanced adipogenesis and fat expansion in programmed offspring of obese dams (Samuelsson et al., 2008; Muhlhausler and Smith, 2009). This is associated with downregulated PPARγ corepressors (Desai and Ross, 2011). In addition to higher amount of TG along with lipoprotein lipase (LPL) activity within WAT (**Figure 1**), obesity prone-rats from cafeteria-diet-fed dams throughout gestation and lactation exhibited modified fatty acid composition (Benkalfat et al., 2011). In rodents, the predisposition of adiposity in offspring following maternal overfeeding occurs in a sex- and species-dependent manner. In accordance with these findings, only female offspring of mice from HF-fed dams throughout gestation and lactation displayed hypertrophied adipocyte with blunted lipolytic capacities (Samuelsson et al., 2008). Using a similar maternal nutritional paradigm, we observed an opposite phenotype in rat offspring (i.e., male obese vs. lean female). Maternal gestational diabetes also predispose adult offspring to adipocyte hypertrophy and obesity (Steculorum and Bouret, 2011), suggesting that modified insulin and/or glucose levels during the perinatal period program adipose dysfunction in offspring (**Figure 1**).

Modified Energy Intake During the Suckling Period

Given that the lactation coincides with the period of maximum adipogenesis in rodents, the modification of milk intake by rearing pups in small (overnutrition) or large litters (undernutrition) has been used extensively to investigate adipose tissue development.

In rats, neonatal overfeeding (i.e., pups reared in small litters) led to rapid weight gain during lactation that remains visible until adulthood. The perinatal period is characterized by hyperinsulinemia, known to participate to the developmental programming of fat accumulation (**Figure 1**). During this period, adipose tissue expansion is due both to hyperplasia (i.e., higher preadipocyte and stromal cell numbers) and hypertrophy (i.e., enhanced lipogenesis such as LPL activity) within WAT. In adult offspring, increased adiposity mainly resulted from adipocyte enlargment [i.e., higher lipogenesis and modified sensitivity of glucocorticoid (GC)] in a depot-specific manner (Boullu-Ciocca et al., 2008) (**Figure 1**). Similar outcomes

(i.e., neonatal hyperinsulinemia, hypertrophied adipocytes, exacerbated lipogenesis and obesity later in life) were also associated with artificially reared rat pups fed a formula high in carbohydrate-derived energy. These findings highlight the importance of modified perinatal glucose and/or insulin levels in programming events (Srinivasan et al., 2008). In contrast, both paradigms of neonatal underfeeding in rats (i.e., pups reared in large litters or breastfed by FR30 dams) resulted in lean phenotype with smaller adipocytes and protection to diet-induced obesity (Patterson et al., 2010; Palou et al., 2011).

Maternal Nutritional Manipulations Impair Sympathetic Activity in Offspring's Adipose Tissue

We showed that perinatal maternal undernutrition (FR50 model during the last week of gestation and lactation) resulted in a delay in the development of gonadal WAT in male rat at weaning. The neonatal WAT was characterized by the appearance of brown-like adipocytes and increased markers of thermogenesis (i.e., UCP1, PGC1α and PPARα). This phenomenon might, at least in part, rely on exacerbated WAT sympathetic activity recruited in offspring to promote browning, thereby increasing the capacity for adaptive thermogenesis (Delahaye et al., 2010). In agreement with these findings, adult rat male offspring from FR20 dams during the first 12 days of pregnancy, but not females, displayed an increase in adipose tissue cellularity along with alterations of WAT sympathetic innervations (García et al., 2011). Reduced BAT mass and UCP1 activity were also closely related to impaired sympathetic activity and lipolysis in adult rats reared in small litters (Xue et al., 2007).

Offspring of Malnourished Dams Show Increased Inflammatory Response in Adipose Tissue

Offspring of malnourished dams displayed chronic low-grade obesity-associated inflammation characterized by elevation of inflammatory factors in plasma (i.e.,TNF-α, IL-6 and MCP-1) and expression of pro-inflammatory mediators in WAT. The latter may be due to increased production of cytokines by adipocytes and/or immune cells that infiltrated within WAT. The hallmark of the WAT inflammatory is the appearance prior to overt obesity. Thus, elevated pro-inflammatory markers in WAT, possibly originating from immune cell infiltration were reported in fetus of obese mice fed a cafeteria diet before mating and throughout gestation (Murabayashi et al., 2013). This phenomenon occurred at an early stage of WAT development in line with increased storage of TG in prenatally undernourished adult sheep (Sharkey et al., 2009) and rat offspring following uteroplacental insufficiency (Joss-Moore et al., 2010). Rat offspring reared in small litters (Boullu-Ciocca et al., 2008) as well as juvenile offspring from obese HF-fed rats (Del Bas et al., 2015) also displayed a early postnatal induction of pro-inflammatory cytokine mRNA expression levels in WAT that were exacerbated under HF diet. However, *in utero* HF diet exposure may result in increased pro-inflammatory markers in WAT of adult mice offspring, independently of maternal obesity (Umekawa et al., 2015). The pro-inflammatory state may also originate, at least in part, from modified GC sensitivity within WAT (Lee et al., 2014). However, it remains to determine whether inflammatory changes are cause or consequence of fat accumulation.

Programming Mechanisms

Numerous studies regarding nutritional manipulations in the perinatal period using different opposite paradigms (undernutrition vs. overfeeding) pointed out the fact that redundant mechanisms rely on the programming of adult offspring's adipose tissue. Some of them occur in a gender-specific manner.

Plasma Hormone Levels and Tissue Sensitivity

Over the past few decades, the adipocytokine leptin has been considered as the main programming factor of the hypothalamus adipose-axis. Originally, leptin was described as a hypothalamic neurotrophic factor involved in the plasticity and hardwiring of the appetite regulatory circuits in the hypothalamus (Bouret et al., 2004). Numerous studies indicated that perinatal leptin manipulations result in increased risk of adult-onset obesity (Breton, 2013). Indeed, altered postnatal leptin surge during lactation observed in undernourished (Delahaye et al., 2008; Palou et al., 2012) or overnourished rodent (Kirk et al., 2009) is associated with fat accumulation in adulthood. In addition, early postnatal leptin blockage increases susceptibility to diet induced obesity in rats (Attig et al., 2008) whereas administration of leptin during the postnatal period reverses obesity in prenatally undernourished adult rats (Vickers et al., 2008). Interestingly, leptin may act directly on adipose tissue via binding to its receptor to increase preadipocyte proliferation (Bol et al., 2008) and has differential morphogenesis effects on male and female adipocytes (Guo et al., 2009). It may also inhibit lipogenesis in already developed adipocytes (Huan et al., 2003) (**Figure 1**). Insulin has been also reported as programming factor on the hypothalamus adipose-axis. Thus, perinatal manipulations of insulin levels (Breton, 2013) and altered insulin signaling in the hypothalamus (Vogt et al., 2014) result in fat accumulation in offspring. Fat cells respond to insulin after binding to its receptor and activation of the PI3K/Akt signaling pathways by activating adipogenesis/lipogenesis and inhibiting lipolysis (Poulos et al., 2010) (**Figure 1**).

The link between chronic excess of plasma GC levels and adiposity as observed in Cushing's syndrome suggested that altered GC metabolism may also predispose to fat expansion. This observation has led to the hypothesis that perinatal hypercorticosteronemia due to disturbed HPA axis feedback might be a key factor in offspring's WAT programming (Breton, 2013). First, GC alone or in interaction with insulin is known to regulate *in vitro* and *in vivo* the differentiation of adipocyte precursors (i.e. increased C/EBP**α**, PPAR**γ**) and lipogenic

genes (**Figure 1**). Second, GC may be the source of chronic inflammatory conditions in WAT by inducing inflammation-related gene expression and pro-inflammatory immune cell infiltration (Lee et al., 2014).

In addition to higher systemic GC levels, local induction of GC activity within WAT due to modified GR, MR, 11β-hydroxysteroid dehydrogenase type 1 (11**β**-HSD1), 11β-hydroxysteroid dehydrogenase type 2 (11**β**-HSD2) relative abundance, may also account for increased adiposity (Lee et al., 2014) (**Figure 1**). Several studies using maternal nutritional manipulation models support this notion. Perinatally undernourished offspring of precocious species (primate, sheep) that are sensitized to weight gain, exhibited increased glucocorticoid receptor (GR) and 11β-HSD1 gene expression, enzyme that regenerates intracellular GC by converting inactive GC metabolites to active GC during WAT development. This phenomenon takes place *in utero* during early fetal development of adipose tissue in female but not male primate (Guo et al., 2013). In low birth weight lambs, the modified GC sensitivity was mainly observed during catch-up growth period along with decreased 11β-HSD2 that modifies active GC to inactive metabolites (Gnanalingham et al., 2005). In rodents, obesity-prone rat reared in small litters and from diabetic dams also exhibited an induction of GR and 11β-HSD1 gene expression in WAT during the postnatal period. The increase in intracellular GC sensitivity paralleled accelerated WAT growth but took place before overt obesity (Boullu-Ciocca et al., 2008). Why postweaning HF diet accentuates the GC sensitivity within WAT remains to be determined.

The action of GC in WAT is far less than clear but mainly depends on local balance of active GC *versus* inactive GC metabolites by 11β-HSD1 and 11β-HSD2 (Lee et al., 2014). In accordance with this notion, we observed a modified 11β-HSD1/11β-HSD2 ratio in adult rat offspring from FR70 dams throughout gestation as well as from HF-fed dams throughout gestation and lactation (Lukaszewski et al., 2011). In particular, the depot-specific decreased 11β-HSD1/11β-HSD2 ratio might be seen as an adaptive mechanism to limit fat accumulation (Lukaszewski et al., 2011).

Epigenetic and Transgenerational Mechanisms

Parental nutritional manipulations during critical developmental time windows may permanently modulate gene expression profiles in progeny via epigenetic mechanisms (i.e., DNA methylation, histone modification and non-coding RNA modifications). Thus, the implication of these epigenetic processes have become increasingly important. It might account for increased risk of adult-onset non-communicable diseases such as obesity (Lillycrop and Burdge, 2012). In particular, perinatally unbalanced maternal diet was showed to induce epigenetic changes in offspring genome resulting in long-lasting modification of the transcriptional control of adipogenesis (Musri and Párrizas, 2012) and/or in inflammation (Toubal et al., 2013). An isocaloric LP diet given to dams throughout gestation and lactation affects the CpG site methylation of the leptin promoter, adipose expression and plasma levels in adult offspring mice (Jousse et al., 2011). A similar LP diet applied to rat dams leads to higher miRNA-483-3p levels, known to reduce adipose tissue expandability in rat offspring (Ferland-McCollough et al., 2012). Maternal obesity in female mice induces increased gene expression of Zfp423, a key transcriptional factor initiating adipogenic commitment, along with lower promoter methylation levels in fetal offspring (Yang et al., 2013). Similarly, weanling rats from obese dams display greater adipocyte differentiation, increased Zfp423, PPARγ and C/EBPβ mRNA expression levels along with specific alterations in DNA methylation of CpG sites (Borengasser et al., 2013). Maternal HF diet during pregnancy also leads to histone modifications within leptin and adiponectin promoter regions that may affect adipocytokine gene expression in mouse offspring (Masuyama and Hiramatsu, 2012).

Perinatal perturbations of fetus/neonate nutrient supply program obesity differently according to the sex of the offspring (Samuelsson et al., 2008; García et al., 2011; Guo et al., 2013). The basis of the sex specific programming effects remains elusive but could reflect direct interactions between maternal nutritional signals and mechanisms and/or factors involved in sex differences in development (i.e., differences in patterns and in timing of development and influence of steroid hormone exposure during the perinatal period) (Aiken and Ozanne, 2013). This phenomenon may be due, at least in part, to gender-specific epigenetic modifications during early stages of the development. In agreement with this notion, the global methylation profile in placenta as well as gene expression pattern was obviously different between male and female offspring from HF-fed obese mice (Gallou-Kabani et al., 2010). Changes to the epigenome associated with first generation phenotype can last through subsequent generations to promote transgenerational inheritance via both maternal and paternal lineages. In the first generation, somatic programming may acutely affect tissue, leading to offspring phenotype. At the same time, the germ cell lineage which transmits genetic and epigenetic information from one generation to the next, may undergo epigenome reprogramming during their development (Dunn et al., 2011). Based on the transgenerational inheritance concept, the multigenerational HF-driven obesity may be seen as a vicious circle that may participate to the transmission of obesity. Accordingly, HF-fed female progeny of obese mice dams displayed WAT inflammation and obesity over three generations. Multigenerational changes in DNA hypomethylation on inflammatory genes contribute, at least in part, to these phenomena (Ding et al., 2014). In humans, epidemiological studies using the Dutch famine birth cohort have also highlighted transgenerational transmission of obesity (i.e., increased neonatal adiposity of the second generation offspring from undernourished mothers) (Painter et al., 2008).

Interestingly, maternal diet supplementation (including methyl donors) during gestation and/or lactation might partially prevent the obese phenotype of offspring (Cordero et al., 2014). Although underlying mechanisms remain elusive, epigenetic processes (i.e., modified methylation status) may account for

deprogramming events (Lillycrop et al., 2005). Thus, maternal diet supplementation via offspring's epigenome changes may constitute a promising strategy (perhaps even prevention) for early intervention in order to alleviate deleterious programming effects of maternal malnutrition.

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Apelin and energy metabolism

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A wide range of adipokines identified over the past years has allowed considering the white adipose tissue as a secretory organ closely integrated into overall physiological and metabolic control. Apelin, a ubiquitously expressed peptide was known to exert different physiological effects mainly on the cardiovascular system and the regulation of fluid homeostasis prior to its characterization as an adipokine. This has broadened its range of action and apelin now appears clearly as a new player in energy metabolism in addition to leptin and adiponectin. Apelin has been shown to act on glucose and lipid metabolism but also to modulate insulin secretion. Moreover, different studies in both animals and humans have shown that plasma apelin concentrations are usually increased during obesity and type 2 diabetes. This mini-review will focus on the various systemic apelin effects on energy metabolism by addressing its mechanisms of action. The advances concerning the role of apelin in metabolic diseases in relation with the recent reports on apelin concentrations in obese and/or diabetic subjects will also be discussed.

Keywords: obesity, type 2 diabetes, apelin, adipokine, insulin sensitivity

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Introduction

Nutrient metabolism and energy homeostasis are tightly regulated by endocrine, paracrine, and autocrine factors. Moreover, skeletal muscle, liver, adipose tissue and pancreatic β-cells play a major role in the maintenance of energy balance. A rapid modification of energy balance leads to obesity that in turn is a crucial cause of insulin resistance. Several mechanisms linking obesity to insulin resistance have been proposed. Among them, adipocyte-secreted factors or adipokines have been shown to play an important role. Alteration of their production (excess or deficit) could directly promote or delay the onset of insulin resistance. The role of leptin and adiponectin has been extensively studied (for review see Lafontan and Viguerie, 2006; Tishinsky et al., 2012; Farooqi and O'Rahilly, 2014). In 1998 Tatemoto et al identified apelin as the ligand of the APJ receptor, a G protein coupled receptor (Tatemoto et al., 1998). Apelin gene encodes for a 77 amino acid preproprotein and the apelin propeptide contains several doublets of basic amino acids implicating potential proteolytic cleavage sites for endopeptidases which give rise to several bioactive carboxy-terminal fragments including apelin-36, apelin-17, and apelin-13 but also the pyroglutamate apelin-13 which is protected from exopeptidase degradation (Masri et al., 2005). The group of Tatemoto has described the presence of apelin in rat adipose tissue (Wei et al., 2005) but the actual secretion from human and murine adipocytes was reported by Boucher et al. demonstrating that apelin was a new adipokine (Boucher et al., 2005). Apelin and its receptor APJ are widely expressed in several tissues (stomach, heart, lung, skeletal muscle, etc.) and in different regions of the brain, including the hypothalamus (O'Carroll et al., 2013). The apelin/APJ system is involved in a wide range of functions. Neither the effects of apelin on the regulation of cardiac and vascular functions, fluid homeostasis and angiogenesis, (Chapman et al., 2014) nor its central actions

on energy metabolism (Knauf et al., 2013) are presented in this article since these aspects were recently reviewed. This mini-review will discuss the recent advances concerning the role of apelin on energy metabolism particularly in pathophysiological situations (obesity, type 2 diabetes) and will try to establish a link between plasma apelin concentrations and metabolic diseases in humans.

Apelin and Glucose Metabolism

One of the first apelin effects observed on glucose metabolism, apart from that on insulin secretion (Sorhede Winzell et al., 2005), is its glucose-lowering effect both in fasted conditions and during a glucose tolerance test (Dray et al., 2008) in standard mice. This decreased glycemia has been shown to be mainly due to increased glucose uptake in target tissues such as skeletal muscle and adipose tissue (Dray et al., 2008). Since the muscles represent the main entry of glucose, apelin effect was studied in isolated soleus muscle. Apelin stimulated glucose transport and its effect was additive to that of insulin (Dray et al., 2008). The signaling pathway was depicted and it was shown that apelin stimulated the phosphorylation of the AMP-activated protein kinase (AMPK) but also the endothelial NO synthase (eNOS) (Dray et al., 2008). The importance of both enzymes has been demonstrated by the use of eNOS$^{-/-}$ mice and DN-AMPK mice (muscle-specific inactive AMPK) respectively. Later on, the study of Yue et al. also reported that apelin was able to stimulate glucose transport *in vitro* in C2C12 muscle cells through a pathway involving AMPK but not eNOS (Yue et al., 2010). This discrepancy could be due to the fact that NOS inhibitors were used in the study of Yue et al. and that these inhibitors are efficient to decrease glucose uptake *in vivo* in muscle cells but not *in vitro* as previously reported (Roy et al., 1998). In addition, apelin also increased Akt phosphorylation in muscle manner both *ex vivo* (Dray et al., 2008) and *in vitro* (Yue et al., 2010). Interestingly, apelin is still able to stimulate glucose uptake in muscle of obese and insulin-resistant mice. This leads to an overall better insulin sensitivity (Dray et al., 2008). The role of apelin in glucose homeostasis was confirmed by the phenotype of apelin null (apelin$^{-/-}$) mice exhibiting hyperinsulinemia and insulin resistance (Yue et al., 2010). The loss of insulin sensitivity in apelin$^{-/-}$ mice was exacerbated by a high fat/ high sucrose diet (Yue et al., 2010).

Even though apelin-induced glucose transport has not yet been reported in isolated mouse adipocytes, apelin stimulates glucose transport in an AMPK-dependent way in human adipose tissue explants (Attane et al., 2011). This has also been observed in 3T3-L1 adipocytes through a mechanism dependent on PI3K/Akt activation but the role of AMPK was not studied (Zhu et al., 2011). In addition, in insulin-resistant 3T3-L1 cells (due to TNFα treatment), apelin increases the insulin-stimulated glucose transport (Zhu et al., 2011). Skeletal muscle and adipose tissue are not the only tissues where apelin stimulates the entry of glucose. *In vivo*, apelin has been shown to increase myocardial glucose uptake and Glut4 membrane translocation in C57BL/6J mice (Xu et al., 2012). Apelin also increases glucose transport *in vitro*, in H9C2 cardiomyoblasts (Xu et al., 2012). A role of apelin has also been shown in intestinal glucose absorption. Ingested glucose can rapidly induce the secretion of apelin in the intestinal lumen in mice (Dray et al., 2013). This study also shows that, when apelin is administered orally, the amount of glucose transporters SGLT1 is decreased in enterocytes, whereas that of Glut2 is increased due to AMPK activation. This results in an increased intestinal absorption of glucose. These data suggest that glucose arrival in the intestine causes its own absorption by inducing the paracrine secretion of apelin. A transient increase in blood glucose levels in the portal vein could induce rapid secretion of insulin (Fukaya et al., 2007), and an improved insulin sensitivity (Burcelin et al., 2000; Delaere et al., 2010). Thus, apelin could also regulate glucose metabolism, by promoting glucose absorption by the enterocytes and then by increasing portal blood glucose and insulin secretion. This could be in agreement with the fact that apelin was shown to increase GLP-1 secretion (Wattez et al., 2013).

Although all studies did not report a significant decrease in fasting blood glucose in obese and insulin resistant mice in response to apelin, decreased insulinemia has frequently been observed. This may be the result of improved insulin sensitivity or a direct effect of exogenous apelin on the pancreas. Accordingly, apelin was shown to decrease insulin secretion stimulated by different glucose concentrations (Guo et al., 2009; Ringstrom et al., 2010). Thus, by activating AMPK and bypassing insulin signaling, apelin exerts direct anti-diabetic effects, which could have an important impact in insulin resistant conditions.

Apelin and Lipid Metabolism

Few publications describe acute effects of apelin on lipid metabolism. In both isolated adipocytes and differentiated 3T3-L1 adipocytes, apelin was shown to inhibit isoproterenol (β-adrenergic agonist) -induced lipolysis through a pathway involving Gq, Gi, and AMPK (Yue et al., 2011). These results were confirmed by Than et al. (2012), who showed that apelin decreases the release of FFA by 3T3-L1 adipocytes through AMPK activation and by increasing the amount of perilipin surrounding the lipid vacuoles, giving them a greater stability and a resistance to lipases (Than et al., 2012). However, in human adipose tissue explants or human isolated adipocytes, apelin had no effect on basal or isoproterenol-stimulated lipolysis (Attane et al., 2011). Effects on adipose tissue and lipolysis were also found *in vivo* after a chronic apelin treatment in standard or high-fat diet (HFD) fed mice. Indeed, Higuchi et al. showed that daily ip apelin injections during 2 weeks decrease the triglycerides content in adipose tissue and the weight of different fat depots in standard mice and in HFD fed mice (Higuchi et al., 2007). Similar results were obtained in transgenic mice over-expressing apelin (Tg-apelin mice) fed a HFD (Yamamoto et al., 2011).

Chronic apelin treatment, in obese and insulin resistant mice, was also shown to increase fatty acid oxidation in muscles through AMPK activation (Attane et al., 2012). More recently, chronic apelin treatment has also been shown to prevent reduction of fatty acid and glucose oxidation in a model of obesity-related decline of cardiac function (Alfarano et al., 2015). In addition to stimulate the utilization of lipids, apelin

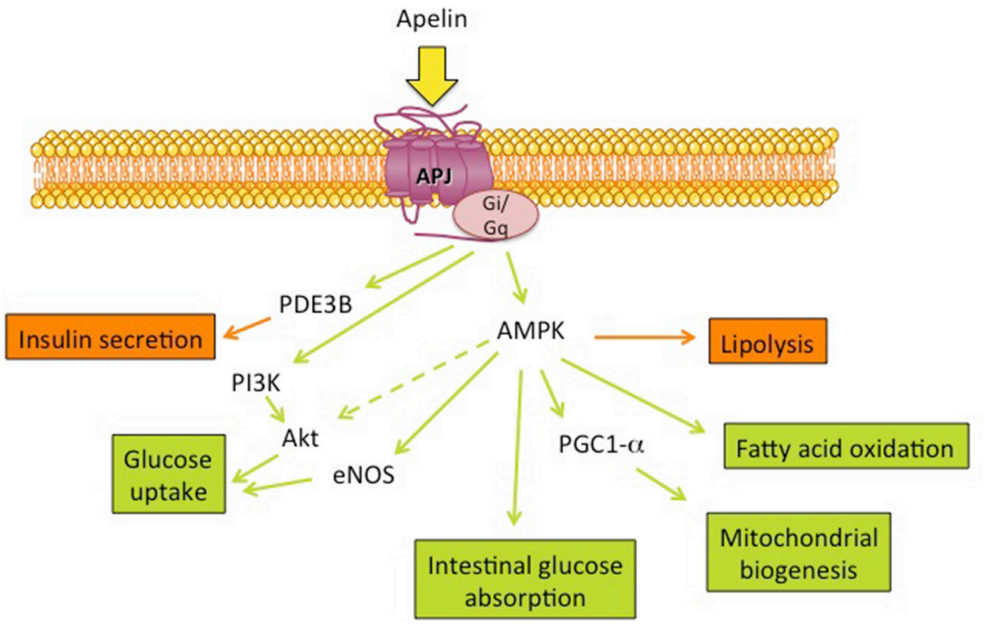

FIGURE 1 | Metabolic effects of apelin and its main signaling targets. Apelin, the ligand of the G protein coupled receptor APJ, can stimulate several metabolic functions (green arrows/boxes) and inhibit (orange arrows/boxes) lipolysis as well as insulin secretion through different signaling pathways: PDE3B, phosphodiesterase 3B; AMPK, AMP-activated protein kinase; PGC1-α, peroxisome proliferator-activated receptor γ co-activator 1α; eNOS, endothelial NO synthase; PI3K, phosphatidylinositol 3-kinase and Akt.

treatment increases mitochondrial biogenesis in skeletal muscle (Attane et al., 2012) and cardiomyocytes (Alfarano et al., 2015) by a mechanism involving increased expression of peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α). Increased mitochondrial DNA content in skeletal muscle was also found in Tg-apelin mice (Yamamoto et al., 2011) in agreement with the effect of apelin on mitochondrial biogenesis. Interestingly, the resistance to obesity of Tg-apelin mice was correlated with an increase in vessel formation in skeletal muscle. The importance of vessels integrity in both blood and lymph vasculature was recently confirmed in apelin$^{-/-}$ mice (Sawane et al., 2013). Indeed, weight gain observed in apelin$^{-/-}$ mice could be related to increased vascular permeability that in turn would induce greater fatty acids uptake in adipose tissue (Sawane et al., 2013). Thus, apelin might also prevent development of obesity through the maintenance of vascular integrity.

Energy expenditure in response to apelin treatment has also been studied via thermogenesis. Higuchi et al. reported that rectal temperature and O_2 consumption were higher in apelin-treated chow-fed mice probably mediated by the increased expression of mitochondrial uncoupling protein 1 (UCP1) observed in brown adipose tissue (Higuchi et al., 2007). O_2 consumption and body temperature were also increased in HFD fed Tg-apelin mice (Yamamoto et al., 2011) but not in obese and insulin resistant mice in response to chronic apelin treatment (Attane et al., 2012). However, food intake was not altered in both models.

All together, these studies clearly show that apelin, by itself, exerts metabolic functions such as glucose uptake but also improves insulin sensitivity since, for example, at the end of chronic apelin treatment, insulin-induced glucose transport was increased in skeletal muscles and there is an overall better insulin and glucose tolerance (Attane et al., 2012). Therefore apelin could be proposed as an interesting therapeutic target in the treatment of type 2 diabetes.

Changes in Apelin Concentrations Related to Human Metabolic Diseases

Numerous studies have reported increased plasma apelin concentrations in obese and/or diabetic subjects (for review see Castan-Laurell et al., 2011). Apelin-17 and [pyr-1]-apelin-13 may represent the predominant forms in plasma (De Mota et al., 2004; Azizi et al., 2008). During the last years, additional information was provided by assays performed especially in diabetic patients and in patients involved in weight loss intervention studies. Interestingly, plasma apelin has been shown to be a novel biomarker for predicting diabetes in Han Chinese subjects (Ma et al., 2014). Plasma apelin concentrations were higher in women than in men but they were associated with a risk of diabetes only in men (Ma et al., 2014). Recent data have also shown that apelin concentrations were significantly higher in type 1 diabetic patients than in control subjects and even higher than in type 2 diabetic patients (Habchi et al., 2014), in line with the previous study of Alexiadou et al. focused on type 1 diabetic subjects (Alexiadou et al., 2012). All together these studies pointed out the role of systemic apelin in metabolic diseases. What is the meaning of elevated apelinemia? Is obesity a main determinant of

elevated plasma apelin concentration? Different elements could be provided. Habchi et al. demonstrated that serum apelin levels were negatively correlated with glycosylated hemoglobin in type 2 diabetic patients, suggesting that circulating apelin is associated with better glycaemic control (Habchi et al., 2014). Increased concentrations of apelin in type 1 diabetes could be an attempt to compensate for the lack of insulin and to overcome insulin resistance. However patients were also treated with insulin, which could as well explain this rise, since insulin is one of the most important regulator of apelin expression and secretion (Boucher et al., 2005). Moreover, type 1 diabetic patients are not obese, suggesting that obesity is probably not the main determinant of increased apelin levels. In line with this point, an absence of correlation between plasma apelin concentrations and BMI has often been described (Castan-Laurell et al., 2011). The recent study of Krist et al. also gives further insights. They aimed to investigate whether changes in circulating apelin, in a context of weight loss, are primarily due to a reduced body fat mass or reflect the improved insulin sensitivity. First, all the different weight loss intervention studies (hypocaloric diet, bariatric surgery or exercise program) reduced the elevated serum apelin concentration determined in different cohorts of obese and diabetic patients as previously reported (Heinonen et al., 2009; Castan-Laurell et al., 2011). Secondly, significant BMI-independent correlations between reduced apelin levels and improved insulin sensitivity were found (Krist et al., 2013). Thus, it could be hypothesized that the increased plasma apelin observed in type 2 diabetic patients, is, as in type 1 diabetes, a compensatory mechanism devoted to directly decrease insulin resistance since apelin exerts different metabolic actions itself. When insulin resistance is decreased, this may lead to decreased apelin levels. It has thus been proposed that lower apelin serum concentrations in healthy lean individuals may be a consequence rather than a cause of normal insulin sensitivity (Krist et al., 2013).

Conclusion

The metabolic effects of apelin (**Figure 1**) described in different mouse models (diet-induced obesity, transgenic models) have underlined the beneficial roles of apelin on both energy metabolism and insulin sensitivity. Still, there remain many questions and many tools need to be developed. Long term apelin treatment studies, in both healthy and pathological conditions, need a more integrative view including cardiac, vascular and central effects. The methods used for apelin quantification include enzyme immunoassays and radioimmunoassays but give a wide range of basal values depending on the studies. More reliable assays, easy to use, are necessary. It will also be important to know whether the elevated serum apelin concentrations correspond to active apelin and what are the predominant forms of apelin in metabolic diseases and their variations. Finally, selective agonists and antagonists for APJ started to be developed but they need to be tested on metabolic tissues and their signaling more largely described. All these points are important in order to validate the promising anti-diabetic properties of apelin.

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Diet impact on mitochondrial bioenergetics and dynamics

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Diet induced obesity is associated with impaired mitochondrial function and dynamic behavior. Mitochondria are highly dynamic organelles and the balance in fusion/fission is strictly associated with their bioenergetics. Fusion processes are associated with the optimization of mitochondrial function, whereas fission processes are associated with the removal of damaged mitochondria. In diet-induced obesity, impaired mitochondrial function and increased fission processes were found in liver and skeletal muscle. Diverse dietary fat sources differently affect mitochondrial dynamics and bioenergetics. In contrast to saturated fatty acids, omega 3 polyunsaturated fatty acids induce fusion processes and improve mitochondrial function. Moreover, the pro-longevity effect of caloric restriction has been correlated with changes in mitochondrial dynamics leading to decreased cell oxidative injury. Noteworthy, emerging findings revealed an important role for mitochondrial dynamics within neuronal populations involved in central regulation of body energy balance. In conclusion, mitochondrial dynamic processes with their strict interconnection with mitochondrial bioenergetics are involved in energy balance and diet impact on metabolic tissues.

Keywords: mitochondrial fusion, mitochondrial fission, dietary fat, caloric restriction, energy balance

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Mitochondrial Dynamics and Bioenergetics

Mitochondria are referred to as the "powerhouses" of the cell due to their prominent role in ATP production and cellular metabolism regulation. Together with their energetic role, mitochondria are very dynamic organelles that continuously divide, collide and fuse with other mitochondria. Therefore, their morphology is highly variable. It can shift between small round punctuated structures or reticulum networks of elongated mitochondria as a result of the balance between fusion and fission processes. Various work has been done to analyze the mechanism of this dynamic behavior and different proteins (a group of large ATPases) have been identified as being involved (James et al., 2003; Ishihara et al., 2004, 2006; Jagasia et al., 2005; Liesa et al., 2009). In particular, the main proteins involved in mammalian mitochondrial fusion processes are mitofusin 1 and 2 (Mfn1 and 2) and autosomal dominant optic atrophy-1 (Opa1). Mitofusin is located on the outer mitochondrial membrane and plays a role in the outer membranes fusion, whereas Opa1 is located on the inner mitochondrial membrane and is involved in the inner membrane fusion process (Malka et al., 2005). In addition, Opa1 controls cristae remodeling and protects from apoptosis (Frezza et al., 2006). It should be noted that also Mfn2 has a dual role, since other than its well-known role in mitochondrial fusion (Zorzano et al., 2010), Mfn2 is also implicated in the structural and functional connection between mitochondria and the endoplasmic reticulum (ER) and it may play a role in ER stress development in conditions of metabolic stress (de Brito and Scorrano, 2008, 2009). As regards fission processes, dynamin related protein 1 (Drp1) and fission protein 1

(Fis1) are the main proteins involved. Drp1 is located mainly in the cytosol and is recruited on the outer mitochondrial membrane by Fis1 that is inserted on the outer membrane (Yoon et al., 2003; Santel and Frank, 2008). Drp1 is also involved in the regulation of apoptosis (Frank et al., 2001).

A correct balance between fusion and fission processes is important for mitochondrial bioenergetics. It has been suggested that mitochondrial fusion processes are induced in conditions in which an optimization of mitochondrial bioenergetics is required, whereas fission processes are associated with mitochondria degradation and therefore they are induced under conditions in which mitochondria are damaged (Westermann, 2012). Deficiency in proteins involved in mitochondrial fusion (Mfn2 and Opa1) reduce respiration in several cell types (Liesa et al., 2009). Mfn2 repression is associated with decreased substrate oxidation and cellular metabolism (Pich et al., 2005). Alterations in OPA1 expression also affect mitochondrial bioenergetics, for example Opa1 depletion causes a reduction in basal respiration (Chen et al., 2005). Therefore, emerging evidence suggests that the balance between mitochondrial fusion and fission processes play an important role in the regulation of mitochondrial energetics.

Several recent reviews analyzed the cellular roles of mitochondrial dynamics and the molecular mechanisms of fusion and fission (McBride et al., 2006; Detmer and Chan, 2007; Hoppins et al., 2007; Liesa et al., 2009; Westermann, 2010, 2012; Elgass et al., 2013; Zhao et al., 2013; Lackner, 2014; da Silva et al., 2014), the present review focused on the impact of diet on mitochondrial dynamic behavior and function in the main metabolic tissues, such as liver and skeletal muscle, as well as the involvement of mitochondrial dynamic processes in body energy balance regulation.

Mitochondrial Dynamics and Bioenergetics in Obesity

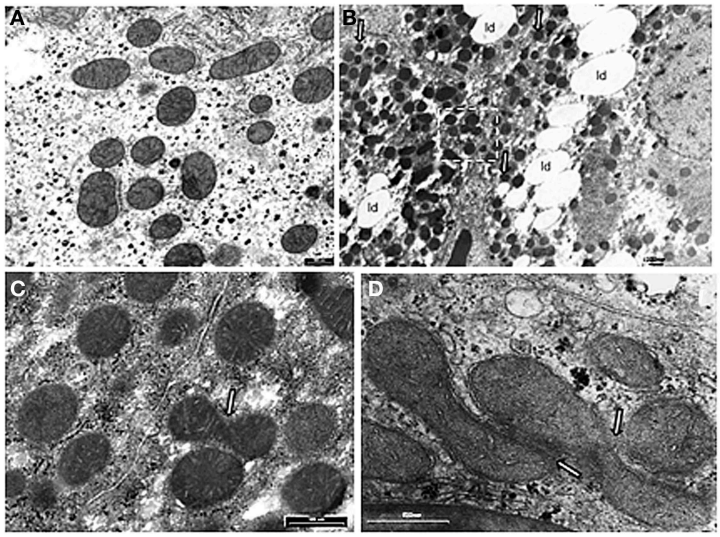
It is well known that mitochondrial dysfunction plays an important role in obesity related diseases, such as insulin resistance and non-alcoholic fatty liver diseases. It has also been shown that impairments of mitochondrial function are associated with changes in mitochondrial network in the main tissues involved in obesity related diseases. Indeed, in obese and insulin resistant Zucker rats, skeletal muscle is characterized by a reduced glucose uptake, insulin resistance and reduced oxygen consumption accompanied by a reduction in Mfn2 expression, mitochondrial size and in the extent of the mitochondrial network (Bach et al., 2003). Reduced Mfn2 expression was also reported in skeletal muscle of obese type 2 diabetic patients. A decreased mitochondrial proton leak and increased bioenergetics efficiency in Mfn2-depleted cells has been demonstrated (Bach et al., 2003). Therefore, it has been suggested that Mfn2 loss-of-function found in obesity conditions enhance bioenergetics efficiency and contribute to obesity development by reducing energy expenditure and increasing fat energy store (Liesa et al., 2009). In line with this suggestion, conditions characterized by increased energy expenditure (such as cold exposure, administration of β3 adrenergic agonist, chronic exercise) are associated with higher Mfn2 expression in skeletal muscle and brown adipose tissue (Cartoni et al., 2005; Soriano et al., 2006). Furthermore, an increase in mitochondrial fission proteins was reported in skeletal muscle in mice with genetically induced obesity (ob/ob) as well as in mice with high fat diet-induced obesity (Jheng et al., 2012). Mitochondrial dysfunction was associated with enhanced fission processes in liver from db/db mouse, animal model of obesity and insulin resistance (Holmström et al., 2012). All these reports suggest that a shift toward fission processes is linked to mitochondrial dysfunction in the main tissues, such as skeletal muscle and liver involved in obesity related metabolic disease. In line with this suggestion, our group recently published data on the impact of high fat diet on mitochondrial function and dynamic proteins content in rat skeletal muscle and liver. In particular, we analyzed the effect of two different fat dietary sources (saturated vs. polyunsaturated omega 3) on the above parameters (Lionetti et al., 2014).

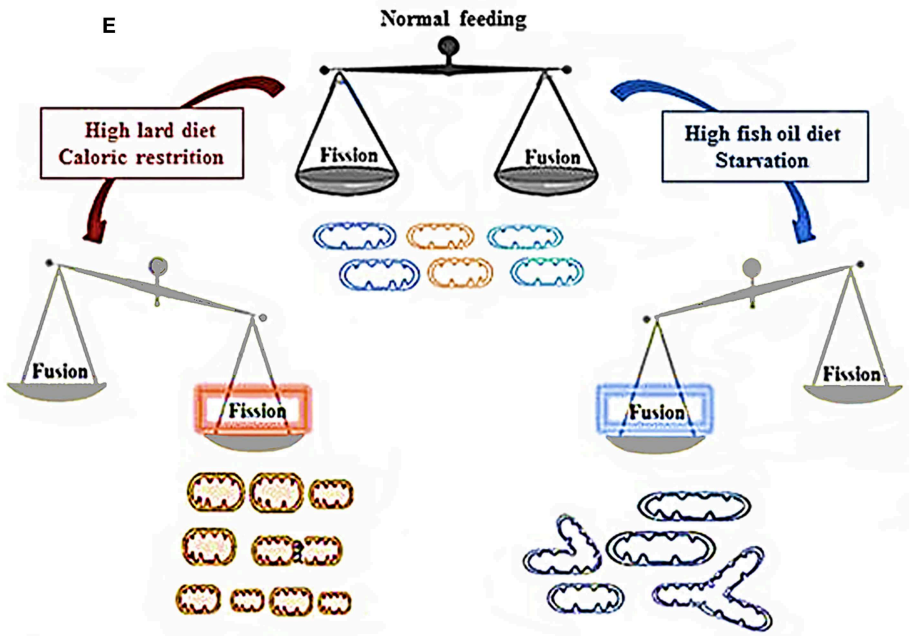
Impact of Dietary Saturated Fatty Acids

High fat diet rich in saturated fatty acids (high lard, HL, diet) elicited hepatic fat accumulation and insulin resistance, which was parallel to impaired mitochondrial function, increased reactive oxygen species (ROS) production and a dysregulated expression profile of mitochondrial dynamics proteins (Lionetti et al., 2014). In particular, HL diet induced an increase in mitochondrial fatty acid utilization, but a decrease in $FADH_2$ linked oxygen consumption and in fatty acid induced proton leak, which caused an increase in mitochondrial energetic efficiency and an increase in ROS content. As regards to mitochondrial dynamics proteins, HL diet elicited a decrease in Mfn2 and an increase in protein involved in fission processes (Drp1 and Fis1) accompanied by the presence of numerous small round mitochondria vs. control diet, as observed by electron microscopy (**Figures 1A–C,E**) (Lionetti et al., 2014).

As regards to skeletal muscle, our results suggest that an HL diet also induced a shift toward fission as observed by electron microscopy and by immunureactivities analysis (Lionetti et al., 2013) which showed weak signal for fusion proteins and strong signals for fission proteins in rats which were fed an HL diet (Lionetti et al., 2013). These findings are in line with previous reports that Mfn2 expression is reduced in skeletal muscle of obese Zucker rats and type 2 diabetic patients (Bach et al., 2005; Hernández-Alvarez et al., 2010). In addition, it has also been suggested that Mfn2 plays a role as a regulator of *in vivo* insulin sensitivity and may be a potential target in diabetes drug development (Sebastián et al., 2012). Indeed, Mfn2 deficiency in mice produces mitochondrial dysfunction, increases oxidative stress and endoplasmic reticulum stress and activates JNK impairing insulin signaling in liver and skeletal muscle (Sebastián et al., 2012). This study suggested a role of Mfn2 in coordinating mitochondria and endoplasmic reticulum function, leading to modulation of insulin signaling *in vivo*.

Moreover, saturated fatty acids have also been reported to induce fission processes *in vitro* in differentiated C2C12 skeletal muscle cells (Jheng et al., 2012) associated with mitochondrial dysfunction. *In vivo*, as previously reported, smaller





	High lard diet vs. High fish oil diet	Caloric restrition vs. Starvation
DRP1, Fis1	↑	↑
MFN2, OPA1	↓	—
ROS	↑	↓
Δψ	↑	↓
Mitochondrial dysfunction	↑	↑
Mitophagy	?	↑

FIGURE 1 | Mitochondrial morphology and dynamics. (A–D) Electron micrographs of ultrathin rat liver sections. **(A)** section of hepatocytes of rat fed a control diet. The mitochondria showed normal features with tubular, ellissoidal and round profiles. **(B)** section of hepatocyte of rat fed a HL diet. Note the abundant lipid droplet (ld) in cytosol and the prevalence of small mitochondria with round profile. **(C)** Higher magnification of insert in B showing an image suggesting a fission process (arrow). **(D)** section of hepatocyte of rat fed a HFO diet showing a cluster of mitochondria in which the fusion process is ongoing (arrows). **(E)** Diet impact on mitochondrial

(Continued)

FIGURE 1 | Continued
dynamics and function. See text for details. Shift toward fission processes has been found in response both to HLD and CR. However, in response to HLD, fission processes are associated to impairment of mitochondrial function and oxidative stress and may be useful to improve substrate intake in mitochondria or remove damaged mitochondria. On the other hand, in response to CR, mitochondrial fragmentation is associated to mitochondrial biogenesis useful to the maintenance of ATP level to meet cellular energetic needs. In addition, during CR, oxidative stress is reduced and mitochondrial fission may contribute to remove damaged mitochondria by mitophagy explaining the prolongevity effect of CR. Noteworthy, in condition of a more stressed nutrient deprivation, such as cell starvation, it has been reported an increase in mitochondrial fusion processes with elongated mitochondria that were spared by mitochondrial degradation and contributed to increase ATP production to face energetic needs. Fusion processes were found to be increased also in condition of HFO diet, where they contributed to an increase in mitochondrial substrate oxidation and to avoid lipid accumulation and oxidative stress. HLD, high fat diet rich in lard; HFO, high fat diet rich in fish oil; $\Delta\psi$, mitochondrial membrane potential; ROS, reactive oxygen species; CR, caloric restriction ⬆= increase, ⬇= decrease; −= no changes.

mitochondria and increased mitochondrial fission machinery have been described in the skeletal muscle of mice with genetic obesity and those with diet-induced obesity (Jheng et al., 2012). Moreover, inhibition of mitochondrial fission improved the muscle insulin signaling and systemic insulin sensitivity of obese mice (Civitarese and Ravussin, 2008).

Mitochondrial fragmentation and increased fission processes in association with ROS formation have also been reported after treatment with high glucose (HG) in both a rat liver cell-line and myoblasts (Yu et al., 2006). Such fragmentation has been suggested to provide metabolically active organelles with increased total surface area that would increase accessibility of metabolic substrate to carrier proteins (Yu et al., 2006). Therefore, it can also be suggested that HL diet induced-mitochondrial fragmentation might be an adaptive cellular response to increase mitochondrial intake and oxidation of surplus of dietary fatty acids, which would result in elevated ROS production (Lionetti et al., 2014).

Impact of Omega 3 Polyunsaturated Fatty Acids

Different sources of dietary fats have been suggested to have different effects on mitochondrial function and dynamic behavior. In fact, in contrast to the effect of saturated fatty acids, omega 3 polyunsaturated fatty acids have been reported to improve mitochondrial function, reduce ROS production, and promote mitochondrial fusion both *in vitro* and *in vivo* experiments. In an *in vitro* steatotic hepatocyte model both eicosapentaenoic and docosahexaenoic acids increased the expression of Mfn2 and the ATP levels, and decreased oxidative stress (Zhang et al., 2011). On the other hand, in Mfn2-depleted steatotic hepatocytes, omega 3 PUFA did not induce the previously described effects (Zhang et al., 2011).

These data obtained *in vitro* were in line with our data showing improvement of mitochondrial function, reduced ROS production and induction of hepatic mitochondrial fusion through fish oil i*n vivo* (Lionetti et al., 2014). Indeed, the comparison of the effect of high fat diet rich in fish oil (HFO diet) and HL diet in rats showed that HFO diet led to less lipid accumulation in liver and higher fatty acid utilization. We also observed in HFO diet fed rats a mild mitochondrial uncoupling due to enhanced expression of uncoupling protein 2. These decreases in mitochondrial efficiency might contribute to increased fatty acid utilization and reduce ROS production. In HFO diet fed rats, a shift toward fusion was found with concomitant increases in Mfn2 and Opa1 as well as decreases in Drp1 and Fis1, in line with an increased number of tubular mitochondria observed by electron microscopy compared to HL diet (**Figures 1D,E**). This fusion phenotype was in accordance with reduced weight gain found in HFO diet vs. HL diet fed rats. With the limitation that the cause-consequence relationship between the leaner phenotype of HFO diet vs. HL diet fed rats and mitochondrial dynamics is not known, it can be suggested that the specific dietary fatty acid composition may play a role in obesity and hepatic steatosis development as well as in mitochondrial bioenergetics and dynamics (Lionetti et al., 2014).

Similar results were found in skeletal muscles of rats fed HFO diet, where, compared to HL diet, reduced fission processes and increased fusion events were suggested by immuoreactivity analysis and electron microscopy (Lionetti et al., 2013). Indeed, skeletal muscle sections from HFO fed rats showed a greater number of immunoreactive fibers for Mfn2 and Opa1 protein as well as weaker immunostaining for Drp1 and Fis1 compared to sections from HL fed rats. The shift toward fusion events in HFO fed rats was associated with the improvement of obesity and systemic and skeletal muscle insulin sensibility (Lionetti et al., 2013).

Differential effects of saturated and unsaturated fatty acids on mitochondrial morphology and dynamics were reported *in vitro* in C2C12 skeletal muscle cells. The results showed that treatment with saturated fatty acids induced mitochondrial fragmentations, whereas unsaturated and polyunsaturated fatty acids protected against palmitate-induced mitochondrial fission (Jheng et al., 2012).

Starvation, Caloric Restriction and Mitochondrial Dynamics

Opposite mitochondrial dynamics behavior has been reported in two different conditions of nutrient deficiency, such as starvation (Rambold et al., 2011) and caloric restriction (CR) (Khraiwesh et al., 2013).

Mitochondrial elongation is a reversible response to nutrient deprivation in many cell culture types (Rambold et al., 2011). It depends on the type of nutrient depleted. Indeed, either glucose or serum elimination increased mitochondrial fragmentation, whereas mitochondrial fusion was induced by a nitrogen-source deficiency (either glutamine or amino acids). However, a combination of nutrient depletions induced a further mitochondrial elongation, suggesting that mitochondrial fusion can be modulated according to type and severity of starvation. Starvation-induced mitochondrial fusion depends on Mfn1 and Opa1 and is mediated by decreased DRP1 fission activity and by preventing Drp1 localization to mitochondria (**Figure 1E**). Mitochondrial fusion has a protective function, leading to an exchange of

mitochondrial DNA and delaying apoptosis (Chen et al., 2010; Rambold et al., 2011). In fact, mitochondrial elongation might be useful to protect mitochondria from mitophagy. In accordance, during the initial period of starvation cytoplasmic components are degraded, whereas mitochondria become substrate much later (Kristensen et al., 2008), because mitochondria spared from degradation may contribute to maximize cellular energy production to sustain cell during nutrient deprivation (Rambold et al., 2011). Accordingly, mitochondrial fusion has been associated with increase in ATP production during stress and starvation (Gomes et al., 2011) producing beneficial effects for cells under conditions of low nutrient supply. Interestingly, it has been demonstrated that mitochondria provide membrane to autophagosomes during starvation (Hailey et al., 2010) and it can be also suggested that sparing mitochondria may be useful to permit them to serve as an autophagosome membrane source in nutrient depletion conditions.

On the other hand, in a study performed to evaluate dynamic mitochondrial behavior in an animal model of CR (mice submitted to 40% CR for 6 months), proteins related to mitochondrial fission (Fis1 and mitochondrial Drp1) increased, but no changes were detected in proteins involved in mitochondrial fusion (Mfn1/Mfn2, and Opa1) (Khraiwesh et al., 2013) (**Figure 1E**). A significant increase in the number of mitochondria per cells as well as in parameters related to mitochondrial biogenesis was also found in CR conditions (Nisoli et al., 2005; López-Lluch et al., 2006). Given that fission is the postulated mechanism for mitochondrial proliferation (Scheffler, 2007), the increase in Fis1 and Drp1 proteins support the idea of increased mitochondrial biogenesis in CR. Moreover, in a model of *in vitro* CR, the greater number of mitochondria was linked to reduced oxygen consumption and membrane potential (López-Lluch et al., 2006). As it has been demonstrated that ROS production by electron leakage increases at high membrane potential (Lambert and Merry, 2004), the decreased membrane potential found in CR conditions is in agreement with the lower ROS production associated with CR. Noteworthy, the levels of ATP production were no different in CR conditions vs. control (Khraiwesh et al., 2013). In effect, CR induced an increase in the number of mitochondria capable to maintain critical ATP levels in conditions of decreased oxidative stress. It is well known that CR attenuates age-dependent oxidative damage and it is correlated with an extension of lifespan in animals as well as with prevention of cancer and diabetes (Sohal and Weindruch, 1996; Colman et al., 2009). It has been suggested that the increase in fission proteins found in CR may be useful in removing damaged mitochondria and to support the prolongevity effect of CR (López-Lluch et al., 2008; Khraiwesh et al., 2013). This suggestion seems to be in contrast with the report that unopposed mitochondrial fission in absence of mitochondrial fusion in the Δmgm1 mutants of S. cerevisiae (yeast Mgm1 is the ortholog of mammalian Opa1) leads to severe lifespan shortening (Scheckhuber et al., 2011). However, it should be noted that the different correlation found between mitochondrial fission processes and lifespan may be due to the different experimental model.

Mitochondrial Dynamics in Regulation of Energy Balance

It is well known that in mammals the NPY/Agrp and POMC neurons within the arcuate nucleus of the hypothalamus regulate hunger and satiety. Recent works suggested that mitochondrial dynamics play an important role in these two neuronal populations, and that Mfn1 and Mfn2 are involved (reviewed in Nasrallah and Horvath, 2014). During positive energy balance (high fat diet exposure) in mice, mitochondrial fusion increased in orexigenic NPY/Agrp neurons to enable elevated neuronal activity and maximize storage of excess energy in fat (Dietrich et al., 2013). The electric activity of NPY/Agrp neurons was impaired when mitochondrial fusion mechanism was altered by cell-selectively knocking down MFN1 or Mfn2. The decreased activity of Agrp neurons was correlated with resistance to fat gain during high fat diet in Agrp-specific Mfn1 or Mfn2 knockout mice (Dietrich et al., 2013). Conversely in anorexigenic pro-opiomelanocortin POMC neurons, Mfn2 selective deletion causes severe obesity and leptin resistance (Schneeberger et al., 2013) probably by mediating ER stress-induced leptin resistance. In fact, ER stress plays a role in the development of obesity and leptin resistance (Zhang et al., 2008; Lionetti et al., 2009; Ozcan et al., 2009; Mollica et al., 2011) and genetic loss of Mfn2 generates ER stress (Sebastián et al., 2012). Indeed, Mfn2 plays an important role in the structural and functional communication between mitochondria and ER (de Brito and Scorrano, 2008). Schneeberger et al. (2013) showed that specific ablation of Mfn2 in POMC neurones causes a decrease in mitochondrial respiratory capacity and an increase in oxidative stress as well as loss of mitochondria-ER contacts, ER stress-induced leptin resistance, hyperphagia, reduced energy expenditure and obesity. Moreover, in diet induced obese mice, it was shown a decrease in mitochondrial network complexity and in mitochondria-ER association due to a reduction in Mfn2 expression in the hypothalamus which precedes the onset of obesity. On the other hand, Mfn2 overexpression ameliorates the diet induced obese phenotype (Schneeberger et al., 2013).

In all, these data suggested that mitochondrial dynamics, namely Mfn1 and Mfn2, in NPY/Agrp and POMC neurons may play a role in the central regulation of energy balance and in etiology of diet induced obesity.

Concluding Remarks

Mitochondrial function varies in accordance to cellular energetic needs and to nutrient supply. Noteworthy, a number of recent works have been focused on the importance of mitochondrial dynamic behavior in terms of fusion and fission processes in determining mitochondrial functionality in diverse diet conditions as well as in the central regulation of energy balance. Therefore, mitochondrial dynamic behavior contributes to bioenergetics physiological adaptation in response to the nutritional status (**Figure 1E**).

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Mitochondrial uncoupling proteins and energy metabolism

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Understanding the metabolic factors that contribute to energy metabolism (EM) is critical for the development of new treatments for obesity and related diseases. Mitochondrial oxidative phosphorylation is not perfectly coupled to ATP synthesis, and the process of proton-leak plays a crucial role. Proton-leak accounts for a significant part of the resting metabolic rate (RMR) and therefore enhancement of this process represents a potential target for obesity treatment. Since their discovery, uncoupling proteins have stimulated great interest due to their involvement in mitochondrial-inducible proton-leak. Despite the widely accepted uncoupling/thermogenic effect of uncoupling protein one (UCP_1), which was the first in this family to be discovered, the reactions catalyzed by its homolog UCP_3 and the physiological role remain under debate. This review provides an overview of the role played by UCP_1 and UCP_3 in mitochondrial uncoupling/functionality as well as EM and suggests that they are a potential therapeutic target for treating obesity and its related diseases such as type II diabetes mellitus.

Keywords: uncoupling protein, energy metabolism, mitochondria, proton-leak, obesity

INTRODUCTION

Mitochondria are the major regulators of cellular energy metabolism (EM). Alterations of mitochondrial functionality have been linked to the pathogenesis of some metabolic disorders, including obesity and type II diabetes mellitus (T2DM).

The principal function of mitochondria is ATP production. Reduced cofactors, such as NADH and $FADH_2$, obtained from oxidizable molecules (carbohydrates, lipids, and proteins) supply electrons to the electron transport chain (ETC) and result in a final reduction of molecular oxygen to water. Electron transfer through the ETC controls pumping of protons (H^+) from the matrix to the intermembrane space, thus creating a proton-motive force (Δp), whose energy is used by ATP synthase for the phosphorylation of ADP.

Mitochondrial oxidative phosphorylation is not perfectly coupled to ATP synthesis, since a portion of the energy liberated from the oxidation of dietary energy substrates is lost as heat instead of being converted into ATP. Indeed, some of the energy present in Δp dissipates as heat by the re-entry of H^+ into the matrix, through pathways independent of ATP synthase (proton-leak). Proton-leak is the sum of two processes: basal- and inducible proton-leak (Brand and Esteves, 2005). The first is not acutely regulated, but rather depends on the fatty-acyl composition of the mitochondrial inner membrane and the presence of adenine nucleotide translocase. On the other hand, inducible proton-leak is acutely controlled, with uncoupling proteins playing a crucial role (Divakaruni and Brand, 2011) (**Figure 1**).

Proton-leak has a marked influence on the entire EM of an organism, and, in rats, accounts for approximately 20–30% of the resting metabolic rate (RMR) (Rolfe and Brand, 1996). Variations in the proton-leak process contribute to the development of obesity or weight loss (Harper et al., 2008), and thus there has been increasing interest in targeting this process in order to increase RMR to treat obesity and other related diseases (**Figure 1**).

The present review discusses the relevance of UCPs on influencing EM. In particular, it focuses on uncoupling protein one (UCP_1) and UCP_3 due to their expression in brown adipose tissue (BAT) and skeletal muscle (SkM), respectively, which significantly contributes to EM.

UNCOUPLING PROTEIN-1 (UCP_1)

UCPs are members of the mitochondrial anion carrier family. The first uncoupling protein identified, UCP_1, is predominantly expressed in BAT where it represents approximately 10% of the mitochondrial protein content and plays a thermogenic role through the catalysis of proton-leak. Recent evidence indicates the existence of two types of thermogenic adipocytes expressing UCP_1: the classical brown adipocytes, which are found in the intercapsular brown adipose organ, and beige/bright adipocytes, which are found in subcutaneous WAT. Only recently it has unequivocally been proven that UCP_1-positive cells found in WAT (i.e., beige adipocytes) are thermogenic-competent (Shabalina et al., 2013) and are actually a distinct subpopulation of white adipocytes that originate from a different lineage (reviewed in Harms and Seale, 2013).

One of the most prominent differences between brown and beige adipocytes is that brown cells express high levels of UCP_1 and other thermogenic genes under basal (unstimulated) conditions, whereas beige adipocytes only express these genes in response to activators, such as β-adrenergic and peroxisome

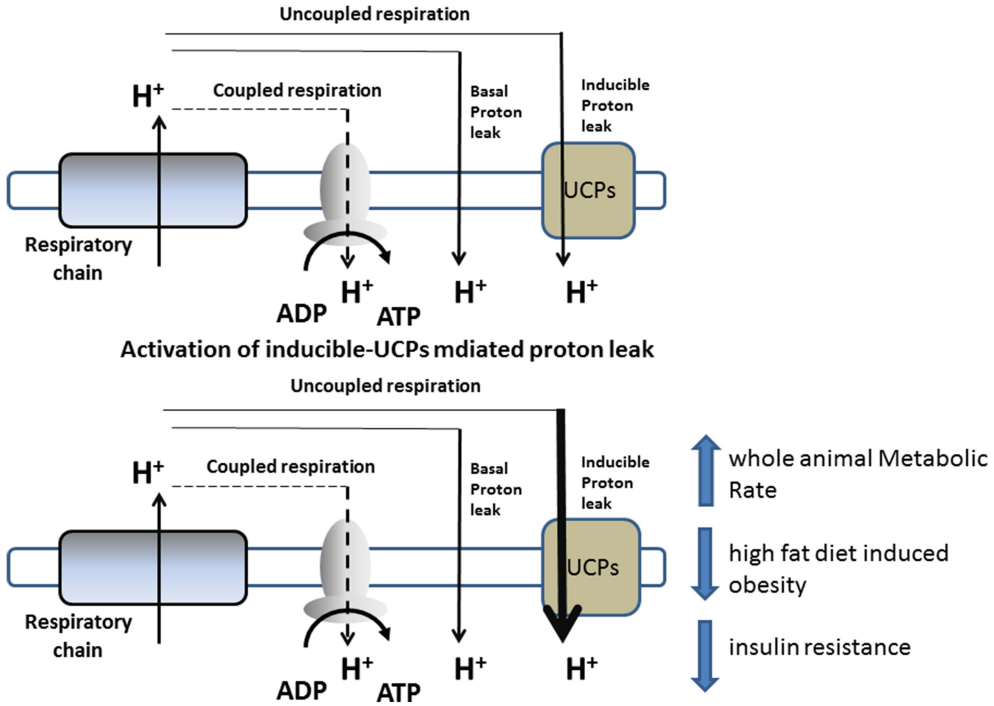

FIGURE 1 | Schematic representation of proton-leak and of the proposed role of UCPs in influencing energy metabolism.

proliferator-activated receptor-γ agonists (Petrovic et al., 2010; Wu et al., 2013; Carrière et al., 2014). Nevertheless, both adipocyte types show comparable levels of UCP_1 upon stimulation, indicating that under specific conditions they may have the same thermogenic capacity (Wu et al., 2012).

Cold and overfeeding are physiological stimuli that influence BAT thermogenesis through the enhancement of sympathetic overflow to BAT (Cannon and Nedergard, 2004). Acute sympathetic nerve activation stimulates heat production by activating UCP_1 function. Indeed, noradrenergic stimulation of brown adipocytes activates cAMP-linked signaling pathways, which results in increased (i) mitochondria number and size, (ii) UCP_1 gene transcription and translation, and (iii) UCP_1 thermogenic activity through activation of lipolysis and release of acute regulators of UCP_1, such as free fatty acids.

Prolonged cold exposure stimulates the proliferation and differentiation of brown precursor cells to expand BAT mass and increase thermogenic capacity (Bukowiecki et al., 1982). Moreover, it also induces beige adipocyte development and function (Vitali et al., 2012). Although sympathetic nerve activity was previously thought to be the major physiological signal that activates BAT thermogenesis and induces beige adipocyte development, numerous hormones and factors have now been shown to regulate such processes. Moreover, brown and beige adipocytes can be selectively recruited and activated (for review Cannon and Nedergard, 2004; Harms and Seale, 2013). The molecular mechanisms of UCP_1 activation have been comprehensively discussed in other recent reviews and will not be elaborated upon here (Klingenberg and Echtay, 2001; Porter, 2008).

ROLE OF UCP_1 IN ENERGY METABOLISM

The presence of UCP_1 provides a marked capacity for BAT to dissipate energy by as much as 20%. In rodents, overeating activates BAT "diet-induced thermogenesis," that preserve energy balance and contrasts obesity by reducing metabolic efficiency (Cannon and Nedergard, 2004). The overexpression of UCP_1 or activation of BAT thermogenesis has been shown to prevent the development of obesity (Krauss et al., 2005). In contrast, the first experiments performed on $UCP_1^{-/-}$ mice housed at a standard temperature (20–22°C) failed to show an enhanced obesity predisposition compared to their wild-type counterparts (Enerback et al., 1997). More recently, Feldmann et al. (2009) reported that UCP_1 ablation induced obesity in mice housed at 30°C, i.e., the thermoneutral temperature at which mice facultative thermogenesis is kept at a minimum. Indeed, when housed at 20–22°C, mice are under chronic thermal stress and inevitably increase their metabolic rate (∼50%) to maintain body temperature (Golozoubova et al., 2006). Therefore, $UCP_1^{-/-}$ mice, that are unable to enhance BAT thermogenesis, have necessarily expend extra energy to defend their body temperature; the activation of alternative compensatory mechanisms, such as shivering thermogenesis, could have masked the role of UCP_1 on EM. The study of Feldmann et al. (2009) also suggests that the impact of housing temperature on EM has been overlooked by most of the studies on animal models, and that cold stress could have confounded many studies. Humans tend to live at thermoneutrality, with the aid of clothing and heating. Thus, to translate the metabolic studies performed in mice to humans, experiments should be conducted under thermoneutral conditions.

Other evidence also support the role for UCP_1 in EM. For example, transgenic mice expressing UCP_1 in white fat depots display a lean phenotype (reviewed in Klaus et al., 2012). Moreover, muscle-specific ectopic expression of UCP_1 leads to increased energy expenditure, delayed diet-induced obesity development, improved glucose homeostasis, increased insulin stimulated glucose uptake, and increased lipid metabolism (Keipert et al., 2013; Ost et al., 2014). This is in agreement with evidence showing that the selective induction of SkM UCP_1-mediated proton-leak leads to an increased whole body energy expenditure as well as decreased adiposity (Adjeitey et al., 2013).

When chronically activated by cold exposure, UCP_1 mediated-BAT thermogenesis enhances the oxidation of metabolic substrates necessary for sustaining enhanced thermogenesis. Under these conditions, BAT not only use stored lipids as substrates, but also large quantities of glucose and triglycerides (the last mainly in the form of chylomicrons) from the circulation (Bartelt et al., 2012; Peirce and Vidal-Puig, 2013). Therefore, by decreasing plasma lipids, lowering plasma glucose, and diminishing obesity, BAT has a potential role in protecting against obesity and T2DM.

UCP_1 EXPRESSING ADIPOCYTES ARE PRESENT IN ADULT HUMAN

It was previously thought that in humans, BAT disappears rapidly after birth. However, more recently the use of radiodiagnostic techniques [positron emission tomography (PET)/computed tomography (CT)] together with histological methods has unequivocally identified the presence of UCP_1-expressing adipocyte depots in adult humans. The tissue is not present in defined regions, but rather scattered within the WAT (Nedergaard et al., 2007). However, it is currently unclear whether the deposits of UCP_1-expressing adipocytes in adult humans are analogous to beige or brown fat.

The enhancement of BAT activity or the browning of WAT in humans has been linked to cold tolerance, enhanced energy expenditure, and protection against metabolic diseases, such as obesity and T2DM (for review Harms and Seale, 2013; Saito, 2013). In fact, the amount of metabolic active BAT in humans positively correlates with RMR and inversely with body mass index (BMI), fat mass (van Marken Lichtenbelt et al., 2009), and the development of T2DM. In addition, it has been shown that genetic variants of UCP_1 are associated with fat metabolism, obesity, and diabetes. Among the UCP_1 polymorphisms, the A3826G SNP in the promoter region has been associated with obesity, weight gain, and resistance to weight loss (reviewed in Jia et al., 2010).

UNCOUPLING PROTEIN-3 (UCP_3)

UCP_3 is primarily expressed in skeletal muscle, but it is also found in BAT and heart tissue. Although UCP_3 was first discovered and described in 1997 (Boss et al., 1997), the mechanisms of activation as well as its physiological role is still under debate (Cioffi et al., 2009). Due to its close homology with UCP_1, UCP_3 was initially implicated in thermoregulation (Boss et al., 1997), as it has been demonstrated to uncouple in a number of experimental models. However, other evidence has questioned the uncoupling activity of UCP_3, including findings that (i) higher expression of UCP_3 is not always associated with increased mitochondrial uncoupling (such as during fasting Cadenas et al., 1999) and (ii) $UCP_3^{-/-}$ mice do not show thermoregulation problems and are not obese (Gong et al., 2000; Vidal-Puig et al., 2000). Indeed, UCP_3 mediated-uncoupling (called mild uncoupling) seems to be activated by specific cofactors, with FFA and reactive oxygen species (ROS) playing crucial and interrelated roles (Brand and Esteves, 2005; Lombardi et al., 2008, 2010), being the absence of one on these cofactors able to musk UCP3-mediated uncoupling.

UCP3 is also thought to play key regulatory roles in mitochondrial fatty acid oxidation and in preventing mitochondrial ROS-induced oxidative damage (Cioffi et al., 2009). Regarding the former, studies relating to the enhancement in SkM UCP3 expression have provided evidence for a close relationship to situations in which there is increased fatty acid oxidation (reviewed in Bezaire et al., 2007; Cioffi et al., 2009). SkM mitochondria isolated from $UCP_3^{-/-}$ mice have a lower ability to oxidize fatty acids than those from $UCP_3^{+/+}$ mice (Costford et al., 2008; Senese et al., 2011), that plausibly is responsible for the greater fat storage observed following long-term high fat feeding in mice lacking UCP_3 (Nabben et al., 2011).

UCP_3 also plays an important role in protecting cells against oxidative damage (Brand and Esteves, 2005; Goglia and Skulachev, 2003; Nabben et al., 2008; Schrauwen and Hesselink, 2004b). UCP_3 may aid in mitigating ROS emission from the ETC; this role seems to be related to UCP_3-mediated uncoupling and the consequent reduction in Δp. In fact, Δp is a key factor in influencing mitochondrial O^{2-} release (Korshunov et al., 1997) and a slight reduction in Δp is associated to a significant depression of O^{2-} release. Interestingly, ROS itself or ROS by-products can induce UCP_3 uncoupling, which provides a negative feedback loop for mitochondrial ROS production (Brand and Esteves, 2005). The sequential molecular mechanisms underlying ROS induced- and UCP_3-mediated uncoupling seems to involve glutathionylation of the protein: a slight increase in ROS production promotes UCP_3 de-glutathionylation, which activates UCP_3-mediated uncoupling and further decreases ROS emission (Mailloux et al., 2011).

Another hypothesis concerning the role of UCP_3 in protecting mitochondria from oxidative damage suggests that UCP_3 is involved in the export of LOOH, which accumulates on the matrix side of the mitochondrial inner membrane (MIM), out of the matrix. This mechanism would reduce or eliminate LOOH from the inner leaflet of the MIM, which could otherwise trigger a cascade of oxidative damage to mitochondrial DNA and enzymes as well as other critical mitochondrial matrix-localized components (Goglia and Skulachev, 2003). This hypothesis has been validated by studies on mitochondria isolated from $UCP_3^{+/+}$ and $UCP_3^{-/-}$ mice, which underscores the ability of UCP_3 to translocate LOOH and mediate LOOH-dependent mitochondrial uncoupling (Lombardi et al., 2010).

ROLE OF UCP_3 IN ENERGY METABOLISM

Many studies have demonstrated that mitochondrial functionality is compromised in obesity and T2DM. In skeletal muscle,

decreased mitochondrial uncoupling together with impaired mitochondrial fatty acid oxidation lead to fatty acids and/or their metabolites accumulation which, associated to increased oxidative stress, are crucial aspects in the development and progression of the above pathologies (Goodpaster et al., 2001; Patti and Corvera, 2010). Indeed, states of insulin-resistance are characterized by increased LOOH levels in SkM, and patients with T2DM exhibit damaged mitochondria with reduced functional capacity.

The involvement of UCP_3 in mild uncoupling, in protection from ROS- and/or LOOH-induced oxidative stress, and in the amelioration of the fatty acid oxidation rate (see above) suggest a protective role for this protein in obesity and T2DM (**Figure 2**). This possibility is strengthened by findings that UCP_3 is expressed in SkM, which is a tissue that represents 40% of metabolic active mass and largely contributes to energy homeostasis.

More experimental evidence supports a role of UCP_3 in EM, including the findings that (i) UCP_3 transgenic mice are less efficient in metabolism than wild-type controls (Costford et al., 2006) and are protected against high fat diet-induced obesity (Son et al., 2004; Costford et al., 2006); (ii) an enhancement of SkM UCP_3 levels (also associated with higher BAT UCP_1 levels) has been observed in obesity-resistant mice compared to obesity-prone mice (Fink et al., 2007); and (iii) modest UCP_3 overexpression in skeletal muscle mimics physical exercise by increasing spontaneous activity and energy expenditure in mice (Aguer et al., 2013). Conversely, studies performed on $UCP_3^{-/-}$ mice argue against a role for UCP_3 in EM, since these mice do not present reduced RMR and are not obese (Vidal-Puig et al., 2000). However, these studies were performed in mice housed at 20–22°C, a condition that, as described above, represents chronic thermal stress [see Uncoupling Protein-1 (UCP_1) section]. Therefore, the occurrence of compensatory thermogenic mechanisms in $UCP_3^{-/-}$ mice could have masked the possible role of the protein in EM.

UCP_3 also shows a protective role in T2DM. However, when studies were performed on transgenic mice, a clear involvement of the protein was evident when UCP_3 overexpressing animals were used as a model (Choi et al., 2007; Costford et al., 2008). Instead, in $UCP_3^{-/-}$ fed a high fat diet regimen (known to induce a state of insulin resistance), insulin sensitivity has been reported to be unaffected (Vidal-Puig et al., 2000), enhanced (Costford et al., 2008), or reduced (Costford et al., 2008; Senese et al., 2011), with the effect being dependent on the age of the mice. Costford et al. (2008). On the other hand, $UCP3^{+/-}$ heterozygous mice show an approximate 50% reduction in SkM UCP_3 protein levels when compared to $UCP3^{+/+}$, and a clear decline in insulin sensitivity has been reported. The effect was observed both when mice were fed with a standard diet or with a high fat diet (Senese et al., 2011). These data are in good accordance with clinical observations reporting that (i) a 50% reduction of UCP_3 protein in human SkM is correlated with the incidence of T2DM (Schrauwen et al., 2001); and (ii) in humans, UCP_3 protein levels are reduced in the pre-diabetic state of impaired

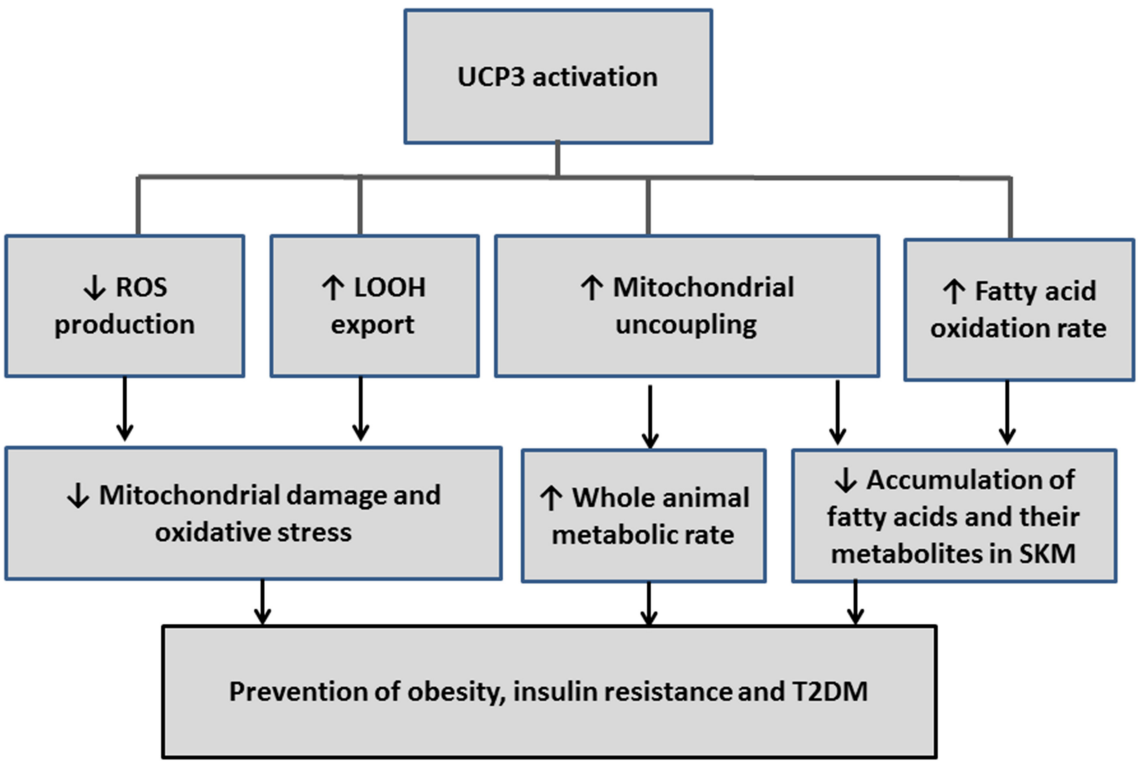

FIGURE 2 | Schematic diagram that illustrates how the reactions influenced by UCP3 could be redirected to prevent or treat obesity, insulin resistance, and type 2 diabetes (T2DM).

glucose tolerance (Schrauwen and Hesselink, 2004a; Mensink et al., 2007).

Human Polymorphisms in the UCP_3 gene also suggest an impact of UCP3 on fat metabolism, obesity, and T2DM. In this context, the UCP_3 gene polymorphism –55T allele has been linked to enhanced UCP_3 mRNA expression and RM (Schrauwen et al., 1999). Heterozygous C/T is associated with decreased obesity and T2DM risk (Meirhaeghe et al., 2000; Herrmann et al., 2003; Liu et al., 2005). Importantly, these findings have been recently confirmed through a meta-analysis showing an association of the polymorphism with protection from obesity in a European patient population (de Almeida Brondani et al., 2014). The association between the −55CT polymorphism of UCP3 and a lower BMI, however, is modulated by energy intake, since it disappears when caloric intake is increased (Lapice et al., 2014).

Heterozygous individuals that have a missense G304A polymorphism show decreased whole body fat oxidation compared to controls (Argyropoulos et al., 1998). They also present reduced levels of plasma lactate (Adams et al., 2009), that can be plausibly due to an impaired consumption of long chain fatty acids for muscle energy and to a greater reliance upon carbohydrates for energy.

Recently, four novel and heterozygous mutations in the UCP3 gene were identified (V56M, A111V, V192I, and Q252X) (Musa et al., 2012). Children carrying these mutations exhibited a higher percentage of fat and BMI, which was associated with dyslipidemia and lower insulin sensitivity (Musa et al., 2012).

CONCLUDING REMARKS AND PERSPECTIVE

Targeting processes that lead to a reduction in mitochondrial coupling/efficiency and ROS production (thus oxidative stress) could be a promising therapeutic strategy to combat obesity and its co-morbidities (such as T2DM). The uncoupling proteins have several hypothesized functions including thermogenesis in certain tissues, protection from ROS, mediation of fatty acids oxidation and export of fatty acids, which are all related to the above pathologies. Hence, an understanding of the mitochondrial processes that lead to uncoupling and, in particular, the elucidation of the exact role played by UCPs at the mitochondrial level and in EM may provide an attractive therapeutic target for diseases rooted in metabolic imbalance.

In this context, the recruitment of thermogenic brown and/or beige adipocytes through activation of UCP_1, and by expanding the tissue, through drugs or other methods, provide a promising approach for enhancing energy expenditure and combating obesity comorbidities. Recent evidence, indicating that brown and beige adipocyte can be recruited by different stimuli, raises the possibility that they may represent separate and distinct targets for therapeutic intervention. Although the roles of UCP_3 are not completely clear, current data in human subjects (living at thermoneutrality) suggest a possible involvement of this protein in EM as well as in counteracting obesity, insulin resistance, and T2DM (**Figure 2**). Therefore, UCP_1 and UCP_3 represent promising therapeutic targets for treating pathologies that result from energy unbalance.

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Brown adipose tissue activity as a target for the treatment of obesity/insulin resistance

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Presence of brown adipose tissue (BAT), characterized by the expression of the thermogenic uncoupling protein 1 (UCP1), has recently been described in adult humans. UCP1 is expressed in classical brown adipocytes, as well as in "beige cells" in white adipose tissue (WAT). The thermogenic activity of BAT is mainly controlled by the sympathetic nervous system. Endocrine factors, such as fibroblast growth factor 21 (FGF21) and bone morphogenic protein factor-9 (BMP-9), predominantly produced in the liver, were shown to lead to activation of BAT thermogenesis, as well as to "browning" of WAT. This was also observed in response to irisin, a hormone secreted by skeletal muscles. Different approaches were used to delineate the impact of UCP1 on insulin sensitivity. When studied under thermoneutral conditions, UCP1 knockout mice exhibited markedly increased metabolic efficiency due to impaired thermogenesis. The impact of UCP1 deletion on insulin sensitivity in these mice was not reported. Conversely, several studies in both rodents and humans have shown that BAT activation (by cold exposure, β3-agonist treatment, transplantation and others) improves glucose tolerance and insulin sensitivity. Interestingly, similar results were obtained by adipose tissue-specific overexpression of PR-domain-containing 16 (PRDM16) or BMP4 in mice. The mediators of such beneficial effects seem to include FGF21, interleukin-6, BMP8B and prostaglandin D2 synthase. Interestingly, some of these molecules can be secreted by BAT itself, indicating the occurrence of autocrine effects. Stimulation of BAT activity and/or recruitment of UCP1-positive cells are therefore relevant targets for the treatment of obesity/type 2 diabetes in humans.

Keywords: UCP1, FGF21, BMP, PTEN, obesity, diabetes, IL-6, gestation

INTRODUCTION

Obesity, well known to be associated with a number of comorbidities, including insulin resistance and type 2 diabetes, has become a major public health problem in recent decades, and has reached epidemic proportions, not only in high-income countries, but also in most middle-income societies. It is defined as an accumulation of adipose tissue that is of sufficient magnitude to impair health (WHO, 2014). Excess weight is usually defined by the body mass index or BMI. The normal BMI range is 18.5–25 kg/m^2, although the range may vary for different countries. Individuals with a BMI above 30 kg/m^2 are classified as obese; those with a BMI between 25 and 30 kg/m^2 are considered to be overweight. In general, the term obesity applies to both the obese and the overweight subjects. More than the total body weight, the distribution of the stored fat is of importance for the development of obesity and its comorbidities. Thus, central or visceral obesity, in which fat accumulates in the trunk and in the abdominal cavity (in the mesentery and around the viscera), is associated with a much higher risk for several diseases than excess subcutaneous fat accumulation. Obesity has profound effects on tissue insulin sensitivity, and therefore on systemic glucose homeostasis. Insulin resistance is present even in simple obesity, without hyperglycemia, indicating a fundamental abnormality of insulin signaling in states of excess adipose tissue mass. The epidemiologic association of obesity, particularly of the visceral type, with type 2 diabetes has been recognized for decades. According to the World Health Organization, 347 millions of people are diabetic in the world, and it is predicted that in 2030, diabetes will be the 7th cause of death considering the worldwide population (WHO, 2014).

Although the pathogenesis of obesity is extremely complex and is far from being unraveled, the key component of the obesity epidemic is long-term dysregulation of energy balance, comprising increased energy intake and/or reduced energy expenditure. Despite active research and impressive improvements in the understanding of the regulation of energy balance, there are only a very limited number of drugs that can be used for the efficient treatment of obesity and its comorbidities. Targeting specific components of the neuroendocrine regulation of energy intake, such as leptin or hypothalamic neuropeptides, has disappointingly revealed unsuccessful as yet. New alternatives focusing on adipose tissue function could potentially be of therapeutic relevance in the future.

Two different types of adipose tissue have been described: brown adipose tissue (BAT), composed mainly of brown adipocytes, and white adipose tissue (WAT), defined by a majority of white adipocytes, both tissues being able to accumulate lipids in intracellular droplets. WAT is an energy-storing tissue that has evolutionary enabled humans to survive for longer periods between meals, storing energy mainly as triglycerides and releasing fatty acids during fasting periods. In recent times, when food has become cheaper and more widely available, excessive WAT storage contributed to the worldwide alarming development of obesity mentioned above (World Health Organization, 2009). White adipocytes are composed of a large single, spherical lipid vacuole and a peripherally located nucleus, together with few mitochondria. WAT has endocrine activity, secreting several factors and hormones, such as leptin and adiponectin. Under certain conditions, another type of adipocytes, named brite or beige cells, can be found dispersed within some of the WAT depots. These cells, which will be discussed below, present a phenotype with metabolic properties that are closest to brown than to white adipocytes.

BAT consists in brown adipocytes, characterized by multiple, small, multilocular lipid droplets with a central nucleus and a high number of mitochondria. BAT is a highly vascularized tissue innervated by the sympathetic nervous system. The mitochondria of BAT are characterized by the presence of uncoupling protein-1 (UCP1) in the inner mitochondrial membrane. When activated, this protein uncouples mitochondrial respiration from ATP synthesis, resulting in heat production, a process that consumes substantial amounts of fuels. BAT, the principal effector organ of non-shivering thermogenesis (i.e., heat production that does not involve skeletal muscle contraction), is present in most mammals and its maturation in the perinatal period varies between species, according to their developmental status at birth (Tews and Wabitsch, 2011).

In humans, BAT develops in the fetus during gestation. Thus, the amount of UCP1 increases during fetal development, peaks at birth, before declining over the first 9 months (Lean et al., 1986; Tews and Wabitsch, 2011). The notion that human BAT is solely apparent during the neonatal stage prevailed for decades (Heaton, 1972; Nedergaard et al., 2007). In 2009, functional human BAT was identified in adults by a combination of CT scans and fluorodeoxyglucose positron emission tomography (FDG-PET) (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). The areas in which BAT is observed in adult humans include supraclavicular, neck, paravertebral, and suprarenal sites (Nedergaard et al., 2007). Estimates of BAT mass and activity from FDG-PET studies suggest that humans have, on average, 50–80 g of BAT (Peirce et al., 2014). Quantitatively, it was estimated that 50 g of BAT can burn as much as 20% of daily energy intake (Rothwell and Stock, 1983). As an example, in a subject with 63 g of supraclavicular BAT, it was calculated that if the depot was fully activated, it would burn an amount of energy equivalent to 4.1 kg of WAT (Virtanen et al., 2009). It can therefore be concluded that, even though the BAT depots are present in small amounts, the activated tissue has the potential to substantially contribute to energy expenditure (Nedergaard et al., 2007).

BAT activity is well known to mostly rely on lipid metabolism, UCP1 being directly activated by fatty acids (Cannon and Nedergaard, 2004). Along this line, it was recently demonstrated that chronic activation of the β3 adrenoreceptor induces coupled increases in lipolysis, *de novo* lipogenesis and fatty acid beta-oxidation not only in white, but also in BAT (Mottillo et al., 2014). Thus, the continuous cycling of triglyceride hydrolysis coupled to resynthesis, which requires large amounts of ATP, could be another important mechanism to increase thermogenesis in BAT, in addition to the role of UCP1 activation in this process (Mottillo et al., 2014). Similarly to what was proposed for skeletal muscle (Dulloo et al., 2004), this may contribute to dissipate excess lipids as occurs during prolonged stimulation of lipolysis (e.g., chronic β3 adrenoreceptor treatment).

In addition to using lipids, BAT also displays a very high rate of glucose uptake, particularly under sympathetic activation (Cannon and Nedergaard, 2004). Interestingly, BAT glucose uptake is close to the values observed for metastasis in cancer in humans (Aukema et al., 2010). This tissue also responds to insulin with a 5-fold increase in glucose uptake, without any change in blood flow (Orava et al., 2011), while under cold exposure, glucose uptake increases by 12-folds, dissipating energy as a function of increased blood flow (Orava et al., 2011). Regarding the fate of glucose in brown adipocytes under anabolic conditions characterized by high insulin levels, it is essentially metabolized to provide glycerol-3-phosphate for triglyceride synthesis or acetyl- CoA for *de novo* fatty acid synthesis (Cannon and Nedergaard, 2004).

To investigate the role of BAT, of UCP1 in particular, studies were carried out in UCP1 knockout mice. Surprisingly, no particular phenotype was noted in these mice when they were kept at 23°C, except for their increased cold sensitivity (Enerback et al., 1997; Kontani et al., 2005). In contrast, when bred under thermoneutral conditions (29°C), UCP1 knockout mice exhibited markedly enhanced metabolic efficiency due to impaired thermogenesis (Feldmann et al., 2009).

Altogether, the existing literature suggests that BAT activation is not only involved in non-shivering thermogenesis, but also in the regulation of insulin-mediated glucose disposal. Whether brown and brite adipocytes display some degree of specialization with regard to these different functions has to be established. The aims of this review are to describe some of the main factors regulating UCP1 activity in brown and brite adipocytes, as well as to discuss the potential role of UCP1 activation for the treatment of insulin resistance and type 2 diabetes associated with obesity.

FACTORS AFFECTING BAT FUNCTION AND ENERGY METABOLISM IMPROVE OVERALL METABOLISM

In rodents, brown adipocytes are found in discrete areas, such as interscapular, cervical, peri-aortic, peri-renal, intercostal and mediastinal depots (Cinti, 2001), which are referred to as "classical" BAT depots. In addition, brown adipocytes can be found scattered in WAT, especially upon cold exposure (Young et al., 1984; Guerra et al., 1998), treatment with β-adrenergic (Himms-Hagen et al., 2000), or with peroxisome-proliferator-activated receptor-γ (PPAR-γ) agonists (Petrovic et al., 2010). These brown-like adipocytes have interchangeably been called "recruitable" (Tseng et al., 2008; Schulz et al., 2013), "beige" (Ishibashi and Seale, 2010;

Auffret et al., 2012; Wu et al., 2012), or "brite" (for brown to white) (Petrovic et al., 2010; Gburcik et al., 2012) cells.

Lineage-tracing studies showed that brown adipocytes in classic BAT areas derive from myogenic factor 5 (*Myf* 5^+)- positive progenitor cells, similarly to skeletal myocytes (Timmons et al., 2007). In contrast, "brite" adipocytes have been shown to originate from Myf-negative (*Myf* 5^-) progenitor cells, much like white adipocytes (Petrovic et al., 2010; Long et al., 2014). Whether "brite" adipocytes descend from unique precursors, or share progenitors with either white or classic brown adipocytes still remains to be established (for rev., see Chechi et al., 2013). Interestingly, the "browning" of WAT (i.e., increased proportion of brown adipocytes) may also involve transdifferentiation of white-to-brown adipose cells (Smorlesi et al., 2012; Frontini et al., 2013), although this issue is still a matter of debate (Wu et al., 2012).

Whatever their developmental origin, white, "brite" and brown adipocytes seem to greatly differ in their function. As mentioned above, BAT is the effector organ of non-shivering thermogenesis (both cold and diet-induced) that, by utilizing large quantities of glucose and lipids from the circulation, can promote negative energy balance. Moreover, the role of BAT activation appears to be broader than solely the promotion of negative energy balance (for rev., see Peirce and Vidal-Puig, 2013). Indeed, BAT is now known to exert anti-type 2 diabetic effects associated with improvments of dyslipidemia and insulin secretion as well as decrease insulin resistance in type 2 diabetes (de Souza et al., 1997; Liu et al., 1998; Frontini et al., 2013; Peirce and Vidal-Puig, 2013). These effects are partly interrelated, but can also be dissociated and exerted by different UCP1-expressing types of adipocytes (i.e., brown and "brite" adipocytes). However, as these different cells are often mixed, such as occurs for classical and "brite" adipocytes in some human depots (Wu et al., 2012; Cypess et al., 2013; Jespersen et al., 2013), only the use of specific cell surface markers (i.e., ASC-1, PAT2, and P2RX5 for white, "brite" and brown adipocytes, respectively) will allow for their identification, as well as for the precise understanding of their respective therapeutic properties (Ussar et al., 2014).

BAT activation by cold exposure, β3-agonist or thyroid hormone treatment was shown to improve glucose tolerance and insulin sensitivity (Cawthorne et al., 1984; Forest et al., 1987; Peirce and Vidal-Puig, 2013). Similar observations were obtained by adipose tissue-specific overexpression of PR-domain-containing 16 (PRDM16) in mice. This Zinc-finger transcription factor induces differentiation of brown adipocytes (Seale et al., 2011). The main mediators of such beneficial effects seem to include fibroblast growth factor 21 (FGF21), interleukin-6 (IL-6), bone morphogenic proteins (BMPs) and prostaglandin D2 synthase. Interestingly, some of these molecules, called batokines, can be secreted by BAT itself, indicating the occurrence of autocrine effects.

Fibroblast growth factor 21 is a member of the fibroblast growth factor (FGF) family that acts as a hormone and that, in contrast to other endocrine FGFs, is devoid of proliferative activity (Itoh, 2014). It is expressed in BAT and WAT, although its main production site is the liver (Nishimura et al., 2000; Muise et al., 2008; Schulz et al., 2013; Zafrir, 2013). Tissue-specific FGF21 regulation was shown to occur in response to chronic cold exposure in mice (Fisher et al., 2012). Under this condition, FGF21 expression was indeed decreased in the liver, but enhanced in BAT, as well as in WAT, where it acted to markedly increase UCP1 expression and the "browning" of subcutaneous tissue (Fisher et al., 2012). Interestingly, in humans, a mild cold exposure (12 h to 19°C) was recently shown to increase the diurnal plasma FGF21 levels, with a positive correlation with the changes in adipose tissue microdialysate glycerol and total energy expenditure (Lee et al., 2013). This suggested that FGF21 could play a similar role in humans as in rodents in promoting cold-induced metabolic changes (i.e., lipolysis and cold-induced thermogenesis). In adipose tissue, it appears that PPARγ transcriptionally controls FGF21, which then acts as an autocrine or paracrine way to increase PPARγ transcriptional activity in a feed-forward loop system (Wang et al., 2008; Dutchak et al., 2012). FGF21 deficiency in mice was shown to result in impaired ability to adapt to long-term cold exposure with diminished "browning" of WAT (Fisher et al., 2012). At the opposite, systemic administration of FGF21 in obese mice resulted in reduced adiposity, improved glycemic control, as well as increased energy expenditure, as mentioned by the authors (Coskun et al., 2008). Altogether, these observations suggest that FGF21 may be a key factor linking UCP1 expression to improved glucose metabolism. It may also exert determinant developmental effects, given the observation that the postnatal maturation of BAT appears to relate to the onset of feeding and initiation of hepatic function, as mediated by the release of FGF21 (Hondares et al., 2010). In addition, it was recently proposed that FGF21 could act within the central nervous system, both at the level of the hypothalamus and the hindbrain to promote a set of responses that occur during starvation (i.e. increase in corticosterone levels, suppression of physical activity, alteration in circadian behavior) (Bookout et al., 2013). This raises the possibility that, in contrast to its beneficial effects on peripheral metabolism, FGF21 may exert deleterious effects by acting centrally.

Interleukin-6 (IL-6), predominantly known as a pro-inflammatory cytokine, is secreted by skeletal muscle (Pal et al., 2014), helper T cells, as well as by WAT and BAT (Mohamed-Ali et al., 1997; Cannon et al., 1998). Several studies implicated IL-6 as a co-inducer of the development of obesity-associated insulin resistance preceding the onset of type 2 diabetes (Pal et al., 2014). This is in keeping with the observation of increased plasma IL-6 levels in obese patients (Cottam et al., 2004). In such patients, IL-6 is preferentially secreted from visceral rather than from subcutaneous adipocytes and may participate in the prevailing increase in sympathetic outflow by exerting central effects (Wallenius et al., 2002; Fain et al., 2004). Paradoxically, central IL-6 delivery was shown to suppress weight gain and visceral obesity, without affecting food intake (Li et al., 2002). The treatment also enhanced UCP1 protein levels in BAT, *via* stimulation of the sympathetic nervous system (Li et al., 2002). This was mediated by phosphorylation of the signal transducer and activator of transcription 3 (pSTAT3). Interestingly, chronic central IL-6 stimulation desensitized IL-6 signal transduction characterized by reversal of elevated pSTAT3 levels (Li et al., 2002). Such desensitization is likely occurring in situations of

chronic elevation in IL-6 levels, such as occurs in human obesity. It should be added that the understanding of the role of IL-6 is more complex, as this cytokine is known to be secreted by skeletal muscle in response to exercise, exerting insulin sensitizing effects (Kelly et al., 2004; Petersen and Pedersen, 2005). Along this line, it was recently shown that BAT transplantation into the abdominal cavity of high fat diet-induced obese mice was able to improve their glucose tolerance, increase their insulin sensitivity, lower their body weight, decrease their fat mass and completely reverse their insulin resistance (Stanford et al., 2013). BAT transplantation also increased insulin-stimulated glucose uptake in BAT, WAT, and heart, but not in skeletal muscle (Stanford et al., 2013). Importantly, the improved metabolic profile was lost when BAT used for transplantation came from IL-6 knockout mice, clearly demonstrating that BAT-derived IL-6 is required for the profound effects of BAT transplantation on glucose homeostasis and insulin sensitivity (Stanford et al., 2013).

Apart from IL-6, another circulating factor, named irisin, was shown to be produced by skeletal muscles during physical exercise in rodents (De Matteis et al., 2013). Irisin, obtained by cleavage from the precursor protein, fibronectin type III domain containing 5 (FNDC5), was described as promoting the appearance/recruitment of "brite" cells in white adipose depots (Bostrom et al., 2012; Lee et al., 2014a). However, the existence of this protein and its role in humans is still a matter of debate (Elsen et al., 2014a).

Thyroid hormones (THs) are well known mediators of overall energy expenditure (Klieverik et al., 2009). Treatment with THs induces UCP1 expression in brown adipocytes in rats, following their binding to TH-responsive elements in the UCP1 promoter (Guerra et al., 1996). Type 2 iodothyronine deiodinase (D2), responsible for the transformation of thyroxine (T4) to triiodothyronine (T3), is also inducing UCP1 expression locally, in BAT (de Jesus et al., 2001). Furthermore, treatment of brown adipocytes and human skeletal myocytes with bile acids (BA) were shown to increase D2 activity and oxygen consumption *via* the activation of UCP1 (Watanabe et al., 2006). In both rodents and humans, this BA-D2-UCP1 pathway appears to be crucial for the fine-tuning of energy homeostasis, improving the metabolic control (Watanabe et al., 2006). Thyroid receptors (TRs) are composed of several isoforms that specifically regulate UCP1 expression and thermogenesis. The α isoform was shown to regulate adaptive thermogenesis, whereas the β isoform appears to modulate UCP1 expression, without increasing thermogenesis (Ribeiro et al., 2001). In humans, a unique patient suffering from extreme diabetes due to a mutation in the insulin receptor gene had to undergo total thyroidectomy because of the presence of a papillary carcinoma. Thirty months after the initial treatment of the thyroid cancer (radioiodine and levothyroxine), remarkable improvements in glycemia were noted and the anti-diabetic treatment could even be discontinued. A PET/CT study revealed the presence of BAT depots in the lower neck, suprascapular, mediastinal, and thoracic paravertebral regions. Interestingly, increased FDG uptake was also noted in the subcutaneous fat, in particular in the pelvic area and over the lower extremities. Overall, the sequence of events in this patient suggests that the metabolic and trophic effects of THs on BAT may play a critical role in non-insulin-mediated glucose utilization, ultimately leading to near-normal glucose levels (Skarulis et al., 2010).

Bone morphogenic proteins (BMPs) are members of the transforming growth factor β superfamily (TGF-β). They were originally thought to be factors inducing bone formation, but were then described to be involved in the development and function of many tissues, such as the intestine, brain and WAT (Hogan, 1996). Some members of the BMP family were shown to play a role in energy homeostasis and the early steps of adipogenesis, in particular. Among the 20 BMP family members, BMP-7 has been implicated in the development of BAT, being able to drive the complete brown fat differentiation program, including PRDM16 expression (Modica and Wolfrum, 2013). BMP-7 can also affect energy homeostasis by acting on mature brown adipocytes, resulting in the induction of UCP1, thereby enhancing thermogenesis. As it is not expressed in mature brown adipocytes, BMP7 appears to exert its action on BAT as an endocrine factor. In addition to its effect on BAT, BMP-7 was also reported to induce the "browning" of WAT and to improve insulin sensitivity (Schulz et al., 2011). Finally, several hypothalamic nuclei were shown to express BMP-7, suggesting that it may regulate BAT function *via* a central mechanism, also responsible for decreased food intake (Modica and Wolfrum, 2013).

BMP8B, another member of the BMP family, was found to be expressed in BAT, as well as in the hypothalamus (Contreras et al., 2014). Central administration of BMP8B induced thermogenesis and increased core temperature, leading to weight loss (Contreras et al., 2014). This effect, exerted within the ventromedial hypothalamus (VMH), was described as being AMPK-dependent, resulting in the activation of the sympathetic outlflow to BAT, without any change in the feeding behavior (Whittle et al., 2012; Contreras et al., 2014).

In contrast to BMP7 and BMP8B, BMP4 was shown to promote the differentiation of mesenchymal stem cells into white adipocytes, inducing fat storage and decreasing energy expenditure in rodents (Modica and Wolfrum, 2013; Contreras et al., 2014). However, in primary human adipose stem cells, both BMP4 and BMP7 induced a white-to-brown adipocyte transdifferentiation (Elsen et al., 2014b), pointing to the occurrence of different effects, depending on the model used for investigation. Further studies are needed to clarify and strengthen the role of BMP proteins in the regulation of BAT or "brite" cells and their consequences on metabolic homeostasis.

Phosphatase and tensin homolog deleted on chromosome ten (PTEN), a well-known tumor suppressor is a phosphatase that specifically catalyzes the dephosphorylation of phosphatidylinositol-3,4,5-triphosphate (PIP_3), in phosphatidylinositol-4,5-diphosphate PIP_2 (Cantley and Neel, 1999). This enzyme counteracts the action of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), resulting in inhibition of the AKT signaling pathway involved in multiple biological processes, including insulin action. Activation of AKT is known to trigger a complex cascade of events that include the inhibition of FOXO transcription factors (Ortega-Molina et al., 2012). Interestingly, mice carrying additional copies of *Pten* ($Pten^{tg}$ mice) are not only protected from cancer and

exhibit extended longevity, but, according to the authors, they also present enhanced energy expenditure that participates in counteracting the development of obesity. This is related to lower BAT levels of phosphorylated AKT and FOXO1, higher BAT and WAT expression of UCP1, as well as of its transcriptional regulator, PGC1-α (Ortega-Molina et al., 2012). In addition, specific deletion of *Pten* in the liver in LPTENKO mice induces a strong hepatic steatosis (Stiles et al., 2004; Peyrou et al., 2015), but improves the overall insulin sensitivity, and decreases the fat mass. "Browning" of WAT could be one of the mechanisms underlying the increased insulin sensitivity of LPTENKO mice, in keeping with the observation of increased WAT glucose uptake (Peyrou et al., 2015). In humans, *PTEN* haploinsufficiency was shown to have divergent effects, as they increase the risk of obesity, while decreasing that of type 2 diabetes by markedly improving insulin sensitivity (Pal et al., 2012). In a very recent study, the grizzly bear was used as a hibernation model, in which obesity is a natural adaptation to survive months of fasting (Nelson et al., 2014). It was remarkably observed that preparation for hibernation was characterized by striking increases in body weight and in fat mass. Animals were shown to exhibit enhanced insulin sensitivity, while they become obese and to develop insulin resistance a few weeks later, during hibernation, to finally recover their insulin sensitivity upon awakening. The modification of insulin sensitivity occurs *via* the effect of the PTEN/AKT-mediated regulation of adipose tissue lipolysis. These results support the notion that adipose tissue is very insulin sensitive in the fed state, while being able to drive insulin resistance in the fasting state, independently from insulin levels (Nelson et al., 2014). In humans, the only physiological recovery of insulin sensitivity after a period of insulin resistance, partially due to an increase in food intake and lipogenesis, is observed in women after pregnancy (Barbour et al., 2007).

The present knowledge on the impact of the main batokines, as well as of the principal other UCP1 modulators on peripheral metabolism is schematized by **Figure 1**.

IMPACT OF PERINATAL NUTRITIONAL CHANGES

Epidemiological evidence in humans strongly suggests that the intrauterine and early postnatal environments have a significant long-term influence on body weight and energy homeostasis in offspring (for rev., see Breton, 2013). Thus, both maternal underfeeding or overfeeding were reported to exert a predisposing effect for the development of later obesity (Breton, 2013). Rodents are commonly used to investigate the mechanisms underlying long-term programming of energy balance in the offspring. High fat feeding of pregnant or lactating mothers was shown to induce glucose intolerance and the development of obesity in the progeny during adult life (Bayol et al., 2008; Samuelsson et al., 2008). The signals that mediate the effects of maternal metabolic disorders in overfed offspring have not been fully identified. They include hormones, such as insulin, leptin and glucocorticoids, proinflammatory cytokines, as well as complex epigenetic modifications (Tamashiro and Moran, 2010; Lukaszewski et al., 2013).

In this context, the sheep appears to be a very good model, because as for humans, the major source of BAT in the fetus is around central organs and is replaced by WAT after birth, whereas in rodents, the primary BAT depot is interscapular and

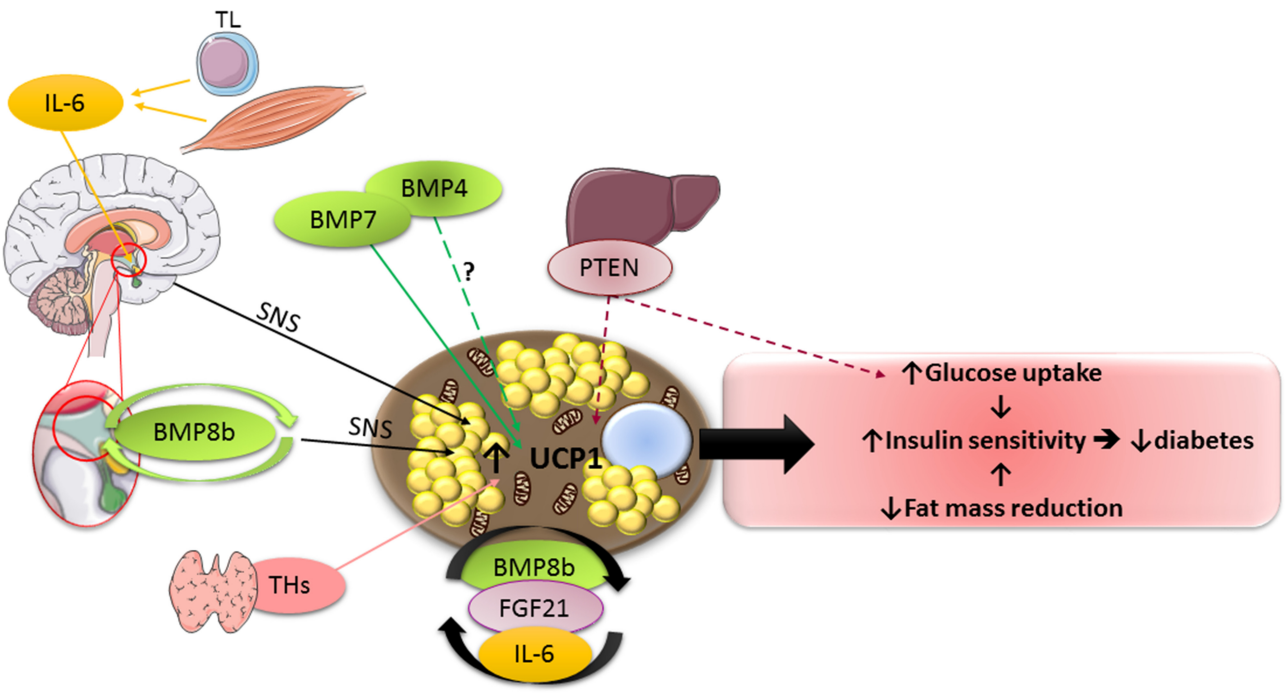

FIGURE 1 | Schematic representation of the main factors described in the review, which modulate UCP1 activity. TL, T lymphocytes; SNS, sympathetic nervous system. For other abbreviations, see text. Full lines indicate stimulatory effects, whereas dotted lines represent inhibitory ones. ? indicates the existence of conflicting data in the literature with regard to the impact of the factor on UCP1 activity.

it remains throughout life (Symonds et al., 2012). In sheep, the mother's diet during pregnancy determines the size of the placenta and can affect both the BAT and WAT mass, depending on the timing and the nature of the diet perturbation. In other words, the respective growth of the BAT and WAT depots depends on the maternal diet during gestation and it may be responsible for the development of obesity, insulin resistance and type 2 diabetes in the offspring, later in life (Symonds et al., 2012). Gestational diabetes in humans is a situation of increased nutrient supply that, together with the high maternal body mass index, can be accompanied by enhanced birth-weight and adverse long-term metabolic consequences, as described by the World Health Organization (2003), as well as in several publications (Larsson et al., 1986; Dabelea et al., 2000; Singh et al., 2006). However, in a recent study, the role of diabetes during gestation on such adverse long-term metabolic consequences has been seriously questioned, as they seem to relate more on known confounders, such as the BMI of either one of the parents (Donovan and Cundy, 2014).

Among the regulators that may link the maternal diet during gestation with the metabolic outcome of the offspring, leptin is one of the main candidates. It is a well-known hormone increasing BAT activity and inducing "browning" of WAT *via* activation of the sympathetic nervous system and resulting increased β3 adrenoceptor signaling (Haynes et al., 1997; Commins et al., 2000). Rodent models of leptin deficiency exhibit marked decreases in BAT thermogenic capacity, as well as activity (Ueno et al., 1998). Moreover, it was shown that leptin injection in the early stage of life in lambs decreases UCP1 expression in BAT, but improves thermoregulation, suggesting a particular role of leptin at such a stage in life in large mammals (Mostyn et al., 2002). Another study revealed that the ability of leptin to increase the metabolic rate early in life is independent from its anorectic action (Mistry et al., 1999).

No data are available as yet with regard to the impact of early leptin administration on the subsequent "browning" of WAT and the related regulation of glucose metabolism, as well as the response to hypercaloric diets later in life in the offspring.

ATTEMPTS AT STIMULATING BAT FUNCTION IN HUMANS

With regard to the relationship between BAT and body weight in humans, an inverse correlation between BMI and the amount of BAT was described, already 5 years ago (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009). In addition, more recent studies indicated that, compared to individuals without BAT, the BAT-positive subjects were younger, had lower body mass index, fasting insulin, insulin resistance, but a greater level of high-density lipoprotein cholesterol (Zhang et al., 2013). During acute cold exposure, BAT was shown as a significant independent determinant of plasma glucose and HbA1c levels (Matsushita et al., 2014). A parallel increase in BAT activity and cold-induced energy expenditure was also observed in response to acute cold exposure in subjects with low BAT activity, demonstrating the possible occurrence of BAT recruitment in humans (Yoneshiro et al., 2013). Very recently, chronic cold acclimation in human subjects was reported to increase the volume of metabolically active BAT, increasing its oxidative capacity, therefore its contribution to cold-induced thermogenesis (Blondin et al., 2014). In another study, the cold-induced increase in thermogenesis was accompanied by a decrease in body weight, mainly affecting the fat mass compartment (Yoneshiro et al., 2013). Cold-acclimation was also shown to increase diet-induced thermogenesis and postprandial insulin sensitivity, without impacting cold-induced thermogenesis (Lee et al., 2014b). These results are in keeping with data showing a physiological role of BAT in whole-body energy expenditure, glucose homeostasis, and insulin sensitivity in humans during prolonged cold exposure (Chondronikola et al., 2014).

Much more work is needed to identify other ways than cold exposure to increase BAT activity in obese subjects. To this end, one of the very useful tools is the use of rodent strains with different sensitivities to diet-induced obesity and insulin resistance. Indeed, resistance to the development of obesity has at least partly been attributed to elevated recruitment of brown adipocytes in skeletal muscle or WAT (Guerra et al., 1998; Almind et al., 2007; Veyrat-Durebex et al., 2009; Harms and Seale, 2013). These studies are highly therapeutically relevant, as BAT activation in overweight or obese subjects will activate thermogenesis and dissipate heat, while at the same time improving glucose metabolism and insulin resistance.

It should be added at that point that, although β3 adrenoceptors are expressed in humans (for rev., see Mund and Frishman, 2013) and β3 agonists are potent UCP1 activators in rodents, the molecules which are active in rodents cannot be used in humans due to inter-species differences. This should be solved in the future by the identification of selective human β3 agonists (Mund and Frishman, 2013; Bordicchia et al., 2014).

CONCLUSION

To conclude, UCP1 is an excellent target to struggle diabetes and decrease body fat mass, improving whole metabolism. Indeed, it negatively regulates the energy balance by increasing energy expenditure. It also secretes several batokines, allowing for inter-organ communication. Finally, it is easily inducible, such as during mild cold exposure with resulting beneficial effects. Finding a molecule with as much efficiency as the β3 agonist in rodents, would be of considerable therapeutic relevance for the treatment of obesity, insulin resistance and type 2 diabetes in humans.

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The effects of 3,5-diiodothyronine on energy balance

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INTRODUCTION

Thyroid hormones (THs) have been known to affect energy metabolism (calorigenic effect) for over a century (Magnus-Levy, 1895; Thompson et al., 1929). In 1985 Magnus-Levy observed that patients with mixedema exhibited an abnormal low oxygen consumption when compared to normal individuals and that unusually higher amount of oxygen was consumed by hyperthyroid patients. 3,3′,5-triiodo-L-thyronine (T_3) is the active form of THs and it is a major regulator of growth and development and of cellular and tissue metabolism (both intermediate and energy metabolism) throughout the body. Metabolic actions include regulation of: basal metabolic rate in homeotherms, synthesis of mitochondrial respiratory enzymes and membranes, oxidative phosphorylation and energy transduction, movement of water and Na^+ ions across cell membranes; calcium and phosphorus metabolism, lipids synthesis and storage, catabolism of fatty acids, cholesterol, carbohydrate; and nitrogen (urea, creatine) metabolism; growth and developmental actions include actions on: rate of postnatal growth of many mammalian and avian tissue, maturation of fetal brain and bone, amphibian larval metamorphosis, and molting in birds. It is now recognized that T_3 affects gene expression in target tissues/cells by binding to its cognate nuclear receptors (TR) which are ligand-inducible transcription factors. Two TR genes α and β encode four T3-binding receptor isoforms (α1, β1, β2, and β3). The transcriptional activity of TRs is regulated at multiple levels. Besides being regulated by T3, transcriptional activity is also regulated: (i) by the type of thyroid hormone response elements located on the promoters of T3 target genes, (ii) by the developmental- and tissue-dependent expression of TR isoforms, and (iii) by a host of nuclear coregulatory proteins (corepressors and coactivators). These nuclear proteins modulate the transcription activity of TRs in a T3-dependent manner. In the absence of T3, corepressors act to repress the basal transcriptional activity, whereas in the presence of T3, coactivators act to activate transcription. The activities regulated via the previous described mechanisms are described as "genomic actions." However, between the mid-1980's and the beginning of the 1990's it became evident that some TH effects are non-genomic in origin. Indeed, high-affinity binding sites for thyroid hormones have for many years been recognized on the plasma membrane and other cellular sites such as mitochondria and cytoplasm (for review see Cheng et al., 2010). Recently, a structural protein of the plasma membrane, integrin αvβ3, has been shown to contain a binding domain for iodothyronines that is an initiation site for hormone-directed complex cellular events, such as cell division and angiogenesis (Bergh et al., 2005) and this qualifies the binding site for characterization as a receptor. Examples of non-genomic action of thyroid hormones are activation of: membrane Ca2-ATPase activity, 2-Deoxyglucose transport, Na, K-ATPase activity, Na^+ current in myocardiocytes, Na^+ current in sensory neuron, Na^+/H^+ exchanger, cancer cell proliferation, angiogenesis (for review see Cheng et al., 2010). In addition to this, it is now recognized that other iodothyronines or THs analogs/derivatives are able to exert relevant biological actions (for recent review, see Moreno et al., 2008; Senese et al., 2014; Zucchi et al., 2014). This article is particularly intended to describe the effects of the 3,5 diiodo-L-thyronine (T_2) on energy balance (Moreno et al., 1997; Goglia, 2005).

3,5-DIIODO-L-THYRONINE (T_2)

T_2, a naturally occurring diiodothyronine, is a product of a currently unknown enzymatic process most probably utilizing T3 as its precursor (Moreno et al., 2002). Some years ago surprising results were published showing that (among a lot of iodothyronines tested) T_2, at a very low concentration (pM), induced a rapid stimulation of oxygen consumption in perfused livers isolated from hypothyroid rats. In the same study, it was shown that T_3 showed a similar effect but this effect was largely abolished by the addition of an inhibitor of D1 deiodinase, while the effect of T_2 was not. Moreover, T_2 exerted its effect more rapidly than T_3 (Horst et al., 1989). Stimulated by that report and another study showing an interaction of a diiodothyronine with mitochondria (Goglia et al., 1981) some laboratories started to investigate more deeply on possible specific biological actions of T_2. Initially, energy metabolism was the major area of interest. Indeed, several reports from various laboratories showed that acute or chronic administration of T_2 to rats resulted in significant changes in energy metabolism. When either T_3 or T_2 were acutely injected to hypothyroid rats, T_2 had a more rapid effect on resting metabolic rate than T_3 (Lanni et al., 1996). The experimental design used in this study was basically the same as that employed by Tata in the early 1960's (Tata et al., 1962; Tata, 1963) and the only difference was that in the study of Lanni

et al. hypothyroidism was achieved by the simultaneous administration of propylthiouracil (PTU) and iopanoic acid (IOP). This treatment produces animals with severe hypothyroidism and at the same time, with a powerful inhibition of all three types of deiodinase enzymes. In such conditions the effects of T_2 were evident as soon as 1 h after its injection reaching the maximal value after 24 h, while that of T_3 became evident only after 24 h reaching the maximal value after 72 h (these effects of T_3 were overlapping to those obtained by Tata, 1963). Moreover, while the effect of T_3 was inhibited by a inhibitor of transcription such as actinomycin D (as shown also by Tata, 1963). the effect of T_2 was not (Lanni et al., 1996; Moreno et al., 1997) (see **Figure 1**).

Following these results studies continued to try to clarify the mechanisms underlying the previous described results. In light of the effects of T2 on energy metabolism, the mitochondria became the obvious candidates to study such mechanisms. In this context, some years ago, by top-down elasticity analysis, it was showed a stimulation of the activity of both cytochrome c-oxidizers and the cytochrome c-reducers components of the respiratory chain, 1 h after the injection of 3,5-T2 (Lombardi et al., 1998). These data indicate a possible direct interaction of T2 with some components of the respiratory chain. Indeed, this hypothesis was in agreement with previous results showing a direct stimulation of the enzyme cytochrome oxidase (COX) activity isolated from bovine heart (Goglia et al., 1994). Arnold and Kadenbach (1997) showed that (in addition to the mitochondrial membrane potential, the substrate pressure in the respiratory chain and the oxygen concentration) the respiration of animal cells is also controlled by the matrix ATP/ADP ratio, via an interaction of nucleotides with COX. In fact, ATP produces an allosteric inhibition of the COX activity. In a further investigation, Arnold et al. (Arnold et al., 1998) showed that 3,5-T2 specifically binds to subunit Va of the COX complex and completely abolishes the allosteric inhibition of respiration induced by ATP. Subunit Va of the COX complex is therefore a mitochondrial site through which T_2 may directly affect mitochondrial activities. In addition to activating mitochondrial substrate oxidation, 3,5-T_2 also stimulates skeletal muscle mitochondrial uncoupling in a very rapid manner (Lombardi et al., 2007, 2009). By discriminating between proton-leak and redox-slip processes, an increased mitochondrial proton conductance has been addressed as the "pathway" underlying the effect of T2 on mitochondrial uncoupling. Thus, activation of COX complex above described associated to changes in the efficiency of the skeletal muscle mitochondrial energy-transduction apparatus, may explain, at least in part, the rapid effect of T2 on metabolic rate.

The stimulatory effects exerted by T_2 on RMR prompted my group to investigate on a possible effect of this iodothyronine in counteracting overweight and lipid accumulation without deleterious side effects in particular on hearth and skeletal muscle such as those showed by T_3 when tested as slimming agent. To test this idea, we administered T2 for 30 days to rats on a high fat diet (HFD) and then we measured the adipose tissue mass, the body weight gain, the liver adiposity, the liver fatty acid oxidation rate, and the serum levels of triglyceride, fatty acids, and cholesterol. In this study we also looked at a possible effect of T_2 on HPT axis (Lanni et al., 2005). The results showed that, except a slight decrease (−20%) in T_4 serum level, no variation in the Hypothalamus-Pituitary-Thyroid axis (HPT) was evident measured by the "TRH-test." In this study rats treated with T2 showed lower body weight, a higher liver fatty acid oxidation rate, less fat mass, an almost complete disappearance of fat from the liver, and significant reductions in the serum triglyceride and cholesterol levels. Recently, most of these results have been confirmed also in animals with a standard laboratory diet and with a prolonged time of treatment (Padron et al., 2014). In addition, several studies from both our and others laboratories showed relevant biological effects of T_2 and some of them reported beneficial effects of T_2, among others:

Moreno et al. (2011) and de Lange et al. (2011) showed that T_2 prevented high-fat-diet-induced insulin resistance in rat whose action involved activation of sirtuin 1 (SIRT1).

Markova et al. (2013) showed an antidepressant-like effect of T2 in rats after a bolus administration of T2 at the doses 75 and 150 μg/100 g b.w.

Shang et al. (2013) showed that T2 was a protective agent against renal damage in diabetic nephropathy in streptozotocin-induced diabetic rats, confirming the involvement of sirtuin 1 (SIRT1).

These effects were observed in absence of deleterious side effects. However, some

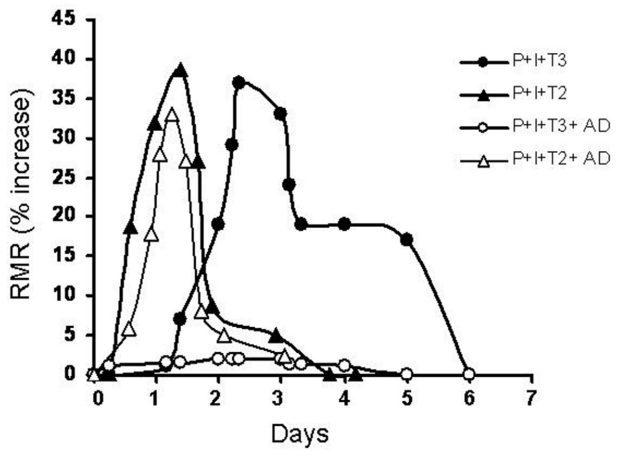

FIGURE 1 | Time course of variation in the resting metabolic rate of hypothyroid rats following administration of a single dose of iodothyronines (25 μg/100 g BW for both T_2 and T_3) with or without a concomitant administration of actinomycin D (8 μg/100 g BW) (AD). Hypothyroidism was induced by combined treatment with PTU and IOP (P + I). Resting metabolic rate (RMR) is reported as % increase [vs. time 0 (immediately before the injection)].

studies have shown some deleterious effect at cardiac level and on HPT axis (Goldberg et al., 2012; Jonas et al., 2014) but in these studies unusual very high doses of T_2 were studied and unspecific interaction of T_2 with nuclear TRs may have occurred.

Further details about the actions of T_2 can be found in some reviews (Goglia, 2005; Coppola et al., 2014; Senese et al., 2014).

Of Note, the understanding of the physiological and pathophysiological role of T2 would benefit from development and standardization of new methods for analytical measurement of T_2 and other TH derivatives. Actually, measurements of T2 in human tissue and sera have been so far taken using immunoassays (Kirkegaard et al., 1981; Nishikawa et al., 1981). However, the lack of labeled iodothyronines with high specific activity as well as of specific antibodies has represented an important limitation to the application of such approach. Promising developments in this field have recently emerged from the optimization of a new competitive chemiluminescence immunoassay (CLIA) based on the use of one selected mouse monoclonal anti-T2 antibody with very low cross-reactivity to structurally related THs and thyronamines (Lehmphul et al., 2014). Mass spectrometry techniques have also drawn attention to the analyses of iodothyronines (Köhrle et al., 2013), however, intrinsic instrumental limits still restrain the application of such approaches as routine tools.

CONCLUSION AND PERSPECTIVES

The data reported and discussed in this article have generated a large interest in the possibility of identifying analogs/derivatives of thyroid hormone that may prove effective as therapeutic agents to counteract some major diseases that are growing in importance worldwide. But, before this perspective can be realized further studies are needed to elucitade the cellular/molecular mechanisms of action of these agents and, in addition, a possible therapeutic use of these agents need deep investigations on possible deleterious side effects especially when administered for a long time. As for T_2 it remains to be established whether it has a physiological role or not and in an affirmative case it remains unclear what are the possible physiological roles that differentiate the actions of T2 and T3. Another aspect is related to the problem of possible TR-mediated genomic effect of T_2. It has been recently proven that T_2 is a specific ligand for a long isoform of TRβ in tilapia (Mendoza et al., 2013) affecting the growing processes in this specie. T3 and T2 both participate in the growth process, however their effects are mediated by different, specific TRβ1 isoforms ($STR\beta_1$ and $LTR\beta_1$ respectively). However, no data are present at the moment showing that a such binding can affect energy metabolism in higher species and further studies are needed to verify this possibility. Further studies may also be useful to try to develop new concepts that could help toward a better understanding of some of the effects of thyroid hormones and those of their analogs/derivatives.

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Mitochondrial efficiency and insulin resistance

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Insulin resistance, "a relative impairment in the ability of insulin to exert its effects on glucose, protein and lipid metabolism in target tissues," has many detrimental effects on metabolism and is strongly correlated to deposition of lipids in non-adipose tissues. Mitochondria are the main cellular sites devoted to ATP production and fatty acid oxidation. Therefore, a role for mitochondrial dysfunction in the onset of skeletal muscle insulin resistance has been proposed and many studies have dealt with possible alteration in mitochondrial function in obesity and diabetes, both in humans and animal models. Data reporting evidence of mitochondrial dysfunction in type two diabetes mellitus are numerous, even though the issue that this reduced mitochondrial function is causal in the development of the disease is not yet solved, also because a variety of parameters have been used in the studies carried out on this subject. By assessing the alterations in mitochondrial efficiency as well as the impact of this parameter on metabolic homeostasis of skeletal muscle cells, we have obtained results that allow us to suggest that an increase in mitochondrial efficiency precedes and therefore can contribute to the development of high-fat-induced insulin resistance in skeletal muscle.

Keywords: mitochondria, type 2 diabetes, insulin, skeletal muscle, proton leak

Obesity and the related metabolic disorders, such as insulin resistance and type 2 diabetes, are growing dramatically all over the world, so that experts are predicting an "obesity pandemic." In particular, it has been estimated that in year 2030 about 400 million people will exhibit type 2 diabetes (Wild et al., 2004). This alarming scenario arises from the prevalence of factors like consumption of high-fat diets and low physical activity.

Because the terms "insulin resistance," "type 2 diabetes" and "mitochondrial efficiency" are central to this discussion, their definition is fundamental. Insulin resistance is defined as "a relative impairment in the ability of insulin to exert its effects on glucose, protein and lipid metabolism in target tissues," so that at physiological concentrations insulin produces a lower biologic response (Kahn, 1978). Therefore, insulin resistance has many detrimental effects on metabolism that are the basis for a number of chronic diseases, including type 2 diabetes, a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both (Alberti and Zimmet, 1998). One fundamental process in the mitochondria is the oxidative phosphorylation, in which the electrons are removed from organic molecules and transferred to oxygen and the energy released is used in the synthesis of ATP. The amount of ATP formed per unit of consumed oxygen is determined by the efficiency of oxidative phosphorylation (Mogensen and Sahlin, 2005).

One of the most deleterious effects of obesity is deposition of lipids in non-adipose tissues, such as liver, skeletal muscle, and heart. It has been proposed that the accumulation of lipids in the muscle cell should interfere with insulin signaling, thereby causing insulin resistance. In agreement with this hypothesis, a strong association between fat accumulation in skeletal muscle (and liver) and insulin resistance has been found in men (McGarry, 2002). In addition, high levels of intramyocellular lipids (IMCL) and muscular insulin resistance have been found in type 2 diabetic patients (Goodpaster et al., 2001) and in high-risk non-diabetic subjects with a family history of diabetes (Jacob et al., 1999; Perseghin et al., 1999). However, high IMCL levels do not necessarily lead to insulin resistance, since they are also present in skeletal muscle from endurance-trained athletes, who are highly insulin-sensitive (Goodpaster et al., 2001; Schrauwen-Hinderling et al., 2006). The emerging idea is that increased intramuscular fat turns to be deleterious when an increase in the supply of lipids to skeletal muscle is not balanced by an increase in the oxidative pathways, so that toxic intermediates, such as ceramides and diacylglycerol, accumulate in the cell and interfere with the insulin signaling system (Kelley and Mandarino, 2000; Shulman, 2000). Therefore, in the above picture, a prominent role is played by the level of cellular oxidative capacity of fatty acids.

Mitochondria are the main cellular sites devoted to fatty acid oxidation. Therefore, a role for mitochondrial dysfunction in the onset of skeletal muscle insulin resistance has been proposed and several studies have dealt with possible alteration in mitochondrial function in obesity and diabetes, both in humans and animal models.

Studies in humans have shown that type 2 diabetes patients exhibited alteration in mitochondrial morphology, as well as a decrease in the activity of the respiratory chain (Kelley et al., 2002; Ritov et al., 2010). Other studies showed a coordinated reduction in the expression of genes encoding key enzymes in

oxidative mitochondrial metabolism in diabetic patients and in high-risk non-diabetic subjects with a family history of diabetes (Mootha et al., 2003; Patti et al., 2003). Petersen et al. (2003) reported a 40% decrease in oxidative metabolism in elderly subjects, that were also characterized by elevated levels of muscular fat and by muscular insulin resistance, thus suggesting that an age-associated decline in mitochondrial function might contribute to the development of insulin resistance. They also found that IMCL and ATP synthase were, respectively, 80% higher and 30% lower in insulin resistant subjects (Petersen et al., 2004). Szendroedi et al. (2007) found that *in vivo* ATP synthesis rate was decreased by 27% in diabetic patients, while in other studies *in vivo* mitochondrial function was compromised by ∼45% in type 2 diabetic patients, although IMCL content was similar between the groups, suggesting that impaired mitochondrial function may be a more important determinant of diabetes than IMCL levels (Schrauwen-Hinderling et al., 2007; Phielix et al., 2008). Taken together, these studies are consistent in showing that *in vivo* mitochondrial function is reduced in insulin resistant subjects and/or type 2 diabetic patients. This decrease could lead to accumulation of fat in muscle, but also provide lesser amount of ATP for membrane transports and signal transduction pathways, thereby contributing to the development of insulin resistance.

However, other observations argue against the hypothesis that mitochondrial dysfunction underlies the development of type 2 diabetes mellitus or muscular fat accumulation (Hancock et al., 2008; Han et al., 2011). In fact, several studies present findings in support of the concept that muscular fat accumulation may precede the development of mitochondrial dysfunction and/or that insulin resistance arises when mitochondrial function is unaffected or even improved (Turner et al., 2007; Hoeks et al., 2008; Ara et al., 2011). For example, an improved or unchanged mitochondrial oxidative capacity has been found after consumption of a high-fat diet in mice or rats exhibiting insulin resistance (Turner et al., 2007; Hoeks et al., 2008). These data suggest that high-fat diets, although leading to insulin resistance in rodents, are not accompanied by mitochondrial dysfunction, but rather they lead to improved mitochondrial oxidative capacity. Other researchers looked at the time course of changes in mitochondrial function in skeletal muscle in response to high-fat feeding. Chanseaume et al. (2007) showed a transiently enhanced activity of the oxidative phosphorylation after 14 days, but a significant decrease at day 40. Laurent et al. (2007) showed in rats that ATP synthesis rates decreased by 50% within 24 h, returned to normal values after 2–3 weeks on the high-fat diet, and again decreased by 30–50% after 1 month. Finally, Bonnard et al. (2008) showed that 1 month of high-fat, high-sucrose diet feeding induced glucose intolerance in mice, without mitochondrial dysfunction, that was evident after 16 weeks. Taken together, these studies are consistent with the hypothesis that mitochondrial dysfunction may be a consequence rather than cause of muscular fat accumulation, but this does not exclude the possibility that mitochondrial dysfunction could in turn induce insulin resistance.

Physical activity is a major regulator of mitochondrial function in muscle, and exercise potently activates mitochondrial biogenesis, while chronic inactivity is associated with reduced mitochondrial number (Hoppeler and Fluck, 2003; Little et al., 2011).

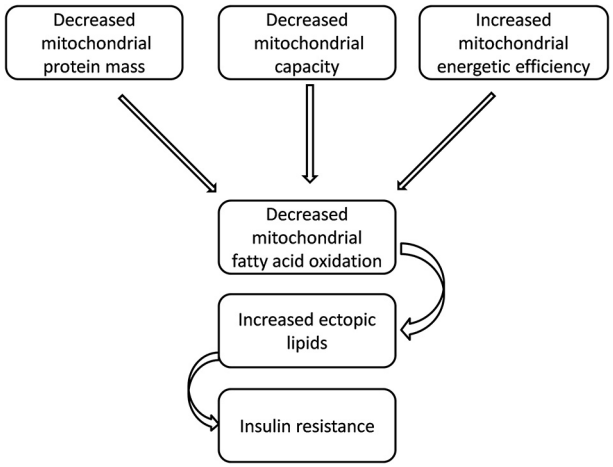

FIGURE 1 | A possible link between insulin resistance and impaired mitochondrial performance.

Obesity and other metabolic disorders are linked with reduced activity levels and increased sedentary behavior (Hamilton et al., 2007; Levine et al., 2008). Thus, it is possible that some mitochondrial defects reported in overweight or obese insulin-resistant subjects can be explained, in part, by low levels of physical activity. In this respect, animal models are very useful tools, since rats kept in laboratory display a sedentary behavior, due to standard housing conditions (Spangenberg et al., 2005), and therefore it is possible to perform studies aiming at the elucidation of the link between insulin resistance and mitochondrial functioning, without the confounding effect of changes in physical activity. Another possible reason of the apparent discrepancy among the various results published on the above issue is the choice of the parameter to be studied in evaluating mitochondrial function. In fact, if the hypothesis is that reduced mitochondrial oxidation of fatty acids causes ectopic fat deposition, that in turn elicits insulin resistance, all the factors contributing to mitochondrial lipid burning must be taken into account. The mitochondrial oxidation of metabolic fuels depends not only on organelle number and organelle activity, but also on energetic efficiency of the mitochondrial machinery in synthesizing ATP from the oxidation of fuels (**Figure 1**). Changes in each of these three factors could theoretically affect lipid oxidation and should be monitored to confirm or reject the hypothesis (**Figure 1**).

Many reported studies on the issue of mitochondrial dysfunction in insulin resistant states have focused the attention on mitochondrial impairment in terms of reduced mass and/or oxidative activity. However, it is well-known that the amount of fuels oxidized by the cell is dictated mainly by ATP turnover rather than by mitochondrial oxidative activity (Boveris et al., 2000) and therefore, in resting skeletal muscle, changes in organelle number and/or activity could be without consequence for cellular bioenergetics, while modifications in mitochondrial energetic efficiency certainly alter the amount of oxidized fuels, even if ATP turnover does not vary. In fact, the efficiency with which dietary calories are converted to ATP is determined by the coupling efficiency

of oxidative phosphorylation. If the respiratory chain is highly efficient at pumping protons out of the mitochondrial inner membrane, and the ATP synthesis is highly efficient at converting the proton flow through its proton channel into ATP (from ADP), then the mitochondria will generate maximum ATP and minimum heat per calorie. These mitochondria are said to be "tightly coupled." In contrast, if the efficiency of proton pumping is reduced and/or more protons are required to make each ATP molecule, then each calorie will yield less ATP but more heat. Such mitochondria are said to be "loosely coupled." Therefore, the coupling efficiency determines the balance of calories used to perform work (ATP) or for heat generation. It remains to be established whether, under high-fat conditions, cellular ATP demand is altered, a parameter that can only be assessed in the living animal (Amara et al., 2008).

To our knowledge, data on the energetic efficiency in skeletal muscle mitochondria in conditions of obesity-induced insulin resistance are scarce. By using a rat model of high-fat diet-induced obesity, we have evidenced that after 1 and 2 weeks of high-fat feeding (Crescenzo et al., 2014a,b), skeletal muscle mitochondrial efficiency is increased, thus giving rise to a reduced burning of energy substrates. This modification of mitochondrial efficiency takes place at a time point when insulin sensitivity is still maintained (Crescenzo et al., 2014a,b). Therefore, these results could be consistent with a role for mitochondrial impairment in the onset of insulin resistance. In fact, if mitochondria are more coupled, less substrates need to be burned to obtain the same amount of ATP. At the same time, high-fat feeding is associated with increased lipid supply to skeletal muscle (Crescenzo et al., 2014b), so that a condition of imbalance could occur, since lipid supply exceeds lipid burning and gives rise to ectopic lipid deposition. In agreement with this suggestion, we have also found increased levels of skeletal muscle triglycerides (Crescenzo et al., 2014b). Interestingly, when high-fat diet intake was carried out for 7 weeks, insulin resistance developed but the alteration in mitochondrial efficiency disappeared (Lionetti et al., 2007). One possible explanation could be related to changes in lipid composition of the mitochondrial membranes induced by the high-fat intake, one of the factors contributing to mitochondrial proton leak and hence to mitochondrial efficiency (Jastroch et al., 2010).

A similar increase in mitochondrial efficiency is also evident after 2 weeks of feeding a high-fat-high fructose diet (Crescenzo et al., 2014b) but in the presence of insulin resistance. Since mitochondrial energetic efficiency is higher both in rats with normal insulin sensitivity (high-fat-fed rats) and in those with decreased insulin sensitivity (high-fat-high fructose-fed rats) we can hypothesize that this mitochondrial modification is not caused by, but could contribute to, the onset of insulin resistance. In agreement with this suggestion, the content of skeletal muscle ceramides (known to be mediators of altered insulin signaling, Coen and Goodpaster, 2012) is higher in rats fed high-fat diet but even higher in rats fed high-fat-high fructose diet and therefore it is possible that in the latter group of rats its concentrations have reached a threshold level to be able to partly block insulin transduction pathway. In addition, in rats fed a low-fat, fructose-rich diet we have found a reduced insulin signaling system in skeletal muscle concomitant to an increase in mitochondrial efficiency and cellular levels of ceramides after 8 weeks of dietary treatment (Crescenzo et al., 2013).

In support of the link between mitochondrial efficiency and insulin resistance in skeletal muscle during high-fat feeding are the results showing that the naturally occurring iodothyronine, 3,5-diiodo-L-thyronine (T2) increases mitochondrial proton leak (Lombardi et al., 2007, 2009), thus decreasing mitochondrial efficiency, and when given to high-fat-fed rats is able to reverse high-fat-induced insulin resistance (de Lange et al., 2011; Moreno et al., 2011).

Another condition of obesity and related insulin resistance is the progression of aging. In agreement with results obtained on diet-induced obesity, when studying age-induced obesity in rats, we have found an increase in skeletal muscle mitochondrial efficiency, in parallel with the development of insulin resistance, in the transition from young (60 days) to middle age (180 days) (Iossa et al., 2004), and a further increase from middle age to old age (2 years) (Crescenzo et al., 2014c).

CONCLUDING REMARKS

In summary, results reporting evidence of mitochondrial dysfunction in type 2 diabetes mellitus are numerous, even though the issue that this reduced mitochondrial function is causal in the development of the disease is not yet solved, also because a variety of parameters have been used in the studies carried out on this subject. By assessing the alterations in mitochondrial efficiency as well as the impact of this parameter on metabolic homeostasis of skeletal muscle cells we have obtained results that allow us to suggest that an increase in mitochondrial efficiency precedes and therefore can contribute to the development of high-fat induced insulin resistance in skeletal muscle.

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Proteomic approaches for the study of tissue specific effects of 3,5,3′-triiodo-L-thyronine and 3,5-diiodo-L-thyronine in conditions of altered energy metabolism

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In vertebrates and, specifically, in mammals, energy homeostasis is achieved by the integration of metabolic and neuroendocrine signals linked to one another in an intricate network hierarchically responding to the tight modulating action of hormones among which thyroid hormones (THs) play a central role. At the cellular level, 3,5,3′-triiodo-L-thyronine (T3) acts mainly by binding to specific nuclear receptors (TRs) but actually it is becoming more and more evident that some T3- actions are independent of TRs and that other iodothyronines, such as 3,5-diiodo-L-thyronine (T2), affect energy metabolism and adiposity. In the postgenomic era, clinical and basic biological researches are increasingly benefiting from the recently developed new omics approaches including, among the others, proteomics. Considering the recognized value of proteins as excellent targets in physiology, the functional and simultaneous analysis of the expression level and the cellular localization of multiple proteins can actually be considered fundamental in the understanding of complex mechanisms such as those involved in thyroid control of metabolism. Here, we will discuss new leads (i.e., target proteins and metabolic pathways) emerging in applying proteomics to the actions of T3 and T2 in conditions of altered energy metabolism in animal tissues having a central role in the control of energy balance.

Keywords: iodothyronine, metabolism, proteomics, obesity, mitochondrion

INTRODUCTION

Strong evidence supports an essential role for thyroid hormones [THs, 3,5,3′,5′-tetraiodo-L-thyronine (T4), and 3,5,3′-triiodo-L-thyronine (T3)] in the physiological regulation of whole-body energy balance and metabolism in homoeothermic species and, specifically, in mammals. Moreover, THs control a bulk of physiological processes such as growth, development, and metabolic rate (for a recent review on thyroid hormone signaling in energy metabolism, see Brent, 2012; Davis et al., 2013; López et al., 2013; McAninch and Bianco, 2014; Mullur et al., 2014). However, the network of events involved in the actions of THs is complicated and still incompletely understood. Integrative omic approaches, such as transcriptomics and proteomics, hold great promise and actually are having increasing applications in the study of complex biological systems. As far as it concerns the metabolic actions of THs, a large amount of data is available from transcriptomic studies which have been quite extensively performed to identify T3-target genes in several models (for review see, Silvestri et al., 2011a, and references therein) actually providing a cornucopia of novel information on the regulation of transcription by THs (e.g., the potential of T3 to regulate miRs, Visser et al., 2009). However, the intrinsic nature of these studies provided no information concerning the status of the corresponding encoded proteins, which, indeed, enable to assess the programs actually executed. On the other hand, proteomic approaches [such as, among the others, two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS)], allow the simultaneous measurement and comparison of the expression levels of hundreds of proteins as well as the identification of other aspects of protein functions (i.e., post-translation modifications), giving rise to a fuller understanding of cellular functions (for recent review, see Johnson and White, 2012; Feeney and Schöneich, 2013; Zhang et al., 2013). However, intrinsic limitations inherent to the more classical approaches—including, among the others, difficulties in the resolution of proteins with extreme isoelectric point values, loss of very hydrophobic proteins, absence of proteins of high and low molecular weights and poor resolution of low-abundance proteins—cannot be ignored (Sriharshan et al., 2014; and for review see, Wittig and Schägger, 2009; Silvestri et al., 2011a,b). In this review, we will outline the new leads emerging from the application of proteomic analyses to the actions of thyroid hormones in the regulation of energy balance in conditions of altered energy metabolism.

PROTEOMIC ANALYSIS PERTAINING TO THE ACTIONS OF T3

Hypothyroidism and hyperthyroidism are two pathological conditions in which energy metabolism is altered leading to hypo- and hyper-metabolic states, respectively. Metabolically active tissues, such as liver and skeletal muscle, retain the ability to

respond, to counteract and to adapt to such alterations by modifying gene/protein expression patterns.

The first application of proteomic tools to the modulations that T3 exerts *in vivo* over tissue proteins can be traced back to 1981. Analyzing the liver of normal, thyroidectomized, and thyroidectomized plus T3 treated rats, by using classical 2-DE, it has been shown that changes in the thyroid state significantly affect the composition of the hepatic nucleoproteins (Nikodem et al., 1981).

To obtain a more comprehensive identification and characterization of molecular events/pathways associated with altered thyroid states, more recently, a high-resolution differential proteomic analysis, combining 2-DE and subsequent matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-ToF MS), was performed, thus providing the first systematic identification of T3-induced changes in the protein expression profiles of liver and skeletal muscle of hypothyroid rats (Silvestri et al., 2006, 2007).

In the liver, among the 600 detected spots, 53 proteins (8% of the analyzed proteome) resulted to be significantly affected by T3 treatment. On the base of their identity, the unambiguously identified proteins were classified as involved in substrate (e.g., aldehyde-dehydrogenase and α-enolase) and lipid (e.g., short chain-specific acyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase) metabolism, energy metabolism (e.g., ATP synthase α-chain), detoxification of cytotoxic products (e.g., catalase and glutathione-S-transferase), calcium homeostasis (senescence marker protein 30), amino acid catabolism (arginase-1), and the urea cycle (ornithine carbamoyltransferase). Interestingly, 76% of these proteins were down-regulated in hyperthyroid rats vs. hypothyroid ones. These proteomic data gave new support to the transcriptome analyses revealing the idea that negative regulation by T3 on gene expression might be much more prevalent than previously thought (Feng et al., 2000). Among the down-regulated proteins, there were ornithine carbamoyltransferase, arginase 1, the peroxisomal catalase, and the cytoplasmic glutathione-S-transferase. This allowed to deeper understand how the thyroid state, in the liver, modulates urea and ammonia production (Marti et al., 1988) and promotes oxidative stress (for recent review, see Videla, 2010).

On the other hand, among the few up-regulated proteins, α-enolase resulted to be strongly induced (+240%) by T3 administration. This further supported the notion that T3 stimulates gluconeogenesis and glucose production in the liver thus opposing to the action of insulin (for recent review, see Brenta, 2011).

Concerning skeletal muscle, the whole-cell protein content of gastrocnemius muscle was analyzed. With the detection limits set, a proteome of about 200 spots was obtained. 33 protein spots (15% of the analyzed proteome) resulted to be significantly affected by T3 treatment. When comparing hyperthyroid vs. hypothyroid rats, 70% of the unambiguously identified proteins (20 protein spots) were up-regulated. The largest group of affected proteins was involved in substrate (e.g., pyruvate kinase muscle isozyme and malate dehydrogenase) and energy metabolism (e.g., creatine kinase M-type and ATP synthase beta chain), another important group was represented by stress-induced proteins (HSPs), and the remainder were implicated in structural features or gene expression (transcription, translation) (e.g., chromodomain-helicase-DNAbinding protein 1 and eukaryotic translation initiation factor 3 subunit 10). Importantly, the thyroid state resulted to induce simultaneous changes in the expression levels of proteins involved in both structural and metabolic features of the gastrocnemius muscle. Specifically, hypothyroidism and hyperthyroidism were found to induce a structural and metabolic shift toward a slower and a faster phenotype, respectively (Silvestri et al., 2007). Indeed, in accordance with a predominant expression of myosin heavy chain (MHC) Ib over MHC IIb in hypothyroidism and a reversal of this ratio after T3 administration, the expression level of myosin regulatory light chain 2, typical of slow-twitch fibers, was strongly increased in hypothyroidism, with hyperthyroidism significantly reducing it. Coherently, and in accordance with a generally increased metabolic dependence on glycolysis in hyperthyroidism, β-enolase, pyruvate kinase, and triosephosphate isomerase protein levels were significantly increased following T3 treatment.

All together, the so far described data allowed to obtain an at glance evaluation of the effects elicited by T3 in hypothyroid rats, highlighting the tissue-specific proteomic response: in the liver T3 produces a general down regulation of affected proteins likely modulating substrate metabolism (i.e., aminoacid catabolism); in the gastrocnemius muscle T3 produces a more pronounced effect of up-regulation likely promoting energy metabolism and glucose utilization.

An even more recent study was published in which proteomic approaches were used to obtain new information on the *in vivo* actions of T3. Specifically, the early changes induced in rat liver by a single mitogen dose of T3 were characterized (Severino et al., 2011). Many enzymes, directly or indirectly involved in energy metabolism and oxidative stress, were identified among the differentially expressed proteins furnishing new insight into the mechanisms by which T3 induces hepatocyte proliferation.

These studies, as a whole, allowed the identification of proteins previously not known to be regulated by T3 and might prompt the scientific community to go further with proteomic-based approaches to increase the awareness of the multiple cell processes and signaling pathways involved in the effects of such iodothyronine.

PROTEOMIC ANALYSIS PERTAINING TO THE ACTIONS OF T2

Overweight and adiposity lead to impaired energy balance with several other metabolic disturbances taking place. T3 exerts both hypolipidemic and hyperlipidemic effects due to its control of lipolytic and lipogenic pathways. Interestingly, accumulating evidence has indicated that several metabolic effects of THs can be attributed to endogenous metabolites of T3 that actually can be considered new discovered arms by which the thyroid gland can control whole body energy homeostasis (Goglia, 2005; Moreno et al., 2008; Piehl et al., 2011; Senese et al., 2014). 3,5-diiodo-l-thyronine (T2) is receiving particular attention in view of its specific excito-metabolic actions (for recent review, see Coppola et al., 2014; Senese et al., 2014 and references within).

The effects and mechanisms underlining the actions of T2 have so far been studied both *in vivo* and *in vitro* (Scapin et al., 2010; Grasselli et al., 2011, 2012) models (for recent review, see Silvestri et al., 2013; Vergani, 2014). Up to now, a controversy exists as far as it concerns the nature of such mechanisms and the possibility that T2 might exert some of its effects in a TR-dependent manner (Mendoza et al., 2013; Jonas et al., 2014; Navarrete-Ramírez et al., 2014; Orozco et al., 2014). However, it cannot be excluded that the putative interaction of T2 with nuclear TRs, at least in mammalian species, might be a consequence of the high doses used (Goldberg et al., 2012; Jonas et al., 2014).

Of the currently described *in vivo* effects of T2, a particular physiological and pharmacological relevance appears to be associated with those that we can define as hypolipidemic and anti-steatotic effects, that have been described in several animal models of overweight and thus of altered energy metabolism [high fat (HFD) or high cholesterol diet-fed rodents] (Lanni et al., 2005; Grasselli et al., 2008; Mollica et al., 2009; de Lange et al., 2011; Moreno et al., 2011; Goldberg et al., 2012; Jonas et al., 2014) and in humans too (Antonelli et al., 2011). Importantly, a more recent study has reported that T2 administration stimulates energy expenditure and reduces body mass gain also in standard diet fed aging rats (Padron et al., 2014).

As far as it concerns the anti-steatotic effect of T2 elicited in the liver of HFD rats (at the pharmacological but not thyrotoxic dose of 25 μg/100 g body weight), to further identify candidate molecules as well as molecular/biochemical pathways linking fat consumption, the pathogenesis of hepatic steatosis, and mitochondrial functions, a high-resolution differential proteomic analysis [by combining 2-DE, MALDI-ToF MS, Blue Native-PAGE (BN-PAGE) and *in silico* analysis] was performed (Silvestri et al., 2010).

Data analysis demonstrated that the steatotic effect of HFD, and the anti-steatotic effect of T2-treatment are strictly associated with altered expression levels of several proteins and enzymes involved in key liver metabolic pathways. These

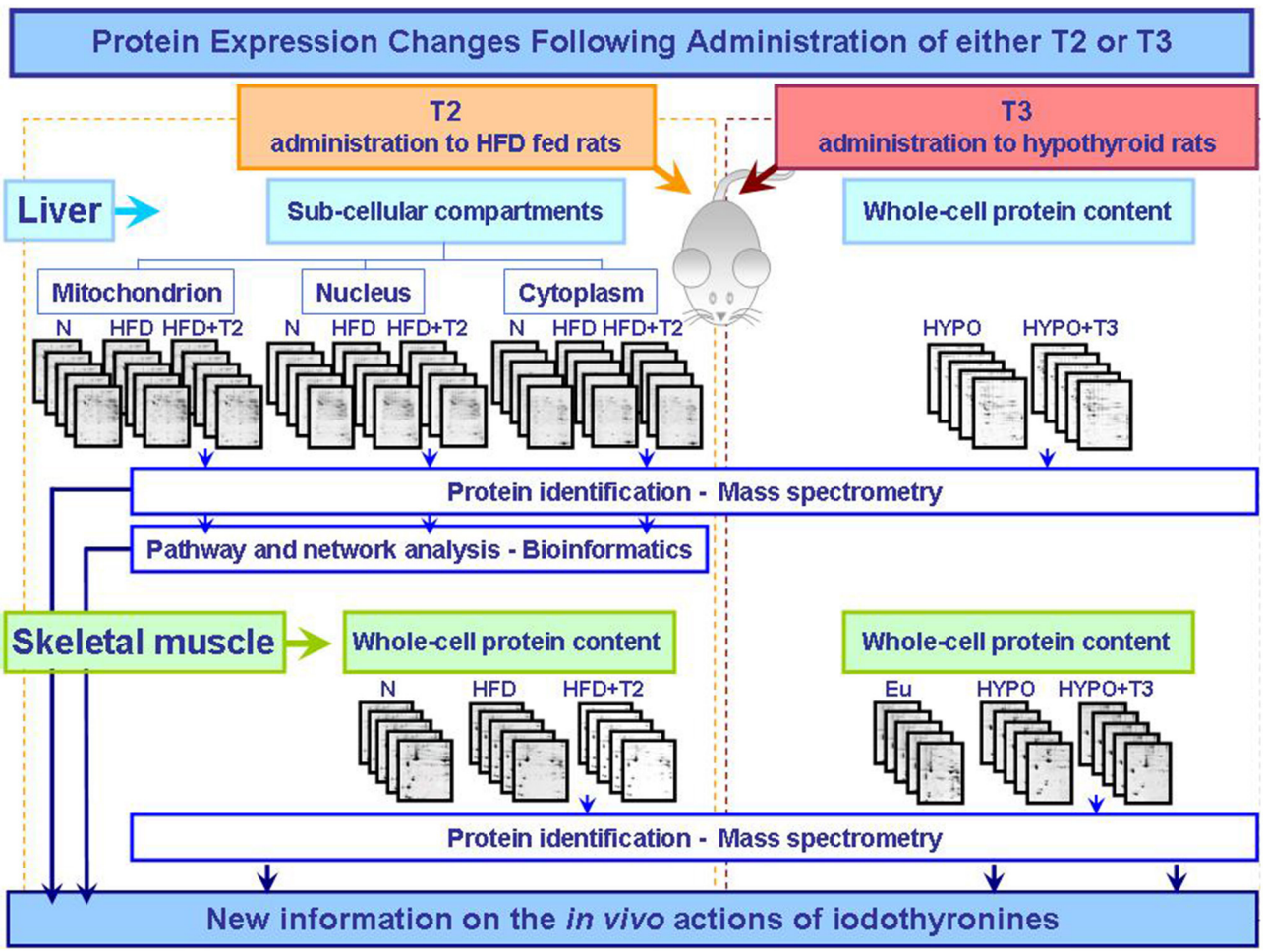

FIGURE 1 | Synoptic of the workflow of the proteomic approaches so far utilized to obtain new information on the actions that T2 and T3 exert *in vivo* in key metabolically active tissues central in the control of energy balance. The described studies were performed as integrated approaches including 2D-E, mass spectrometry, and bioinformatic tools (Silvestri et al., 2006, 2007, 2010; Moreno et al., 2011). Abbreviations: N, standard diet fed control rats; HFD, high fat diet fed rats; HFD+T2, high fat diet fed rats treated with T2; Eu, euthyroid rats; Hypo, hypothyroid rats; Hypo+T3, hyperthyroid rats.

pathways included: fatty acid metabolism [e.g., carnitine O-palmitoyltransferase (CPT) 2 and long-chain fatty acid-CoA ligase], ketone-bodies [e.g., hydroxymethylglutaryl-CoA (HMG-CoA) synthase] and energy metabolism (e.g., ATP synthase subunit alpha), amino acid and nitrogen metabolism (e.g., glutamate dehydrogenase 1), the urea cycle (e.g., carbamoyl-phosphate synthase), the stress response (e.g., HSP60 and catalase) and protein turnover (e.g., proteasome component C2 and proteasome iota chain). Importantly, after T2-treatment, the majority of these proteins exhibited alterations in their expression level that were opposite to those observed in HFD and normalized vs. those observed in standard diet fed control rats (Silvestri et al., 2010). For example, long-chain fatty acid-CoA ligase and ATP synthase subunit alpha, both having a central role in lipid biosynthesis and energy metabolism, were significantly up-regulated by HFD (thus positively correlating with the steatotic condition present in HFD rats) and normalized in their expression levels by T2 treatment, thus highlighting some of the biochemical bases of the hypolipidemic effect of T2. The obtained proteomic data were corroborated by the *in silico* analysis (i.e., pathway and network analysis by using the Ingenuity® Systems, www.ingenuity.com). Of note, the network concerning the effects of T2 contained two external coupling namely HMG-CoA synthase and CPT, supporting, once again, the centrality of lipid metabolism in the action of T2 in the liver. Moreover, as mitochondria appeared to be a major target for the metabolic and energy adaptations induced by fat-overload, and displayed a significant response, in terms of their proteome, to T2-treatment, a BN-PAGE-based approach was also used to characterize the profile of the liver OXPHOS and to measure their individual in-gel activities. In summary, T2 induced vs. HFD, an enhancement of OXPHOS activities (complex I, II, and IV) well suggesting reduced oxidative damage to the respiratory chain itself and a success in efficiently catabolizing the extra load of fatty acids, likely "sparing" other metabolically active tissue, specifically the skeletal muscle, counteracting, at the same time, the fat-induced insulin resistance (IR) attributable to HFD (de Lange et al., 2011). To test this hypothesis, by using the same animal model as in Silvestri et al. (2010), an integrated approach was designed to assess the effects of T2 on skeletal muscle insulin sensitivity and protein profile (Moreno et al., 2011). Schematically, without inducing sarcopenia, T2 administration to HFD rats produced in gastrocnemius muscle an increase in: type II (glycolitic) fibers, glucose transporter 4 (GLUT4) membrane content, glucose utilization (through Akt activation), and glycolytic enzymes activity, while preventing the HFD-induced increase in fatty acid uptake (through FAT/CD36) and intramyocellular lipid (triglyceride) accumulation. The 2-DE/nano-liquid chromatography-electrospray ionization-linear ion trap-tandem mass spectrometry (nLC-ESI-LIT-MS/MS)-based analysis revealed that T2 treatment, while inducing a shift toward a fast phenotype, significantly altered the gastrocnemius muscle protein expression profile of HDF rats. In particular, structural proteins such as the fast isotypes of myosin light chains (i.e., MLC1f, MLC2f) and the tropomyosin α chain fast increased significantly following T2 treatment whereas the content of the slow isotypes MLC1s, MLC2s, and the tropomyosin α chain slow decreased. Coherently, in T2-treated HFD rats, other glycolytic enzymes (i.e., α- and β-enolase, and triosophosphate isomerase) were up-regulated while enzymes involved in oxidative metabolism (i.e., carbonic anhydrase III and myoglobin) were significantly down-regulated vs. HFD control rats (Moreno et al., 2011).

Overall, these data indicated, for the first time, that T2, at least at the used dose and in rats, without thyrotoxic effects, can significantly impact the proteome profile of responsive tissues, such as liver and skeletal muscle, thus ultimately influencing fuel utilization, and energy metabolism in a functional cross-talk between target organs. In particular, in terms of tissue specific effects of T2, the liver might be mainly involved in lipid metabolism derangement while the skeletal muscle might be mainly implicated in glucose utilization.

CONCLUSIONS AND PERSPECTIVES

The biochemical and cellular mechanisms that underlie tissue specific actions of T3 and T2 are only beginning to be elucidated. However, the proteomic studies so far conducted separately analyzed the effects of T3 and T2 in different states of altered energy balance: changed thyroid state and over-nutrition, respectively (**Figure 1**). To further characterize and compare the molecular and biochemical pathways that underlie T3 and T2 metabolic actions, T3 and T2 themselves should be used in the same experimental design in comparative approaches so to highlight putative common effects or iodothyronine-specific one.

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Adipose tissues and thyroid hormones

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The maintenance of energy balance is regulated by complex homeostatic mechanisms, including those emanating from adipose tissue. The main function of the adipose tissue is to store the excess of metabolic energy in the form of fat. The energy stored as fat can be mobilized during periods of energy deprivation (hunger, fasting, diseases). The adipose tissue has also a homeostatic role regulating energy balance and functioning as endocrine organ that secretes substances that control body homeostasis. Two adipose tissues have been identified: white and brown adipose tissues (WAT and BAT) with different phenotype, function and regulation. WAT stores energy, while BAT dissipates energy as heat. Brown and white adipocytes have different ontogenetic origin and lineage and specific markers of WAT and BAT have been identified. "Brite" or beige adipose tissue has been identified in WAT with some properties of BAT. Thyroid hormones exert pleiotropic actions, regulating the differentiation process in many tissues including the adipose tissue. Adipogenesis gives raise to mature adipocytes and is regulated by several transcription factors (c/EBPs, PPARs) that coordinately activate specific genes, resulting in the adipocyte phenotype. T3 regulates several genes involved in lipid mobilization and storage and in thermogenesis. Both WAT and BAT are targets of thyroid hormones, which regulate genes crucial for their proper function: lipogenesis, lipolysis, thermogenesis, mitochondrial function, transcription factors, the availability of nutrients. T3 acts directly through specific TREs in the gene promoters, regulating transcription factors. The deiodinases D3, D2, and D1 regulate the availability of T3. D3 is activated during proliferation, while D2 is linked to the adipocyte differentiation program, providing T3 needed for lipogenesis and thermogenesis. We examine the differences between BAT, WAT and brite/beige adipocytes and the process that lead to activation of UCP1 in WAT and the presence of BAT in humans and its relevance.

Keywords: BAT, thermogenesis, adipogenesis, lipogenesis, lipolysis, deiodinases, brite adipocytes, "browning"

Abbreviations: ACC, acetyl-CoA carboxylase; AKT or PKB, protein kinase B; aP2, fatty-acid binding protein; BAT, brown adipose tissue; BMP7, BMP4, Bone morphogenetic protein 7 or 4; cAMP, cyclic AMP; c/EBPα, β, δ, CAAT/enhancer binding protein α, β, δ; CD137, Tumor necrosis factor receptor superfamily 9; ChREBP, carbohydrate responsive element binding protein; Cidea, Cell death activator CIDE-A; D1, dio1, deiodinase 1; D2, dio2, type 2 deiodinase; D3, dio3, deiodinase 3; ERK, Extracellular-signal related kinase; FAS, fatty acid synthase; FFA, Free fatty acid; aFGF and bFGF, acidic and basic fibroblast growth factor; FGF10, Fibroblast growth factor 10; Glut4, Glucose transporter 4; GPD, glycerophosphate dehydrogenase; IBMX, 3-Isobutyl-1-methylxantina; IGFI, Insulin growth factor I; IGFBP, Insulin growth factor binding proteins; LDH, lactic dehydrogenase; LPL, Lipoprotein lipase; MCSF, Macrophage colony-stimulating factor; ME, malic enzyme; Myf5, myogenic factor 5; NE, norepinephrine; PEPCK, phosphoenolpyruvate carboxykinase; PET, positron emission tomography; PGC1α, PPARγ coactivator 1α; PI3K, phosphoinositol-3-kinase; PKA, protein kinase A; PPARγ, Peroxidase proliferator-activated receptor gamma; PPARE, Peroxidase proliferator-activated response element; PRDM16, PR domain containing 16; Pref1, preadipocyte factor 1; Pten, phosphatase and tensin homolog; RARE, retinoic response element; RIP140, receptor-interacting protein 140; SC, subcutaneous white adipose tissue; SCD, stearoyl-CoA desaturase; SLC, Solute carrier transporter; SNS, sympathetic nervous system; Spot14, spot14 or Trsp, Thyroid responsive protein; SVF, stromal vascular fraction; T3, triiodothyronine; T4, thyroxine; TBX1, T-box transcription factor 1; Tcf21, transcription factor 21; Tmem26, Transmembrane protein 26; TNFα, Tumor necrosis factor α; TRα, TRβ, Thyroid hormone receptor α, β; TRE, Thyroid Response Element; Triac, triiodothyroacetic acid; TSH, Thyrotropin; TSHr, TSH receptor; UCP1, uncoupling protein 1; V, visceral white adipose tissue; WAT, white adipose tissue.

INTRODUCTION

Thyroid hormones regulate multiple physiological systems in many tissues and are of maximal importance during developmental processes. T3 regulates the development of many tissues (Bernal, 2002; Morreale De Escobar et al., 2004) by acting in specific cells, for example in the cochlea or the retina (Forrest et al., 1996; Roberts et al., 2006). The supply of thyroid hormones is finely tuned and regulated in a time- and dose-specific way in specific areas of the brain, through sequential increases or decreases in D2 and D3 deiodinases, as studied in human fetal brain (Kester et al., 2004), in the cochlea (Ng et al., 2004) or during the metamorphosis of amphibians and fishes (Brown et al., 1996; Isorna et al., 2009). Thyroid hormones act by regulating genes involved in the differentiation program of many tissues. During adult life thyroid hormones regulate the function of many tissues, as brain, muscle, heart, liver, adipose tissue or skin by controlling the metabolism of carbohydrates, lipids, the transcription of many proteins (Mullur et al., 2014) and the basal metabolic rate. T3 acts through their nuclear receptors, which are encoded by two genes TRα and TRβ, with different isoforms: TRα-1, TRα-2, and TRβ-1. They bind to thyroid response elements (TREs) present in the promoters of the target genes, forming heterodimers with

RXR. T3 actions are modulated by corepressors and coactivators. Thyroid hormone concentrations are modulated in tissues by the action of the deiodinases D1, D2, and D3 that control the amount required in each tissue.

The adipose tissue is one important target of thyroid hormones. The adipose tissue is the main place for lipid storage, besides its function in lipid transport, synthesis and mobilization. The adipose tissue stores energy in the form of fat, so that this metabolic energy can be used in times of hunger or illness. In addition, adipose tissue works as a homeostatic mechanism regulating energy reserves and releasing many substances that keep the homeostasis of the organism, some of them, like leptin, act as adipostats regulating the amount of fat stored.

Mammals have two types of adipose tissue: white and brown adipose tissue (WAT and BAT), with different phenotype, function and regulation. The white adipose tissue (WAT) was considered for many years a site for lipid storage. White adipocytes have a characteristic large lipid droplet that fills the cellular space, while the cellular structures (nuclei, mitochondria) are placed near the cellular membrane. WAT is distributed in different anatomical locations that have been grossly divided into subcutaneous and visceral fat o intra-abdominal fat. Both locations have different lipolytic sensitivity in response to hormones and its abundance is associated to a different health risk, because an increase in visceral fat is associated to insulin resistance, metabolic syndrome and cardiovascular diseases (Wajchenberg, 2000). Other organs as the kidney, heart and the gonads (perirenal or perigonadal depots) are also cover by fat. Those adipose locations are not pure WAT and some of them are located in the primitive BAT locations as found in hibernating animals. In humans WAT is one of the largest tissues and is found in many depots all along the body, it accounts for about 10–15% of the total body weight in control subjects, and this percentage increases up to 50% in obese subjects.

The brown adipose tissue (BAT) is responsible for the adaptive or facultative thermogenesis. BAT is activated in response to cold exposure or fat diets providing extra heat in demanding situations to maintain energy balance. BAT is abundant in small rodents, hibernating animals and specially in newborns (Cannon and Nedergaard, 2004); it is found in small pads in the interscapular and cervical region, protecting organs as the heart, aorta, kidneys and other organs that should be heated up during the arousal from hibernation. The main function of BAT is to produce heat. This is possible by the activation of the uncoupling protein 1 (UCP1), a mitochondrial protein that acts as proton channel, uncoupling the oxidative phosphorylation and producing heat, instead of ATP. This activation is switch on by the adrenergic stimulation that increases after cold exposure. BAT is a highly innervated and irrigated tissue. BAT morphology is characterized by multilocular lipid droplets that can be easily mobilized and multiple and active mitochondria which number and activity increases under cold exposure (mitochondriogenesis). Today, the activation of BAT is considered as a possible therapeutic tool to fight obesity.

The analysis of the lineage of white and brown adipocytes reveals that both cells have a distinct embryological origin. Brown adipocytes have a myogenic origin, different from white adipocytes, defined by the expression of the myogenic marker, myogenic factor 5, Myf5+ that is also found in myoblasts (Gesta et al., 2007; Timmons et al., 2007). Several genes have been identified to trace the presence of white and brown adipocytes (see below and **Figure 1**), as well as markers of its terminal differentiation: UCP1 and D2.

In addition another type of adipose tissue has been identified recently identified, called "beige" or "brite" adipose tissue. Under certain circumstances WAT contains small clusters of brown-like adipocytes that express UCP-1, which have been called "brite" (brown-white) or beige adipocytes. They are multilocular and express UCP-1, Cidea (Cell death activator CIDE-A) and other markers of brown adipocytes as PGC1α (PPARγ Coactivator 1α). They are more frequently in certain anatomical locations, e.g., the inguinal fat. "Brite" adipocytes seem to come from different embryonic precursors than brown adipocytes and express distinct gene signatures (Petrovic et al., 2010). Its presence, abundance and increase in activity are regulated different than brown adipocytes (Macotela et al., 2012; Walden et al., 2012).

PROLIFERATION AND DIFFERENTIATION OF ADIPOCYTES. T3 ACTIONS

The adipose tissue was often considered a place for lipid storage. Recently it has been a renewed interest on the study of adipose tissue, the adipocyte-specific genes and its regulation, the secretion of adipocytes and the signaling pathways altered in pathological situations as obesity or diabetes. This has lead to a better knowledge of the adipose tissue. The adipocyte is the functional unit of the adipose tissue and is specialized in the storage of lipids. It acquires its full capacity during adipogenesis that involves the proliferation of mesenchymal-type cells and its differentiation that allows the adipocyte to accomplish its specific functions.

The study of adipose tissue was hampered by the lack of good cell culture systems and by the high lipid content of the adipose tissue. The differentiation of the adipocytes was studied in the eighties using preadipose cells lines derived from NIH 3T3 fibroblasts (3T3-L1 and 3T3-F442) (Spiegelman et al., 1983; Lin and Lane, 1994). Several enzymes were identified that increase exponentially during the process of differentiation. Many of them were lipogenic and glycolytic enzymes: the glycerophosphate dehydrogenase (GPD), the lactic dehydrogenase (LDH), the acetyl-CoA carboxylase (ACC), the fatty-acid binding protein aP2, the stearoyl-CoA desaturase (SCD), the fatty acid synthase (FAS) (Mackall et al., 1976; Spiegelman et al., 1983; Bernlohr et al., 1984; Ntambi et al., 1988), the lipoprotein lipase (LPL), malic enzyme (ME), phosphoenolpyruvate carboxykinase (PEPCK) and some new genes as adipsin (Cook et al., 1987; Flier et al., 1989) and adipoQ, nowadays called adiponectin (Hu et al., 1996). There was a progression in the appearance of these proteins, some are expressed as early markers of differentiation and others appear later on (Ailhaud et al., 1992). The transcription factors C/EBPs were identified as one of the earlier molecules necessary for the adipocyte phenotype (Christy et al., 1989, 1991).

The nuclear receptors peroxisome proliferator-activated receptors (PPARs) were identified later on as critical for the adipocyte phenotype, as will be review later on.

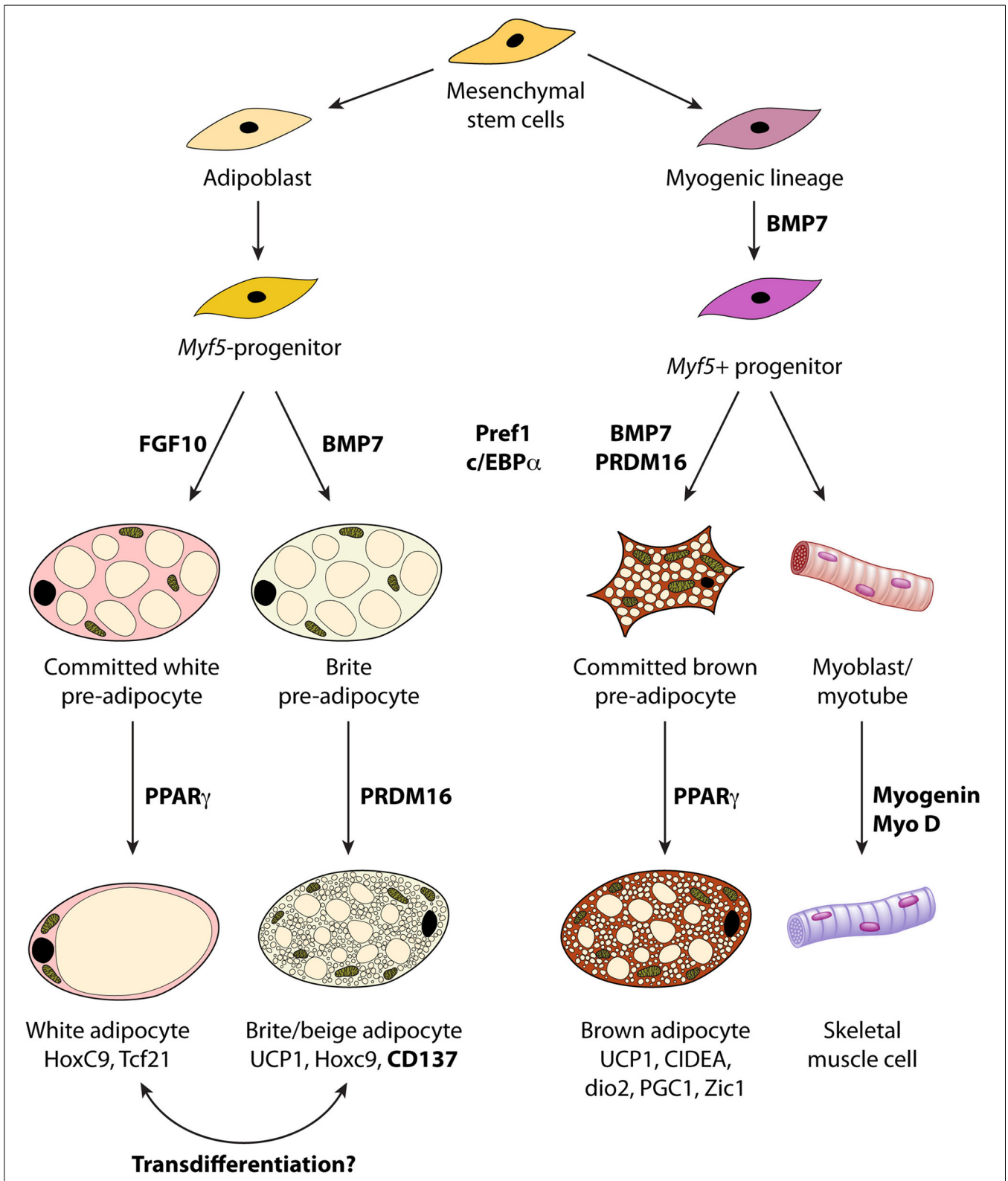

FIGURE 1 | Lineage of white, brown and "brite/beige" adipocytes from stem cells and molecular markers involved in the developmental program. BMP, bone morphogenetic protein; UCP1 uncoupling protein 1; PGC1, Peroxisome proliferator-activated receptor gamma coactivator 1.

Proliferation of preadipocytes. Role of T3 and D3

Pluripotential stem cells give rise to preadipocytes, a mesenchymal cell predetermined to be adipocyte. It is not clear which are the specific factors that trigger the transition from pluripotential stem cells into predetermined preadipocytes. A common mesenchymal stem cell produces adipocytes, myoblasts and osteoblasts (Cornelius et al., 1994; Falconi et al., 2007). PPARγ2 activation itself induces the differentiation of mesenchymal cells into adipocytes (Chen et al., 2007), and several HOX genes have been identified as transcription factors that trigger the transition, several HOX genes display specific expression in WAT and 4 HOX genes (HOXA4, HOXB4, HOXC4, HOXD4) discriminate between WAT and BAT (Cantile et al., 2003). Leukemia inhibitory factor (LIF) could be a marker of these initial steps inducing, together with PPARγ2, the adipocyte phenotype (Falconi et al., 2007). The preadipocyte factor 1, Pref-1, also called Dlk1, is an imprinted gene found in preadipocytes and a potent inhibitor of adipogenesis (Swick and Lane, 1992). Pref-1 activates ERK phosphorylation (Smas and Sul, 1993; Kim et al., 2007) and is a marker of proliferation (**Figure 1**). Using microarrays, Pref-1 was identified as marker of proliferation of brown preadipocytes, while C/EBP and Necdin were expressed during the proliferation of brown as well as white preadipocytes (Timmons et al., 2007). Necdin is an imprinted gene expressed in the paternal allele, that inhibits the activation of PPARγ1 promoter (Macdougald and Burant, 2005).

The preadipocytes are mesenchymal-type cells found in the stroma-vascular fraction (SVF) of the adipose tissue. These precursor cells allowed to set primary cultures of preadipocytes, which proliferate and differentiated in culture (Nechad et al., 1983). Several growth factors present in serum, mainly fibroblast growth factors are mitogenic for brown preadipocytes (Garcia and Obregon, 1997). Cold exposure induces the proliferation of brown preadipocytes as studied *in vivo* using 3H-thymidine, and this proliferation is beta-adrenergic (Bukowiecki et al., 1986; Geloen et al., 1988; Rehnmark and Nedergaard, 1989), while insulin was proposed as a mitotic factor for white adipocytes (Geloen et al., 1989). The increases in DNA synthesis were confirmed in primary cultures of brown preadipocytes using β1 adrenergic agonists (Bronnikov et al., 1992). In our hands norepinephrine (NE) is a poor mitogen itself, but increases the mitogenic action of serum, growth factors and vasopressin (Garcia and Obregon, 1997) producing true brown adipocytes that express UCP1 (Garcia and Obregon, 2002). Therefore, NE is important for brown adipocyte proliferation, besides its role in thermogenesis increasing UCP1 expression. The fatty acid arachidonic is a good mitogen for brown adipocytes (Garcia et al., 2012). Recent studies have reported the role of activin in the proliferation of white adipocytes (Zaragosi et al., 2010).

Thyroid hormones seem to be anti-mitogenic, as T3 inhibits bFGF and aFGF mitogenic effect in brown preadipocytes (Garcia and Obregon, 2002). Moreover, type III deiodinase (D3) activity and mRNA are strongly induced by growth factors in brown adipocytes (Hernandez and Obregon, 1995; Hernandez et al., 1998) as in other proliferating cells, suggesting the physiological importance of low T3 levels during proliferation. D3 activity and mRNA increases abruptly when serum is added to cultures of brown adipocytes (Hernandez et al., 2007). So, we propose that D3 is a mitogenic marker in brown preadipocytes. On the contrary, D2 activity is low during proliferation, having a role during differentiation, therefore establishing that both deiodinases have an opposite role in BAT.

Few proliferation studies have been done in white preadipocytes, but serum stimulates DNA synthesis and proliferation in white preadipocytes in primary cultures (unpublished results). The specific growth factors governing proliferation of white preadipocytes require further research. White preadipocytes require only T3, insulin and transferrin to proliferate in serum-free medium (Deslex et al., 1987a,b). Moreover, preadipocytes from obese people produce mitogenic factors that induce a higher proliferation rate than those produced by control subjects (Lau et al., 1987). Proteins secreted by macrophages have been proposed to be mitogens in human preadipocytes (Lacasa et al., 2007), but the specific growth factors or adipokines have not been defined although fatty acids have been proposed to be mitogens for adipocytes. FGF10 was proposed as a mitogen for WAT, because in FGF10−/− mouse embryos the development of WAT is greatly impaired, due to a decreased proliferative activity of WAT, indicating that FGF10 and not C/EBPα is required for the proliferation of white preadipocytes (Asaki et al., 2004). Adipose tissue is a source of several growth factors as IGF-I, IGF binding proteins, TNF alpha, angiotensin II, and MCSF that could stimulate proliferation (Hausman et al., 2001, 2008).

Differentiation of adipocytes. The role of transcription factors and T3 regulation

The differentiation of adipocyte was first studied in preadipose cells lines (3T3-L1 and 3T3-F442). Differentiation was induced using dexamethasone and IBMX, an agent that increase cAMP levels. T3 was also included in the "differentiation cocktail." So, we do not know if the effects observed are due to the action of the T3 added or the process of differentiation itself. During adipocyte differentiation the transcription of specific genes and the synthesis of numerous proteins increase, specially the lipogenic enzymes GPD, FAS and ME, and many others as described above (Mackall et al., 1976; Spiegelman et al., 1983). The activation of these proteins and enzymes follows a temporal pattern with different timings for each transcriptional increase. LPL or IGF-1 are early markers, after the transcription factors C/EBPs and PPARγ; those are followed by the lipogenic proteins: FAS, GPD, ME, Glut4, aP2, ACC, the beta-adrenergic receptors and many others. Later markers are PEPCK, the alfa-2 adrenergic receptors, leptin, or adipsin (Ailhaud et al., 1992). Recently, studies using microarrays (Soukas et al., 2001) show that the adipocyte differentiation is different *in vivo* and *in vitro* and is more complex than previously though. Some genes were expressed only in cell lines and others only in cells derived from tissues "*in vivo*" suggesting that some genes were activated only "*in vivo*" to generate the adipocytes.

So, the differentiation of adipocytes is achieved by the coordinate activation of several adipose-specific genes (Rosen and Spiegelman, 2000), regulated by the C/EBPs and PPARs families of transcription factors that are keys for the activation of the genes required for adipogenesis.

The CCAAT/enhancer-binding proteins (C/EBP) belong to the basic leucine zipper family. The C/EBP family (C/EBPα, C/EBPβ,

C/EBPδ) recognizes a common DNA-binding element and has tissue-specific expression patterns. C/EBPα is expressed in brown and white adipose tissues, placenta and liver and is a master regulator of adipose tissue development. C/EBPα overexpression induces the differentiation of 3T3-L1 preadipocytes. It works as antimitotic inducing growth arrest (proteins GADD45 and p21) (Mandrup and Lane, 1997). C/EBPα in preadipocytes increases several adipocyte-specific genes (aP2, Glut4) and triglycerides accumulation (Lin and Lane, 1992; Mandrup and Lane, 1997).

C/EBPβ and C/EBPδ are expressed before than C/EBPα and activate C/EBPα, while PPARγ and C/EBPα induce the differentiation into adipocytes. Many genes (SCD1, aP2, S14, PEPCK, Glut4, UCP1, D2) have C/EBP binding domains in their promoters and are activated together during adipogenesis (Christy et al., 1989). During the development of brown adipose tissue during fetal life, C/EBPβ and C/EBPδ increases precede C/EBPα expression (Manchado et al., 1994).

Mice with a deletion in C/EBPα (C/EBPα KO mice) die shortly after birth due to hypoglycemia, defective hepatic glycogen storage and gluconeogenesis (Linhart et al., 2001). C/EBPα KO mice had no WAT and little BAT; UCP1 mRNA was very low, showing that C/EBPα is essential for the liver and adipose tissue developmental program. Our studies using these mice showed that UCP-1 expression was very low, adipogenesis was impaired and the mitochondria number and function reduced (Carmona et al., 2002). The expression of PGC-1 and thyroid hormone receptors were delayed. BAT D2 activity and BAT T3 were very low indicating that C/EBPα is critical for a correct BAT thyroidal status. It seems that BAT D2 is crucial for the differentiation and activity of fetal BAT and possibly T3 is absolutely necessary for BAT function.

Neonatal hypothyroidism decreases C/EBPα and C/EBPβ expression in liver, but not in BAT (Menendez-Hurtado et al., 1997). In the PEPCK gene C/EBPs and TREs are related, as the activation of C/EBPs is required for a functional TRE (Park et al., 1997).

Besides the C/EBPs, the differentiation of adipocytes is regulated by the PPARs, especially by the PPARγ isoform. PPARs are nuclear receptors acting as transcription factors that regulate gene expression through nutritional stimuli and that control lipid metabolism. Fatty acids, especially arachidonic acid and its metabolites, are natural ligands that activate PPARs. PPARs family has several members: PPARα (activated by fibrate, regulates lipid catabolism), PPARδ and PPARγ, quite specific of adipose tissue. PPARs activate the PPAR response elements (PPRE) present in the promoter of specific target genes, and form heterodimers with the X receptor of retinoic acid (RXR). FAS, aP2, PEPCK, LPL, SCD have PPREs in their promoters. The PPRE sequence of a given gene can bind different isoforms in different tissues, e.g., PPARα in the liver and PPARγ in adipose tissue. PPARs play important roles in adipogenesis, inflammation, atherogenesis, glucose homeostasis and cancer.

During adipogenesis PPARγ is activated (Tontonoz et al., 1995). The ectopic expression of PPARγ induces differentiation into adipocytes upon the stimuli of PPARγ agonists (thiazolidinediones, glitazones) (Tontonoz et al., 1995). PPARγ knockout mice presented several alterations with opposite results: some studies showed a reduced fat formation, and protection against obesity and insulin resistance with lipodystrophy (Jones et al., 2005). The mice with targeted deletion of PPARγ2 have insulin resistance indicating that PPARγ2 is necessary for the maintenance of insulin sensitivity (Medina-Gomez et al., 2005).

The specific coactivator of PPARγ, PGC-1, was identified in 1998 (Puigserver et al., 1998). Under cold exposure PGC-1 increases in BAT. PGC-1 increases the transcriptional activity of PPARγ on the UCP1 promoter and the overexpression of PGC-1 in white adipocytes results in UCP-1 increases as well as mitochondrial enzymes; so PGC-1 is considered a marker of brown adipocytes and activator of BAT (Puigserver et al., 1998). It is also fundamental for hepatic gluconeogenesis, heart function and inflammation (Puigserver and Spiegelman, 2003; Handschin and Spiegelman, 2006; Uldry et al., 2006). Hepatic steatosis, increase in body fat, lower amount of mitochondria, lower respiratory capacity and abnormal cardiac function are found in mice with targeted deletion of PGC1α (Leone et al., 2005).

T3 REGULATES GENE EXPRESSION IN ADIPOCYTES

T3 regulates adipogenesis and the related processes, lipogenesis and lipolysis *in vivo* as well as in cultures of adipocytes (Oppenheimer et al., 1991; Ailhaud et al., 1992). All the isoforms of thyroid hormone receptors TRα-1, TRα-2, and TRβ-1 are present in WAT and BAT and in white and brown adipocytes, and TRα-1 is more abundant (Bianco and Silva, 1988; Teboul and Torresani, 1993; Tuca et al., 1993; Hernandez and Obregon, 1996a). T3 and other hormones regulate the different isoforms. Certain mutations in the TRα gene (P398H mutation) induce increased body fat, visceral adiposity, elevated basal glucose, impaired lipolysis, hyperleptinemia and a reduced adaptive thermogenesis (Liu et al., 2003). This mutation in TRα reduces the binding of PPARα to PPRE elements, interfering with PPARα signaling (Liu et al., 2007).

Many genes expressed during the differentiation program are regulated by T3 and have been extensively studied. The list of genes includes GPD, ME, PEPCK, S14 (Kinlaw et al., 1995), FAS (Moustaid and Sul, 1991), GLUT4, and LPL among many others (Mariash et al., 1980; Blennemann et al., 1995; Bianco et al., 1998). T3 may bind the TREs present in the gene promoters (Petty et al., 1990; Giralt et al., 1991) of those genes. In fact, functional TREs have been identified in several genes as well as its interactions with other nuclear receptors, as PPARs or retinoic acid receptors (Silva and Rabelo, 1997), and also with insulin (IRE) and cAMP response elements (CREs). There is a cross-talk among the regulatory elements (TREs, RAREs, PPARs) of the nuclear receptors as many of them share RXR as pattern of dimerizacion and coactivators, as described in several genes: UCP-1, ME, ACC and others (Mullur et al., 2014). We studied the regulation by T3 of ME and Spot14 (S14) in cultured brown adipocytes (Garcia-Jimenez et al., 1993; Hernandez et al., 1993; Perez-Castillo et al., 1993). ME is a lipogenic enzyme important during differentiation and S14, a T3-target gene, is present in many lipogenic tissues. Both genes increase during differentiation of adipocytes, and are activated by T3. T3 acts at the transcriptional level but also stabilizes the mRNAs produced and T3 effects synergize with insulin.

The effect of norepinephrine and retinoic acid was also examined (Hernandez et al., 1993).

The effect of T3 on lipid metabolism also affects other genes as adiponutrin (Adpn, Pnpla3, also called acylglycerol-O-acyltransferase or calcium-independent phospholipase A2 epsilon, iPLA2-epsilon), is a triacylglycerol lipase strongly upregulated by T3 in rat and human white and brown adipocytes (Calvo and Obregon, 2009). Adiponutrin is a lipase with double action as lipase involved the hydrolysis of triacylglycerol and in acyl-CoA transacylation of acyl-glycerols, thereby involved in lipid homeostasis. Other lipases with high impact in BAT are ATGL/desnutrin/Pnpla2 (Ahmadian et al., 2010, 2011) and LPL, that is down regulated by T3 but upregulated when T3+NE is added (Medina-Gomez et al., 2003, 2008).

The fatty acids oxidation is also regulated by T3 in BAT as clearly shown in the D2KO mice (Christoffolete et al., 2004; Castillo et al., 2011) and the FAT-D2KO (Fonseca et al., 2014), with specific deletion of D2 in adipose tissue. D2KO mice show that T3 generated in BAT via D2 is important to accelerate fatty acid oxidation and lipogenesis, as measured by the lipogenic enzymes ACC and ME blunted in BAT. The lack of T3 in D2KO mice impairs thermogenesis and mice survive thanks to compensatory mechanisms in the sympathetic system and increased lipolysis.

Besides its actions on lipid metabolism T3 exert multiple actions on glucose and carbohydrate metabolism, e.g., Glut4 has a TRE in its promoter and the carbohydrate responsive element binding protein (Chrebp) important for Glut4 expression, both are up regulated by T3 in brown adipocytes; other T3 actions include the regulations of gluconeogenesis and glycolysis (**Figure 2**). T3 also increases ion transport, by increasing several transporters (SLCs) as tramdorin in the plasma membrane as well as mitochondrial transporters, increasing structural genes as keratins (Martinez-De-Mena and Obregon, 2014) and enzymes involved in degradation of metabolites and decreasing TSH receptors.

T3 ACTIONS ON THERMOGENESIS

In white adipocytes or 3T3-L1 adipocytes the differentiation process is tested by the increase in lipids, measuring the increase in lipid droplets or increases in lipogenic enzymes. The differentiation in BAT includes an increased thermogenic capacity, measured as UCP-1 increases.

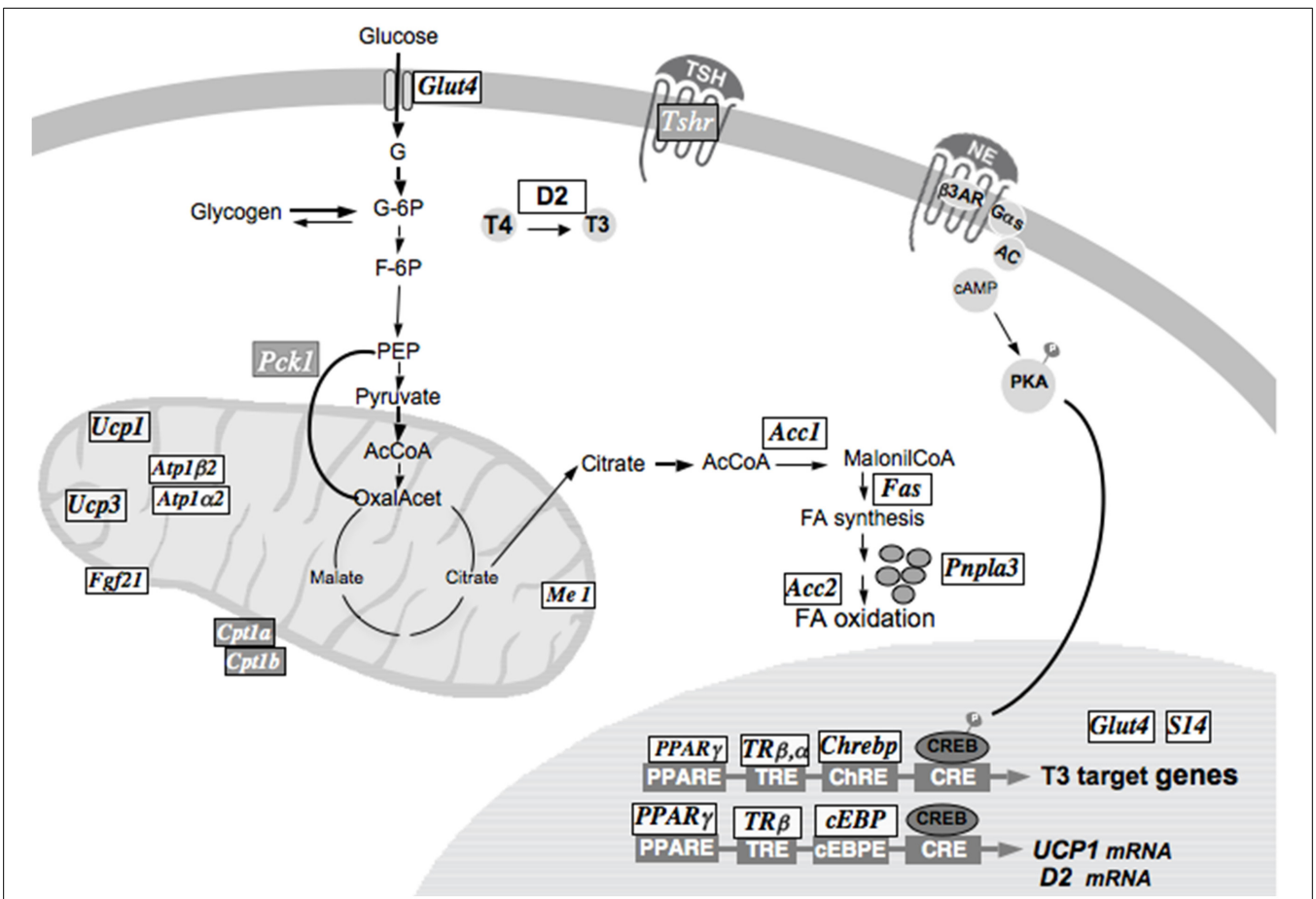

FIGURE 2 | T3 actions on gene expression in brown adipocytes are represented. The genes upregulated by T3 are shown in black with white background (UCP1, UCP3, Glut4, Me1, Spot14, Pnpla3, Chrebp). The genes down regulated in the presence of T3 (TSHr, Pck1, Cpt1) are represented in white with a dark background.

BAT function is the production of heat under cold exposure (facultative thermogenesis). This function is accomplished by the mitochondrial uncoupling protein (UCP-1), which uncouples the oxidative phosphorylation. After activation of the sympathetic nervous system (SNS), NE is released (Ricquier et al., 1986). NE binds to the adrenergic receptors and the adenylyl cyclase is activated increasing cAMP levels; this activates lipolysis, producing FFA which activate UCP1 (Cannon and Nedergaard, 2004). The amount of UCP-1 is the index of the thermogenic capacity of BAT. UCP-1 transcription is activated by NE or cold exposure (Bouillaud et al., 1984; Bianco et al., 1988). T3 increases the adrenergic stimulation of UCP1 (Obregon et al., 1987; Bianco et al., 1988, 1992; Giralt et al., 1990). In thermoneutral conditions and during the intrauterine life, T3 is required for the expression of UCP1 mRNA and euthyroidism is required during the first postnatal days for the increases in UCP1 mRNA (Obregon et al., 1987). In cultured brown adipocytes T3 is required for UCP-1 adrenergic increases, and the stabilization of mRNA transcripts (Hernandez and Obregon, 2000). UCP1 is induced by T3 in fetal rat brown adipocytes in primary culture (Guerra et al., 1996). The effect of T3 on UCP1 is mediated through the TRβ1 isoform (Ribeiro et al., 2001; Martinez De Mena et al., 2010).

The UCP-1 promoter have CRE sequences (Kozak et al., 1994; Yubero et al., 1998; Rim and Kozak, 2002) in the proximal promoter and an enhancer element containing TRE elements (Cassard-Doulcier et al., 1994; Rabelo et al., 1995), RAREs (Alvarez et al., 1995; Rabelo et al., 1996) and a PPRE in the distal promoter (Teruel et al., 1999). These sequences are promiscuous for its binding to the UCP-1 promoter. Negative regulators of UCP-1 expression are serum and mitogens that activate c-jun (Yubero et al., 1998). Other hormones as glucocorticoids and sexual hormones regulate UCP-1 mRNA.

We studied the effect of Triac, triiodothyroacetic acid, a natural metabolite of T3 produced in the liver, in cultured brown adipocytes (Medina-Gomez et al., 2003). Triac, is a better agonist than T3 for the TRβ isoform; Triac is 10–50 more potent than T3 in stimulating the adrenergic increases of UCP1 and D2, and also down-regulates LPL mRNA in the same fashion. So, Triac is a potent thermogenic agent. The role of Triac was also studied in rats (Medina-Gomez et al., 2008). Triac, in equimolar dosages, was more potent than T3 in rats in the stimulation of UCP-1, LPL, in reducing leptin and low doses of Triac induced ectopic expression of UCP-1 in inguinal WAT (Medina-Gomez et al., 2008), that today are called "beige/brite" adipocytes.

The adrenergic input also increases D2 deiodinase in BAT (Silva and Larsen, 1983), leading to increases in BAT T3. This suggests that T3 has an important role in thermogenesis. Moreover the conversion of T4 to T3 was required for the thermogenic function of BAT (Bianco and Silva, 1987). This is also true for the stimulation of D2 (Hernandez and Obregon, 1996b; Martinez-Demena et al., 2002) that does not occur but in the presence of T3. D2 participates in the formation of BAT, as described above in C/EBPα knockout mice (Carmona et al., 2002), with low UCP1 mRNA, D2 activity, and low mitochondriogenesis. D2 is also implicated in the process of lipogenesis under adrenergic stimuli (Bianco et al., 1998). In D2 knockout mice there is a hyper-adrenergic stimulation compensatory for the lack of T3 production in BAT. Lipogenesis is not providing the FFA levels required during cold exposure, resulting in an impaired adaptive thermogenesis (Christoffolete et al., 2004). Indeed, D2 is a marker of BAT activation (thermogenesis).

Little is known about the role of the deiodinases in white adipocytes. It is evident that they have a role in lipogenesis and in the expression of genes involved in the differentiation program. D1 was found in WAT (Leonard et al., 1983) and human WAT (Ortega et al., 2012) and both, D1 and D2 are found in rat WAT as measured by activity and mRNA (Calvo and Obregon, 2011) and in human preadipocytes (Nomura et al., 2011), but its role has not been established. It remains to be seen if D2 and D1 have different roles from those described in brown adipocytes.

WHITE, BROWN ADIPOCYTES AND "BRITE" ADIPOCYTES. A ROLE FOR BAT IN HUMANS

Studies comparing brown and white adipocytes in primary culture (Nechad et al., 1983) established that precursor cells from WAT (epididymal fat) and from BAT differentiate into white and brown adipocytes, respectively, with different phenotypes and regulation. The work done during 30 years using primary cultures of precursor cells confirms that precursor cells in each of these tissues are already committed to become brown or white adipocytes. Nowadays it is clear that BAT and WAT derive from different precursor cells. Using microarrays to study both preadipocytes in culture (Timmons et al., 2007), a myogenic signature was found in brown preadipocytes, Myf5, not found in white adipocytes, in which Tcf21 is present, a transcription factor that inhibits myogenesis. Some genes are specific of brown adipocytes or are only found only in white adipocytes.

The lineage of both adipocytes shows that they have a different embryological origin. Brown adipocytes have a myogenic origin defined by the expression of Myf5+, myogenic marker also found in myoblasts (Gesta et al., 2007; Timmons et al., 2007). PRDM16 controls the switch from skeletal muscle to BAT (Seale et al., 2008) activating BAT phenotype (Seale et al., 2007) as well as several markers of BAT as UCP1, D2 and PGC1α. Several genes have been identified that indicate the presence of brown adipocytes such as Myf5, PRDM16, BMP7, BMP4, and Zic1, while others, as Tcf21 is a marker of white adipocytes (**Figure 1**).

The "beige/brite" adipocytes are found in certain locations of WAT. They are multilocular and express UCP-1. They are found in small amounts and more frequently in certain anatomical locations, e.g., the inguinal fat in rodents. "Brite" adipocytes seem to come from different embryonic precursors than brown adipocytes and express distinct gene signatures (Petrovic et al., 2010), mainly CD137, TBX1, TMEM26 (Walden et al., 2012; Wu et al., 2012) while PRDM16 and PGC1α are markers of "browning." Its presence, abundance and increase in activity are regulated different than brown adipocytes (Macotela et al., 2012; Walden et al., 2012).

A recent review (Harms and Seale, 2013) lists several experimental models, including knockout mice, in which leaner mice have more active BAT or "brite" adipocytes induced, while a low BAT function is associated to increases in body fat or insulin resistance. When BAT activity increases mice are resistant to weight

gain meaning that by increasing BAT activity a reduction in metabolic diseases can be achieved.

Many groups have tried to identify the processes that induce the transition from WAT to "brite" adipocytes and weather BAT and "brite" adipocytes are the same or different cells and the mechanisms of reactivation or induction of BAT activity (Giralt and Villarroya, 2013). The conversion into "brite" adipocytes can be followed using CD137 as a marker of "browning" in human adipocytes (Elsen et al., 2014).

Nowadays the activation of BAT or the "Browning" of WAT is considered a possible strategy to fight obesity. Under extreme cold exposure conditions, a reactivation of BAT adipocytes in inguinal WAT was observed and this was called "convertible" adipose tissue (Loncar, 1991). In this sense, many attempts have been done and there is a number of models in which "browning" of WAT is found (Harms and Seale, 2013), indicating that many signals are able to activate brown fat or to induce "browning" of WAT. Other possible explanation is that a common pathway is activated in all these situations or models, e.g., activation of the adrenergic pathway or also the possible extra-cold experienced by the mice by changes in the fur or skin that augments the cold experienced.

The increases in UCP1 is the golden rule to assess the "browning, as observed using some drugs and also in mice with targeted deletion of a certain genes. Beta 3 adrenergic agonists induce UCP1 in muscle and this provides a mechanism against weight gain (Almind et al., 2007). Brown adipocytes were also found in WAT (Guerra et al., 1998; Xue et al., 2005, 2007). The same effect is observed using models of hyperleptinemia (Commins et al., 1999; Orci et al., 2004) or tungstate (Claret et al., 2005) and we observed that low doses of Triac induce UCP-1 expression in rat inguinal WAT (Medina-Gomez et al., 2008). In mice with targeted deletion of the co-repressor RIP140 (Leonardsson et al., 2004), increases in UCP1 were observed: the mice were lean and resistant to diet-induced obesity. An increasing number of reports find leaner phenotypes associated to increased BAT activity or the presence and activation of "brite" adipocytes, e.g., overexpression of UCP1 in WAT (Kopecky et al., 1995, 1996). More recently the overexpression of Pten results in a phenotype with high UCP1, uncovering the role of Pten promoting energy expenditure (Ortega-Molina et al., 2012). Therefore, when leaner phenotypes are observed in mice, BAT activation or "browning" of WAT should be searched.

Human BAT

Due to the implication of BAT in the maintenance of energy balance it has been a growing interest in its possible role in humans, as the reactivation of BAT activity in humans will provide therapeutic tools to fight obesity. BAT was always considered to have minimal importance in humans, though the presence of human BAT was fully accepted under certain conditions: in newborns (Houstek et al., 1993; Krief et al., 1993), patients with pheochromocytoma or outdoors workers or even at all ages (Tanuma et al., 1975, 1976; Huttunen et al., 1981; Zingaretti et al., 2009). There was a growing evidence for the presence of active BAT in humans, as identified using [18F]-fluorodeoxy-glucose-based positron emission tomography (PET) for diagnostic purposes (Nedergaard et al., 2007). In 2009, three papers were published in the NEJM (Cypess et al., 2009; Van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009) where BAT was unequivocally identified and analyzed in humans, including analysis of genes markers of BAT. During the last 5 years it has been a surge of studies on the possible function of BAT in humans and its regulation by cold, diet, obesity and drugs (Zingaretti et al., 2009; Vijgen et al., 2012, 2013; Jespersen et al., 2013; Van Der Lans et al., 2013; Borga et al., 2014; Broeders et al., 2014; Chechi et al., 2014). Several possibilities have been explored as the induction of brite adipocytes in human adipocytes using BMP7 (Pisani et al., 2011). Besides cold, thyroid hormones also regulate human BAT as hyperthyroidism increases BAT metabolism in humans with higher glucose uptake and higher lipid oxidation rate (Lahesmaa et al., 2014).

CONCLUSIONS

In summary, adipogenesis is a complex process that involves a sequential activation of many genes and enzymes, in a cascade of events regulated by transcription factors (C/EBPs, PPARs, PGC1a) that govern the differentiation of adipocytes. T3 regulates many of the enzymes involved in the process of adipogenesis, either directly through the interaction of its nuclear receptors (TRs) with TREs or through the interaction with other nuclear receptors as PPARs or coactivators. The deiodinases, especially D2, play a crucial role producing the T3 required or limiting its levels. D3 increases during proliferation, while D2 plays a crucial role in adipogenesis, thermogenesis and lipid metabolism. The number of genes regulated by T3 in adipocytes continues to grow, not only for lipid metabolism and carbohydrates but also regulating other process unknown to be important for the biology of the adipocyte. Several genes have been identified as markers of brown, white and "beige/brite" adipocytes, establishing that they are distinct cells. "Browning" occurs in some WAT depots under specific conditions or drugs and specific markers of "beige/brite" adipocytes have been identified. Additionally, BAT has been identified in humans and its presence and regulation is being actively studied. The reactivation of BAT and the induction of beige/"brite" adipocytes in humans could represent a therapeutic option to fight obesity.

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Hormonal regulation of the hypothalamic melanocortin system

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Regulation of energy homeostasis is fundamental for life. In animal species and humans, the Central Nervous System (CNS) plays a critical role in such regulation by integrating peripheral signals and modulating behavior and the activity of peripheral organs. A precise interplay between CNS and peripheral signals is necessary for the regulation of food intake and energy expenditure in the maintenance of energy balance. Within the CNS, the hypothalamus is a critical center for monitoring, processing and responding to peripheral signals, including hormones such as ghrelin, leptin, and insulin. Once in the brain, peripheral signals regulate neuronal systems involved in the modulation of energy homeostasis. The main hypothalamic neuronal circuit in the regulation of energy metabolism is the melanocortin system. This review will give a summary of the most recent discoveries on the hormonal regulation of the hypothalamic melanocortin system in the control of energy homeostasis.

Keywords: hypothalamic melanocortin system, arcuate nucleus, pro-opiomelanocortin (POMC), neuropeptide Y (NPY), agouti-related peptide (AgRP), hormones, obesity

HYPOTHALAMIC MELANOCORTIN SYSTEM

Energy homeostasis is a tightly regulated process and the imbalance between its components, food intake and energy expenditure, causes metabolic dysfunctions including obesity, a major risk factor for comorbidities including type 2 diabetes, hypertension and stroke, and cardiovascular diseases. A major player in the regulation of energy homeostasis is the Central Nervous System (CNS) and specifically the hypothalamus. By monitoring, processing and responding to peripheral signals, such as hormones, the hypothalamus in turn will regulate peripheral organ functions. Within the hypothalamus several neuronal populations have been identified as important players in metabolism regulation. The central melanocortin system consists of three neuronal populations: the pro-opiomelanocortin (POMC)-expressing neurons, the neuropeptide Y (NPY) and agouti-related peptide (AgRP)-co-expressing neurons (Cowley et al., 1999; Elmquist et al., 1999) located in the hypothalamic arcuate nucleus and the melanocortin 4 receptor (MC4R)-expressing neurons located in the hypothalamic paraventricular nucleus. While the anorexigenic POMC neurons, by activating MC4R neurons, induce decreased food intake and increased energy expenditure, the orexigenic NPY/AgRP neurons, by antagonizing POMC action on MC4R, increase food intake and decrease energy expenditure, thus increasing body weight.

The POMC gene encodes a protein precursor that generates a number of bioactive peptides, including adrenocorticotrophin (ACTH), α-, β-, and γ-melanocyte stimulating hormone (α-MSH, β-MSH, and γ-MSH) and β-endorphin, via several post-translational modification processes. Among these peptides, α-MSH is the most well-known anorexigenic peptide which mediates the effects on food intake and energy expenditure through its binding and activation of MCRs (Ollmann et al., 1997).

The critical role of POMC in the regulation of metabolism has been evidenced by studies showing that in humans, individuals with POMC gene mutations display early-onset obesity (Krude et al., 1998; Krude and Gruters, 2000). A similar obese phenotype occurs also in POMC-deficient mice (Yaswen et al., 1999). However, while the acute ablation of POMC neurons in adult mice results in an obese phenotype with hyperphagia (Gropp et al., 2005), postnatal ablation of POMC neurons leads to an obese phenotype with reduced energy expenditure but no hyperphagia (Greenman et al., 2013). On the other hand, reactivation of central POMC at different stages of development in neural-specific POMC deficient mice reduces food intake and weight gain and attenuates comorbidities such as hyperglycemia and hyperinsulinemia (Bumaschny et al., 2012).

Contrary to POMC mutations, NPY, and AgRP gene mutations produce a negligible phenotype with no difference in food intake and body weight (Erickson et al., 1996; Qian et al., 2002; Gropp et al., 2005; Luquet et al., 2005), suggesting that compensatory mechanisms may occur during development (Wu and Palmiter, 2011). Indeed, with the use of the diphtheria toxin and diphtheria toxin receptor (DTR)-mediated cell specific knockout system (Saito et al., 2001), acute ablation of AgRP neurons in adult mice causes a significant metabolic effect (Gropp et al., 2005; Luquet et al., 2005). Because these neurons also produce and release the inhibitory amino acid neurotransmitter,

γ-aminobutyric acid (GABA), its role in the development of this metabolic phenotype regulation has been postulated. Indeed, AgRP-specific vesicular GABA transporter knockout mice have a lean phenotype and are resistant to high fat diet-induced obesity (Tong et al., 2008). GABAergic signaling by NPY/AgRP neurons inhibits POMC neurons in the ARC by direct synaptic innervations (Horvath et al., 1997; Cowley et al., 2001) and also inhibits the parabrachial nucleus (PBN) in the hindbrain (Wu et al., 2009). Moreover, the direct delivery of bretazenil (a GABA receptor partial agonist) into the PBN prevented anorexia caused by AgRP neuronal ablation, suggesting that GABAergic signaling in the PBN is important for the regulation of feeding (Wu et al., 2009).

Recently, advanced technologies such as the optogenetic and DREADD (designer receptors exclusively activated by designer drugs) systems have provided stronger evidences on the central role of POMC and NPY/AgRP neurons in the regulation of feeding behavior (Aponte et al., 2011; Krashes et al., 2011, 2013; Atasoy et al., 2012; Zhan et al., 2013). For example, Zhan and collaborators recently showed that POMC neurons regulate feeding by integrating long-term information from the ARC and short-term information from the NTS in the brainstem (Zhan et al., 2013). On the other hand, activation of AgRP neurons acutely promotes feeding behavior but suppression of POMC neurons is not required for this acute effect (Aponte et al., 2011; Krashes et al., 2011; Atasoy et al., 2012). One study, using DREADD system, has addressed the temporal effect of the acute activation of AgRP neurons on food intake in single, double or triple knockdown of NPY, AgRP-specific vesicular GABA transporter and MC4R (Krashes et al., 2013). This study proposes that NPY and GABA are required for the short-term feeding response while AgRP is responsible for the long-term feeding response.

α-MSH and AgRP exert their metabolic effects through direct interaction with MCRs. In the CNS, MC3R, expressed in the hypothalamic arcuate POMC nucleus, and MC4R, expressed in several brain areas including the paraventricular nucleus of the hypothalamus, play a critical role in mediating the agonistic and antagonistic effect of α-MSH and AgRP, respectively (Ollmann et al., 1997). Both MC3R and MC4R deficient mice displayed an obese phenotype, however, while MC3R are not hyperphagic (Butler et al., 2000; Chen et al., 2000; Renquist et al., 2012), MC4R deficiency mice show hyperphagia and reduced energy expenditure (Huszar et al., 1997; Marsh et al., 1999; Butler et al., 2001). In addition, mutations in the human *MC4R* gene are associated with non-syndromic obesity (Vaisse et al., 1998; Yeo et al., 1998).

The importance of MC4R-expressing neurons in the PVN in regulating metabolism has been recently showed by studies in which restoration of MC4Rs in PVN Single-minded 1 (SIM1) neurons of MC4Rs deficient mice induced a reduced obese phenotype (Xu et al., 2013; Shah et al., 2014). Of note, SIM1 seems to play also an important role in metabolism regulation both in humans and mice (Holder et al., 2000; Michaud et al., 2001).

The activity of the melanocortin neurons is regulated by many peripheral signals including hormones such as leptin, ghrelin, insulin, glucocorticoids, and thyroid hormones. By either activating or inhibiting these neurons, these peripheral signals convey information on the metabolic status of the organism.

LEPTIN

Leptin is an anorexigenic hormone produced and released by the white adipose tissue in the amount proportional to the mass of fat in the body (Zhang et al., 1994; Frederich et al., 1995; Maffei et al., 1995). Leptin interacts with six types of receptors (*Ob-Ra, -Rb, -Rc, Rd, Re, and Rf*) encoded by a single leptin receptor gene (*Ob-R*) (Lee et al., 1996; Wang et al., 1996). However, only the long form of leptin receptors (*Ob-Rb*), consisting of an extracellular and an intact cytoplasmic domain, mediates the anorexigenic effect of leptin (De Luca et al., 2005). Deficiency in leptin or leptin receptors induces a morbid obese phenotype characterized by hyperphagia, hyperglycemia, hyperlipidemia, and reduced energy expenditure in both rodents and humans (Halaas et al., 1995; Chen et al., 1996; Montague et al., 1997; Clement et al., 1998). Neuron-specific Ob-R-deficient (*db/db*) mice displayed an obese phenotype while hepatocyte-specific *db/db* mice are normal (Cohen et al., 2001; De Luca et al., 2005), suggesting that the direct effect of leptin in the brain is essential for metabolism regulation. Mice lacking Ob-Rb in POMC neurons, AgRP neurons or both POMC/AgRP neurons displayed increased body weight and fat mass (Balthasar et al., 2004; Van De Wall et al., 2008), although their phenotypes are milder compared to those of whole brain neuron-specific Ob-Rb deficient mice, suggesting the involvement of other leptin receptor-expressing neurons in mediating the effect of leptin on energy homeostasis.

Leptin regulates POMC and NPY/AgRP neurons at different levels. For examples, leptin increases POMC mRNA levels while decreasing NPY/AgRP mRNAs (Mizuno et al., 1998; Mizuno and Mobbs, 1999). Besides the transcriptional regulation, leptin directly depolarizes (activates) POMC neurons while simultaneously hyperpolarizing (inactivates) NPY/AgRP neurons (Cowley et al., 2001). In addition, systemic leptin administration to leptin deficient (ob/ob) mice rapidly induced synaptic input reorganization onto NPY/AgRP and POMC neurons (Pinto et al., 2004), suggesting that the synaptic rearrangement is an important event for leptin-induced behavioral changes (Horvath, 2006). A recent study demonstrated that AgRP neurons are critical in mediating metabolic syndrome in ob/ob mice since ablation of Agrp neurons in leptin deficient (*ob/ob*) mice showed reduced food intake and improved glucose tolerance (Wu et al., 2012).

INSULIN

Insulin, produced from pancreatic β-cells, plays a fundamental role in the regulation of glucose homeostasis by modulating glucose uptake in peripheral organs (Bagdade et al., 1967). However, since insulin receptors (IR) are widely expressed in the brain (Havrankova et al., 1978), accumulated evidences have indicated a role for insulin in the CNS. Indeed, brain specific-IR deficient mice develop obesity with increased body weight, fat mass, and food intake (Bruning et al., 2000). However, mice with selective deletion of IR in the arcuate melanocortin system, either POMC or AgRP neurons, did not display alteration in energy homeostasis, while only a defective suppression on hepatic glucose production was found in mice with selective deletion of IR in AgRP neurons (Konner et al., 2007). In addition, re-expression of IR in either POMC or AgRP neurons of L1 mice, a genetic mouse model with significant reduction of IR in the

ARC (Okamoto et al., 2004), suggested that insulin signaling in AgRP neurons negatively regulates hepatic glucose production while IR activation in POMC neurons positively regulates hepatic glucose production and energy expenditure (Lin et al., 2010). Interestingly, when both leptin and IR were ablated in POMC neurons, systemic insulin resistance despite increased pancreatic insulin secretion was observed in these mice (Hill et al., 2010). In addition, the obese phenotype observed in POMC-specific ObR knockout mice was ameliorated when both ObR and IR were selectively deleted from POMC neurons, suggesting that insulin and leptin signaling in POMC neurons may have opposing effects in the regulation of body weight but additive effects on glucose homeostasis. In support of this, leptin and insulin responsive neurons are expressed in distinct subpopulations of POMC neurons (Williams et al., 2010). Thus, the divergent effects of leptin and insulin on energy homeostasis may due to the activation of different POMC subpopulations.

GHRELIN

Ghrelin, the hunger hormone, is predominantly secreted by specialized endocrine cells of the stomach when the stomach is empty. Ghrelin is synthesized as a preprohormone and several processes are required to generate its active form. After removing the signal peptide from the preprohormone, ghrelin precursor is acylated at the third serine with n-octanoic acid by an enzyme called ghrelin o-acyltransferase (GOAT) (Gutierrez et al., 2008; Yang et al., 2008) and then it is cleaved by prohormone convertase 1/3 to produce the active 28-amino-acid acylated ghrelin (Zhu et al., 2006). Ghrelin exerts its orexigenic effects through the growth hormone secretagogue receptor (GHSR) (Sun et al., 2004). The physiological function of Ghrelin and GHSR on feeding has been demonstrated by ghrelin and GHSR deficient mice. Both ghrelin- and GHSR-deficient mice are resistant to high-fat diet-induced obesity but only when mice are exposed to the diet shortly after post-weaning and not in adulthood (Sun et al., 2003; Wortley et al., 2004, 2005; Zigman et al., 2005).

The strongest expression of GHSR has been observed in the hypothalamus (Willesen et al., 1999). This observation, together with ghrelin-induced high c-fos expression in the hypothalamus, indicated this site of the CNS as main site of ghrelin's action. In the ARC, GHSR is predominantly expressed in NPY/AgRP neurons. Accordingly, peripheral and central administration of ghrelin induced c-fos expression in NPY/AgRP neurons (Nakazato et al., 2001; Wang et al., 2002). In addition, ghrelin increases NPY and AgRP mRNA expression levels and the electrical activity of NPY/AgRP neurons (Kamegai et al., 2001; Shintani et al., 2001; Cowley et al., 2003; Seoane et al., 2003; Van Den Top et al., 2004). Although ghrelin inhibits POMC neuronal activity, no GHSR expression has been reported in POMC neurons (Willesen et al., 1999) thus suggesting that this inhibitory effect may be mediated by the activation of NPY/AgRP neurons. In addition, ghrelin positively regulates prolyl carboxypeptidase, the enzyme responsible for α-MSH degradation (Kwon Jeong et al., 2013) thus further increasing the orexigenic tone. Ghrelin-induced food intake is mediated by NPY/AgRP neurons (Nakazato et al., 2001; Shintani et al., 2001; Chen et al., 2004). For example, administration of neutralizing antibodies or antagonists of both NPY and AgRP blunted the orexigenic effects of ghrelin. Consistently, ghrelin's effect on feeding is abolished in NPY/AgRP double-deficient mice (Chen et al., 2004). NPY/AgRP neurons-mediated ghrelin's effect is evident in diet-induced obese mice (DIO). DIO mice show decreased expression of *Npy* and *AgRP* mRNA levels, decreased *Goat* mRNA levels in the stomach and decreased hypothalamic *GHSR* expression and they are ghrelin resistance (Briggs et al., 2010). Interestingly, while ghrelin resistance has been observed in DIO mice, *ob/ob* mice retain their sensitivity to ghrelin, suggesting that elevated leptin levels may be involved in the development of ghrelin resistance. In support of this, a recent study showed that central leptin administration in *ob/ob* mice induced ghrelin resistance (Briggs et al., 2014). AgRP-selective re-expression of GHSR in whole body of GHSR-deficient mice partially restores the orexigenic response to ghrelin (Wang et al., 2014). In addition, AgRP-specific vesicular GABA transporter knockout mice showed impaired ghrelin-mediated feeding response and impaired inhibitory postsynaptic potentials in POMC neurons, indicating that GABA release from AgRP neurons is an important mediator of ghrelin's effect on food intake (Tong et al., 2008). Of note, uncoupling protein 2 (UCP2) is a mediator of ghrelin's action on feeding behavior in both the hypothalamus and the ventrotegmental area (VTA). In *Ucp2* deficient mice, intrahypothalamic administration of ghrelin showed a blunted effect on food intake and intra-VTA ghrelin injection also attenuated food intake (Andrews et al., 2008).

THYROID HORMONES

Thyroid hormones play an important role in metabolism regulation affecting nearly all the tissues in the body. Thyroxine (T4) produced by the thyroid gland is converted in target tissues in the active form of thyroid hormone, Triiodothyronine (T3), by a process called 5′ deiodination. Thyroid hormones affect metabolism acting on both food intake and energy expenditure (Vijayan and Mccann, 1977; Suzuki et al., 1982; Lin et al., 1983; Choi et al., 2002; Herwig et al., 2008; Klieverik et al., 2009). The hyperphagia, induced by increased thyroid hormones levels, is mediated by the CNS. Indeed, central T3 administration induced increased food intake by increasing and reducing NPY and POMC mRNA levels, respectively (Ishii et al., 2003). Changes in central T3 levels occur during different metabolic states (Van Haasteren et al., 1995). For example, elevated levels of T3 occur in the hypothalamus during starvation (Coppola et al., 2005, 2007). This increased T3 is due to the elevated activity of the enzyme responsible for the conversion of T4 in T3 (Diano et al., 1998). Similar to the effect of T3 in the brown adipose tissue in increasing uncoupling protein 1 (UCP1) activity, T3 in the hypothalamus regulates UCP2 levels, which in turn, will affect the activity of NPY/AgRP neurons and thus increase food intake. Via this signaling pathway involving NPY/AgRP neuronal activity, T3 is also responsible for fasting-induced suppression of TRH mRNA expression in the PVN (Coppola et al., 2005, 2007; Vella et al., 2011).

GLUCOCORTICOIDS

Glucocorticoids are known regulators of energy balance (Nieuwenhuizen and Rutters, 2008). For example, Cushing' syndrome, one pathology characterized by hypercortisolism,

displays several symptoms including hypertension, insulin resistance, hyperglycemia as well as rapid weight gain (Hankin et al., 1977). Conversely, Addison's disease, condition of hypocortisolism, causes weight loss (Lovas and Husebye, 2007). Similarly, hypocortisolism induced by adrenalectomy (ADX) reduces food intake, fat stores and body weight (Dallman et al., 2004). Since ACTH, which is generated from POMC-expressing cells in the pituitary gland, stimulates the production of glucocorticoids from the adrenals, pituitary POMC's effects on energy balance were addressed in neural-specific POMC deficient mice (Smart et al., 2006). Interestingly, increased glucocorticoids by re-expression of pituitary POMC in neural-specific POMC deficient mice exacerbates obesity with severe insulin resistance, suggesting that central POMC's role is not substituted by peripheral POMC (Smart et al., 2006). In further support of the role of glucocorticoids in the regulation of energy balance, many obese mouse models including diet-induced obesity and genetic mouse models were characterized by elevated corticosterone levels, and ADX to these mice has been shown to ameliorate their obese phenotype (Okada et al., 1993; Makimura et al., 2000). The mechanism by which ADX ameliorates obesity involves the CNS. For example, corticosterone has been shown to affect the hypothalamic melanocortin signaling by regulating POMC and AgRP mRNA expression in leptin-deficient mice (Makimura et al., 2000). In addition, studies from our group have shown that ADX directly influences neuronal activity of the arcuate POMC neurons by affecting the synaptic input organization of these neurons (Gyengesi et al., 2010). Furthermore, ADX has been reported to enhance leptin's effect on feeding by reducing the responsiveness of melanocortin receptors to its ligands (Drazen et al., 2003).

CONCLUSIONS

The hypothalamic melanocortin system is an integrative center in the regulation of energy balance. Numerous studies have shown that many peripheral signals including hormones can directly influence the activity of the hypothalamic melanocortin system. Besides hormones, other signals including nutrients such as glucose (Thorens, 2012), lipids (Lam et al., 2005; Moulle et al., 2014), and amino acids (Cota et al., 2006; Schwartz, 2013) also function as signal molecules for the melanocortin system.

All of these signals have been shown to regulate the melanocortin system via extracellular and intracellular morphological changes that will affect the activity levels of the different component of the system. These changes include synaptic input organization (Pinto et al., 2004; Zeltser et al., 2012), neuron-glia interaction, and intracellular organelles alterations including mitochondrial and peroxisomal density and function (for review see Koch and Horvath, 2014).

In conclusion, further studies on the mechanisms controlling the melanocortin system are crucial to make advancement in our abilities to develop new therapies for the treatment of metabolic disorders.

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New avenues for regulation of lipid metabolism by thyroid hormones and analogs

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Weight loss due to negative energy balance is a goal in counteracting obesity and type 2 diabetes mellitus. The thyroid is known to be an important regulator of energy metabolism through the action of thyroid hormones (THs). The classic, active TH, 3,5,3′-triiodo-L-thyronine (T3) acts predominantly by binding to nuclear receptors termed TH receptors (TRs), that recognize TH response elements (TREs) on the DNA, and so regulate transcription. T3 also acts through "non-genomic" pathways that do not necessarily involve TRs. Lipid-lowering therapies have been suggested to have potential benefits, however, the establishment of comprehensive therapeutic strategies is still awaited. One drawback of using T3 in counteracting obesity has been the occurrence of heart rhythm disturbances. These are mediated through one TR, termed TRα. The end of the previous century saw the exploration of TH mimetics that specifically bind to TR beta in order to prevent cardiac disturbances, and TH derivatives such as 3,5-diiodo-L-thyronine (T2), that possess interesting biological activities. Several TH derivatives and functional analogs have low affinity for the TRs, and are suggested to act predominantly through non-genomic pathways. All this has opened new perspectives in thyroid physiology and TH derivative usage as anti-obesity therapies. This review addresses the pros and cons of these compounds, in light of their effects on energy balance regulation and on lipid/cholesterol metabolism.

Keywords: thyroid hormones, lipid metabolism, energy balance, insulin resistance development, obesity

INTRODUCTION

The thyroid plays a crucial role in the control of energy metabolism through action of thyroid hormones (THs) in metabolically active tissues such as liver, skeletal muscle and brown adipose tissue. Knowledge on TH action has increased significantly after the cloning of the TH receptors (TRs) termed TRα (including splice-variants α1 and α2 of which α2 does not bind to TH) and TRβ (including transcripts β1 and β2, with β1 predominantly involved in metabolic control, see for a more detailed review: Mullur et al., 2014). TRα1 (henceforth: TRα) is expressed in the brain and to a lesser extent in kidney, skeletal muscle, lungs, heart, and liver, whereas TRβ1 (henceforth: TRβ) is expressed predominantly in the kidneys and liver, and at lower levels in brain, heart, thyroid, skeletal muscle, lungs, and spleen (Williams, 2000). In absence of hormone, transcriptional regulation through the TRs is blocked through association with co-repressors (Astapova and Hollenberg, 2013). An example comes from a recent study reporting expression of an inactive mutant of nuclear receptor co-repressor (NCoR), NCoRΔID, in mouse liver which, by favoring coactivator recruitment and receptor activation, resulted in increased expression of genes encoding enzymes involved in bile acid metabolism that are under transcriptional control of TRβ (Astapova et al., 2014). Interestingly, one coactivator of TRβ has been recently discovered to be a sirtuin, namely SIRT1 (Singh et al., 2013; Suh et al., 2013; Thakran et al., 2013). To exert their action, TRs commonly heterodimerize with the retinoic X receptor (RXR) but may also homodimerize (Forman et al., 1992). In addition, TRs interact with other nuclear receptors including peroxisome proliferator activated receptors (PPARs), (Bogazzi et al., 1994; Buroker et al., 2007; de Lange et al., 2007). TR-PPAR interactions are of particular importance in regulation of lipid metabolism. The *in vivo* association of unliganded TRα with PPARα has been shown to inhibit PPARα signaling in the liver, a process abolished by T3 (Liu et al., 2007). A dominant negative TRα mutant associated with PPARα in a way that could not be abolished by T3, resulting in hepatic steatosis (Liu et al., 2007). Thus, these results imply that T3 "unblocks" PPARα action by relieving TRα association with PPARα. In accordance, TRα knock-out mice were shown to be protected from diet-induced hepatic insulin resistance (Jornayvaz et al., 2012). TRβ isoforms reduce serum lipids *in vivo* (Johansson et al., 2005; Angelin and Rudling, 2010; Pramfalk et al., 2011; Shoemaker et al., 2012). TRβ disruption in mice has been shown to impair fatty acid oxidation (Araki et al., 2009) which persisted with TRα overexpression (Gullberg et al., 2000, 2002). This implies that T3 increases lipid metabolism both by binding to TRα and TRβ and through PPARs, however through different underlying mechanisms. Physical interactions between TRβ and PPARα in mouse heart (Buroker et al., 2007), and PPARδ in rat skeletal muscle (de Lange et al., 2007) have been suggested to increase expression of common target genes involved in lipid metabolism.

THE DEVELOPMENT OF SPECIFIC TRβ AGONISTS AS LIPID/CHOLESTEROL-LOWERING AGENTS

Hyperthyroidism can lead to thyrotoxicosis, with increased heart rate, atrial arrhythmias and heart failure, muscle wasting, postmenopausal osteoporosis in women, fatigue, anxiety, and preference for decreased temperatures (Webb, 2004). Basic studies have revealed that TRα is responsible for TH's effects on heart rate: mice lacking TRα showed reduced heart rate and body temperature (Wikström et al., 1998), and echocardiograph studies in mice deficient in TRα or TRβ have revealed that the effects of THs on heart rate are TRα-dependent (Weiss et al., 2002). Cardiac contractile functions and the expression of genes involved therein have been shown to be all TRα-dependent (Gloss et al., 2001).

Initial studies on TH analogs date from before the cloning of the TRs and showed that analogs binding strongly to hepatic TRs but weakly to heart TRs (Ichikawa et al., 2000) were effective in reducing body weight and serum lipid/cholesterol levels but did not cause evident cardiac arrhythmias. L-94901, the first described selective thyromimetic compound with 50% of the binding affinity of T3 to hepatic TRs and only a minimal affinity to cardiac TRs (Underwood et al., 1986), has been reported to lower plasma cholesterol in experimental animals without inducing cardiotoxic side effects. A subsequent compound, CGS 23425, lowered total serum and LDL cholesterol and increased synthesis of apolipoprotein A1 (APOA1). Indeed, after the cloning of the TRs, CGS 23425 was confirmed to be weakly TRβ-selective (Taylor et al., 1997).

Because of TRα-associated unwanted side effects of TH, the nineties saw the development of TRβ-specific ligands [these comprise GC-1 (Chiellini et al., 1998), its derivative GC-24 (Borngraeber et al., 2003), and KB141 (Grover et al., 2005)], or, alternatively, ligands targeted to the liver. The first such cholesterol-reducing compound with high liver specificity was CGH-509A (derived from conjugation of L-T3 and cholic acid). Other drugs developed for targeting to the liver were MB-07811, a prodrug of the active metabolite MB07344 (Erion et al., 2007) and KB-2115 (Berkenstam et al., 2008). Several compounds that were intentionally designed to be liver-specific were subsequently also found to be TRβ-selective, and *vice versa*. For instance, the selective TRβ-agonist GC-1 has been found to be preferentially taken up by the liver (Baxter and Webb, 2009) and, in turn, MB-07811 resulted to be more than ten-fold TRβ-selective. TRβ affinity varies between compounds: GC-1 (Chiellini et al., 1998) has equal TRβ affinity with respect to T3, GC-24 (Borngraeber et al., 2003) and MB-07344 (Erion et al., 2007; Fujitaki et al., 2008) have a relative twofold-lower affinity, and KB-141 has a sixfold lower relative affinity (Erion et al., 2007). In addition, the synthetic agonists have different pharmacokinetic and pharmacodynamics properties with respect to T3 (Erion et al., 2007). Finally, effective tissue uptake differs between the compounds, which could be related to their relative affinities for the classic TH transporters such as monocarboxylate transporter-8 (MCT-8) (Erion et al., 2007).

TH ANALOGS AND CHOLESTEROL METABOLISM

A large number of TH analogs ameliorate lipid and lipoprotein metabolism through the lowering of plasma total and LDL cholesterol levels and the stimulation of reverse cholesterol transport (RCT) in different animal models (Baxter and Webb, 2009; Tancevski et al., 2009; Pramfalk et al., 2011). The LDL cholesterol-lowering effect exerted by TRβ1- and liver-selective thyromimetics such as KB-141, GC-1, KB-2115, MB-07811, T-0681, CGS-23425, and DITPA (Underwood et al., 1986; Taylor et al., 1997; Grover et al., 2003, 2004; Erion et al., 2007; Tancevski et al., 2009) is ascribed to increased hepatic LDL clearance via stimulation of LDL receptor (Erion et al., 2007; Tancevski et al., 2009, 2010) and the stimulation of cholesterol 7 alpha-hydroxylase (CYP7A1) expression and activity (Gullberg et al., 2000; Johansson et al., 2005; Erion et al., 2007; Tancevski et al., 2010) enhancing hepatic cholesterol uptake and its conversion into bile acids to be excreted by feces. Moreover, T0681 induces the expression of ABCG5 and ABCG8, a tandem pump promoting the biliary excretion of free cholesterol (Tancevski et al., 2010). Recent mechanistic studies in mice showed the LDL receptor (LDLr) expression to be crucial for the hypocholesterolemic effects of MB-07811 and for T-0681 (Erion et al., 2007; Tancevski et al., 2010) which was not the case for that of T3, GC-1 and KB-2115 (Goldberg et al., 2012; Lin et al., 2012). These data suggest that patients with familial hypercholesterolemia may benefit from a treatment with specific TH analogs.

TRβ AGONISTS AND PREVENTION OF HEPATIC STEATOSIS: SIDE EFFECTS INCLUDING INSULIN RESISTANCE

At first the use of TRβ agonists to ameliorate lipid profiles was considered unfavorable due to their potential causing, in analogy with T3, of adipose lipolysis, and induction of hepatic lipogenesis and thus steatosis. In fact, T3 poorly reduces hepatic steatosis in rodent obesity models (Cable et al., 2009). Genetically obese/diabetic rodent models or models of rodents placed on high-fat diets have increasingly been used to investigate the efficiency of these compounds to lower lipid profiles and to counteract non-alcoholic fatty liver disease (NAFLD) (Cable et al., 2009; Vatner et al., 2012) and hepatic insulin resistance (Vatner et al., 2012), with varying outcomes.

MB-07811 efficiently reduced hepatic steatosis as well as plasma FFA and triglycerides in various rodent models including male ob/ob mice, male Zucker diabetic fatty (ZDF) rats, and male C57Bl/6 mice placed on a high-fat rodent diet (60% fat by kcal) for 3 months. This compound, in contrast to T3, did not cause adipose lipolysis, and efficiently reduced hepatic steatosis by inducing hepatic fatty acid oxidation and mitochondrial respiration rates, phenomena known to be related to hepatic TR activation. Unlike T3, MB-07811 did not increase heart weight and neither did it decrease pituitary thyroid-stimulating hormone beta (TSHβ) expression (Cable et al., 2009). Additional TRβ agonists, namely GC-1 and KB-2115, tested in similar systems have proven to be effective in depleting liver lipids, (Cable et al., 2009; Vatner et al., 2012), but recent evidence has shed doubt on the potential use of these compounds to ameliorate insulin sensitivity (Vatner et al., 2012). Male Sprague-Dawley rats treated daily with GC-1 while being placed on a commercial high-fat diet showed a 75% reduction of hepatic triglyceride content, but developed fasting hyperglycemia and hyperinsulinemia due to increased glucose production and diminished hepatic insulin sensitivity. In addition, white adipose lipolysis was increased,

which the authors suggest to contribute to endogenous glucose production due to the increased glycerol flux (Vatner et al., 2012). Rats being fed the same diet period but treated daily with KB-2115 displayed a reduction of hepatic steatosis without evident fasting hyperglycemia, increased glucose production or diminished hepatic insulin sensitivity. Instead, insulin-stimulated muscle glucose uptake was diminished with concomitant reductions in glucose transporter 4 (GLUT4) protein content (Vatner et al., 2012). The induction of insulin resistance at various levels by several TRβ agonists may put their therapeutic potential into question. Nevertheless, KB-2115 recently entered phase III clinical trials which were discontinued when cartilage damage was observed in a long-term study on dogs (Sjouke et al., 2014).

Despite these drawbacks, the need to develop agents that counteract dyslipidemia persists, thus the search for effective TRβ agonists remains justified. An overview of the metabolic effects of the described compounds is given in **Table 1**.

ACTION OF T3 AND OTHER TH METABOLITES BY NOT DIRECTLY INTERACTING WITH NUCLEAR TR-TREs

In recent years, it has become ever more clear that T3 exerts its effects not only through nuclear TR-TRE interactions, but also through cytosolic TRs or even independent of TRs. Ten percent of the TR pool is localized within the cytosol (Baumann et al., 2001) and T3 has been shown to interact with these cytosolic TRs to influence phosphatidyl inositol 3- kinase (PI3K)-Akt signaling, with phosphorylated Akt subsequently influencing transcription of genes involved in glucose metabolism via mammalian target of rapamycin (mTOR) (Moeller et al., 2006). T3-induced phosphorylation of Akt in rat skeletal muscle causes glucose transporter 4 (GLUT4) translocation to the sarcolemma (de Lange et al., 2008). Truncated forms of TRs are also present in mitochondria. A variant of TRα in mitochondria (Wrutniak-Cabello et al., 2001) has been suggested to directly stimulate oxidative phosphorylation upon interaction with T3 (Oetting and Yen, 2007).

Other receptors have been shown to interact with T3: (Davis et al., 2005). TH interacts with integrin alpha V beta 3 in the cell membrane. This event triggers the MAPK/ERK pathway with phosphorylated MAPK translocating into the nucleus and associating with TRβ (Plow et al., 2000). This causes phosphorylation of the TRβ receptor, enhancing its action on transcription rate (Davis et al., 2000). Thus, TR activation can be enhanced through pathways other than solely TH binding.

Furthermore, T3 causes activation of non-receptor proteins. AMP-activated kinase (AMPK), which plays a central role in lipid and glucose metabolism homeostasis (Hardie et al., 2012), has been shown to be a target of transient and rapid activation (within hours) by T3 in skeletal muscle (de Lange et al., 2008; Irrcher et al., 2008). Sirtuins are NAD+ activated deacetylases that have been shown to play a role in metabolic homeostasis (Chang and Guarente, 2014). T3 has been shown to activate SIRT1 by various groups, and this depends on interaction with TRβ (Singh et al., 2013; Suh et al., 2013; Thakran et al., 2013). Some effects of T3 through SIRT1 require binding of TRβ to TREs (Suh et al., 2013;

Table 1 | From thyromimetics to TRβ ligands and TH metabolites: reported TRβ affinities and metabolic effects.

Ligand	Displays TRβ binding affinity (with respect to T3)	Reduces Plasma LDL cholesterol	Acts LDL receptor-dependent	Prevents hepatic steatosis	Causes insulin resistance	Increases heart weight/rate
THYROMIMETIC						
L-94901	Weak	Yes				No
CGS-23425	Weak	Yes				No
TRβ AGONIST						
GC-1	Equal	Yes	No	Yes	Yes	No
GC-24	2-fold lower	Yes	Yes	Yes		No
KB-141	6-fold lower	Yes	Yes			No
T-0681		Yes	Yes			No
DITPA		Yes	Yes			No
LIVER-TARGETED						
CGH-509A		Yes				No
MB-07811		Yes		Yes		No
MB-07344	2-fold lower	Yes		Yes		No
KB-2115		Yes	No		Yes	No
TH METABOLITE						
TRIAC	3-fold higher however no (or weak) TRβ specificity					No
T1AM	None	No	No		Yes	No
T2	60-fold lower (human TRβ) with no TRβ specificity	Yes	No	Yes	No (rats) ND (mice)	No/No (rats) Yes/ND (mice)*

*Abbreviations: ND, not determined; *dose of T2 used in mice is 10-fold higher.*

Thakran et al., 2013). However, one target of T3-TRβ-SIRT1 action is FOXO1, which upon deacetylation triggers the expression of genes involved in gluconeogenesis in mice that do not necessarily contain a TRE in their promoters (Singh et al., 2013). Some of the above mentioned mechanisms of action exerted by T3 are shared by other TH metabolites. AMPK activation in skeletal muscle has been found also to occur by 3,5-diiodo-L-thyronine (T2) (Lombardi et al., 2009), as well as SIRT1 activation in liver (de Lange et al., 2011) and in kidney (Shang et al., 2013). These results have added to the realization that some of the actions of T3 (including those on energy metabolism) can overlap with those of the TH metabolites with low affinity for TRs. The mechanistic aspects of the metabolic effects of the thyroid hormone analogs and metabolites are highlighted in **Figure 1**.

NATURAL TH METABOLITES, PROMISING LIPID-REDUCING AGENTS?

Besides T3, the thyroid is a source of other iodothyronines, which are either produced within the thyroid itself or are deiodinated periferically, the so-called "non-classical" THs (see for an more extensive overview Senese et al., 2014). One non-classical TH with high TR affinity, Triac (3,5,3′-triiodothyroacetic acid), has been shown to be weakly TRβ-selective and to lower cholesterol without reduced effects on heart rate (Moreno et al., 2008). Other TH metabolites, due to their weak binding to TRs, do not predominantly act through binding to nuclear TRs and by modulating TRE-mediated gene transcription. An example is T1AM, an amine present in serum of rodents and humans (Saba et al., 2010; Hoefig et al., 2011), and in rat liver and brain (Saba et al., 2010). T1AM has no affinity for TRβ and TRα (Chiellini et al., 1998). T1AM, however, is a potent agonist of trace amine-associated receptor 1 (TAAR1), an orphan G protein-coupled receptor (GPCR) (Zucchi et al., 2006). Contrary to T3, T1AM does not ameliorate lipid profiles and may cause insulin resistance depending on the dose: intracerebroventricular (icv) injection of T1AM into short-term fasted male mice in a dose of 130 ng/100 gBW (Manni et al., 2012) causes hypophagia as well as peripheral effects namely raised plasma glucose levels and reduced peripheral insulin sensitivity despite increased pancreatic insulin production. This would classify this compound as unsuitable for improving metabolic profiles. Another TH metabolite which has been under study in recent years is T2. This compound

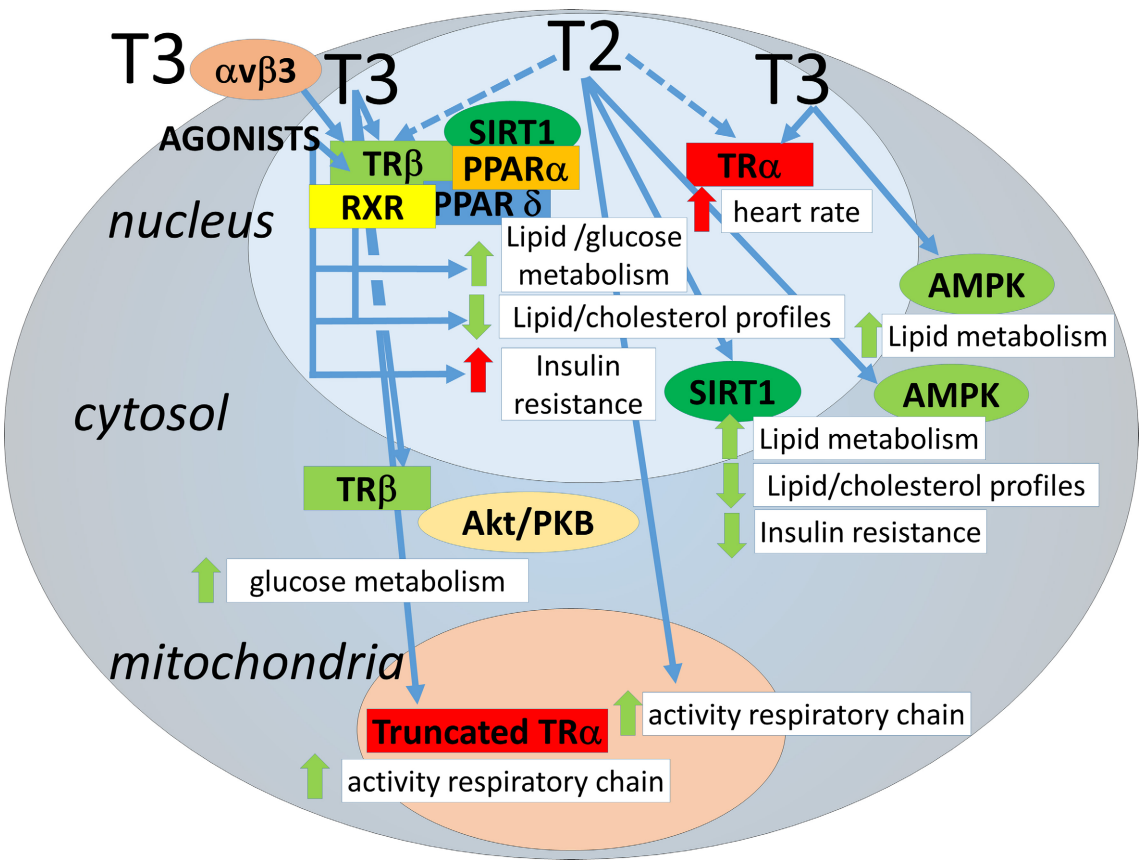

FIGURE 1 | Avenues for regulation of lipid metabolism by thyroid hormones and analogs. THs may act through transcriptional regulation by nuclear TR binding but also through interactions involving cytosolic/mitochondrial TRs or other proteins, each of which influencing lipid metabolism. For clarity, the TH analogs have not been depicted separately, negative effects on insulin sensitivity have only been demonstrated for a subset (see text). Of the thyroid hormone metabolites only T2 is depicted, since T2 is the only TH metabolite thus far with low affinity for TRα and positive effects on metabolism. Avenues are depicted as blue arrows. Dotted arrows: yet to be determined/confirmed. Effects are depicted as red arrows (negative) or green arrows (positive).

has a 60-fold reduced binding capacity to human TRβ (Mendoza et al., 2013). In line with this, it has been shown that T2 has a weak transactivating capacity of TRβ target genes in different systems, *in vitro* and *in vivo* (Ball et al., 1997; Cioffi et al., 2010; de Lange et al., 2011; Mendoza et al., 2013). Interestingly, T2 has an affinity for TRβ in fish that is similar to that of T3 (Mendoza et al., 2013), indicating an evolutionary role for this iodothyronine as a genuine classic TH. In Wistar rats housed at thermoneutrality and placed on a high-fat diet (50% fat), T2 (25 μg/100 g BW) induces a protein profile favoring a shift toward fast-twitch, type II skeletal muscle fibers, and a preference for glucose as fuel (Moreno et al., 2011). Under the same conditions, T2 has strong lipid lowering effects and can effectively prevent hepatic steatosis, ameliorating tissue and systemic insulin sensitivity (de Lange et al., 2011). T2, apart from binding directly to subunit Va of mitochondrial cytochrome-c oxidase, enhancing its activity (Arnold et al., 1998), acts by activating hepatic nuclear SIRT1, which deacetylates PGC-1α and SREBP-1c, thus inducing the expression of genes involved in mitochondrial fatty acid oxidation, and repressing genes involved in lipogenesis, respectively, confirmed by proteomic profiling (de Lange et al., 2011). Thyroid hormones, ameliorating metabolic parameters, may improve healthy aging (see for review de Lange et al., 2013). Indeed, very recently, Padron et al. (2014) reported that T2 administration (25, 50, and 75 μg/100 g BW) to aging Wistar rats reduced body- and retroperitoneal fat mass gain, increased resting metabolic rate (RMR), ameliorated glucose tolerance, did not alter heart mass and heart rate, and only lowered serum T4 and T3 levels at the two higher doses (Padron et al., 2014). A similar effect was recently observed in HFD-fed mice which showed increased heart weights only when treated with unusual very high doses of T2 (250 μg/100 g BW) (Jonas et al., 2014). Reaching a "safe" dose of T2 may thus be feasible in humans as well, which warrants further investigation.

CONCLUSIONS AND PERSPECTIVES

There has been much progress in identifying "novel" mechanisms of action of T3 and T2 (**Figure 1**) and TH metabolites and in developing "safe" (that is: non-thyrotoxic) TH-related compounds to reduce lipid profiles. The discovery of thyroid-related compounds which could be implied in the regulation of energy balance may thus pave the way to strategies for their use in the clinic.

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Update on 3-iodothyronamine and its neurological and metabolic actions

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3-iodothyronamine (T1AM) is an endogenous amine, that has been detected in many rodent tissues, and in human blood. It has been hypothesized to derive from thyroid hormone metabolism, but this hypothesis still requires validation. T1AM is not a ligand for nuclear thyroid hormone receptors, but stimulates with nanomolar affinity trace amine-associated receptor 1 (TAAR1), a G protein-coupled membrane receptor. With a lower affinity it interacts with alpha2A adrenergic receptors. Additional targets are represented by apolipoprotein B100, mitochondrial ATP synthase, and membrane monoamine transporters, but the functional relevance of these interactions is still uncertain. Among the effects reported after administration of exogenous T1AM to experimental animals, metabolic and neurological responses deserve special attention, because they were obtained at low dosages, which increased endogenous tissue concentration by about one order of magnitude. Systemic T1AM administration favored fatty acid over glucose catabolism, increased ketogenesis and increased blood glucose. Similar responses were elicited by intracerebral infusion, which inhibited insulin secretion and stimulated glucagon secretion. However, T1AM administration increased ketogenesis and gluconeogenesis also in hepatic cell lines and in perfused liver preparations, providing evidence for a peripheral action, as well. In the central nervous system, T1AM behaved as a neuromodulator, affecting adrenergic and/or histaminergic neurons. Intracerebral T1AM administration favored learning and memory, modulated sleep and feeding, and decreased the pain threshold. In conclusion T1AM should be considered as a component of thyroid hormone signaling and might play a significant physiological and/or pathophysiological role. T1AM analogs have already been synthetized and their therapeutical potential is currently under investigation. 3-iodothyronamine (T1AM) is a biogenic amine whose structure is closely related to that of thyroid hormone (3,5,3′-triiodothyronine, or T3). The differences with T3 are the absence of the carboxylate group and the substitution of iodine with hydrogen in 5 and 3′ positions (**Figure 1**). In this paper we will review the evidence supporting the hypothesis that T1AM is a chemical messenger, namely that it is an endogenous substance able to interact with specific receptors producing significant functional effects. Special emphasis will be placed on neurological and metabolic effects, which are likely to have physiological and pathophysiological importance.

Keywords: T1AM, 3-iodothyronamine, thyroid hormones, neuromodulation, lipid metabolism, diabetes mellitus, learning, memory

DISTRIBUTION AND METABOLISM OF T1AM

The gold standard technique for detecting T1AM is represented by mass spectrometry, coupled to an appropriate separation technique, usually HPLC. While the initial reports of endogenous T1AM in brain (Scanlan et al., 2004), heart (Chiellini et al., 2007), and blood (Braulke et al., 2008) were admittedly non-quantitative, subsequent technical improvements allowed the assay of T1AM in virtually every tissue in rodents (Saba et al., 2010), as well as in human blood (Galli et al., 2012). Tissue concentrations were found to be on the order of 1–90 pmol/g, and the highest values were detected in liver, brain, and muscle (Saba et al., 2010). The presence of endogenous T1AM at this concentration range has been subsequently confirmed in liver (Hackenmueller et al., 2012; Ghelardoni et al., 2014) and brain (Musilli et al., 2014).

In serum T1AM was measured at a concentration on the order of 0.2–0.3 nM both in rat and in human, and so it was significantly lower than tissue concentration. In another investigation T1AM was not detected in blood (Ackermans et al., 2010), but in that study a different preparation procedure was used and method sensitivity was probably too low (0.25 nM) to get positive results. On the other hand the presence of T1AM in human blood was confirmed with a chemiluminescence immunoassay (Hoefig et al., 2011), and its concentration was estimated to be much higher, namely 66 nM. While it has been hypothesized that the immunological assay may detect a fraction of T1AM

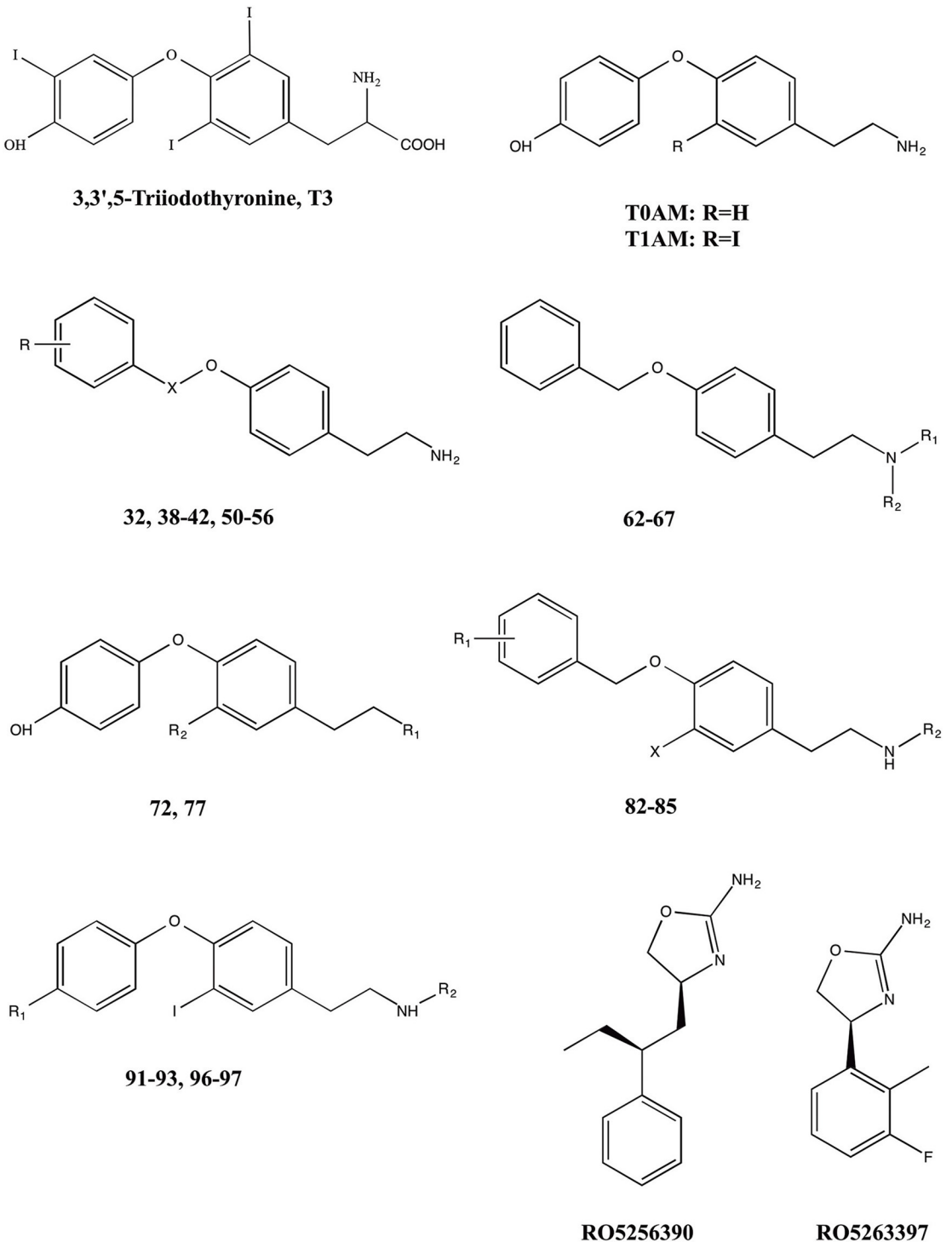


FIGURE 1 | Chemical structures of 3,5,3′-triiodothyronine (T3),endogenous thyronamines (T1AM–T0AM) and synthetic analogs.

which is not extracted by the preparation procedure used for mass spectrometry assays, an alternative explanation of this discrepancy is the subsequent observation that T1AM binds with high affinity to the plasma protein apoB-100 (Roy et al., 2012 see below). In fact the immunological assay was a competition assay in which T1AM labeled with horseradish peroxidase was used as a reporter and calibration curves were not obtained in whole serum (Hoefig et al., 2011). Therefore, binding of labeled T1AM to apoB-100 may have caused overestimation of endogenous T1AM.

Experiments in which cell cultures were exposed to exogenous T1AM, or isolated organs were perfused with exogenous T1AM, confirmed that T1AM is accumulated by many different cells types, including hepatocytes, cardiomyocytes, and thyrocytes (Saba et al., 2010; Agretti et al., 2011; Ghelardoni et al., 2014), although the molecular identity of the T1AM transporter(s) still remains to be clarified (Ianculescu et al., 2009; Saba et al., 2010). Consistently, radiolabeled T1AM reached virtually every organ after intravenous injection (Chiellini et al., 2012; Lee et al., 2013). Acute uptake prevailed in liver, kidney, stomach, and intestine, while after 24 h most residual T1AM was detected in liver, muscle, and adipose tissue.

The close chemical similarity with T3 induced to speculate that T1AM may be synthetized from the T3 through decarboxylation and deiodination (Scanlan et al., 2004; Ianculescu and Scanlan, 2010; Piehl et al., 2011). However, only trace amounts of T1AM were produced in cardiomyocytes exposed to T3 (Saba et al., 2010), while administration of deuterated T4 was not associated with detection of deuterated T1AM in the liver of mice treated with perchlorate and metimazole (Hackenmueller et al., 2012). In human blood, a significant correlation between T3 and T1AM was observed when these substances were assayed by mass spectrometry (Galli et al., 2012), while in thyroidectomized patients treated with synthetic T4 normal serum T1AM values were detected by chemiluminescence immunoassay, supporting the hypothesis that T1AM may be an extrathyroidal metabolite of thyroid hormone (Hoefig et al., 2011). Therefore, the biosynthetic pathway responsible for T1AM production is still uncertain.

Tissue T1AM metabolism includes oxidative deamination to 3-iodothyroacetic acid, deiodination to thyronamine, N-acetylation and esterification with sulfate or glucoronate (Pietsch et al., 2007; Piehl et al., 2008; Wood et al., 2009; Saba et al., 2010; Agretti et al., 2011; Hackenmueller and Scanlan, 2012). Either T1AM or its catabolites undergo biliary and urinary excretion, as shown by the observations performed after administration of radiolabeled T1AM (Chiellini et al., 2012; Lee et al., 2013).

T1AM RECEPTORS AND BINDING SITES

T1AM is not a ligand for nuclear thyroid hormone receptors, but it was found to stimulate with high affinity trace-amine associated receptor 1 (TAAR1), a G protein-coupled membrane receptor (Scanlan et al., 2004). TAAR1 was identified in 2001 on the basis of its affinity for the so-called trace amines, namely β-phenylethylamine, p-tyramine, triptamine, and octopamine (Borowsky et al., 2001; Bunzow et al., 2001; Grandy, 2007). Nine different TAAR subtypes exist, and they are widely expressed in several tissues (Zucchi et al., 2006).

So far investigations on the physiological role of TAARs have focused on the central nervous system effects of TAAR1, which has been hypothesized to act as a physiological regulator of monoaminergic neurotransmission. TAAR1 is expressed in several brain areas, particularly the limbic regions and nuclei containing monoaminergic cells (Lindemann et al., 2008). In mouse, dopaminergic neuron firing in the ventral tegmental area was modulated by p-tyramine. This effect was lost in TAAR1 knockout mice (Lindemann et al., 2008) and inhibited by N-(3-ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoromethyl-benzamide (EPPTB), a selective TAAR1 antagonist (Bradaia et al., 2009). In addition, type 2 dopamine receptor (D2R) antagonists enhanced TAAR1-mediated increase in cAMP, possibly by disrupting TAAR1-D2R interaction (Espinoza et al., 2011). Since D2R is the main target of antipsychotic drugs such as haloperidol (Strange, 2001), the observed functional TAAR1-D2R interaction might have potential therapeutic implications for dopamine-related disorders (Revel et al., 2011). It has also been reported that TAAR1 is a target of psychotropic agents like amphetamine, methamphetamine, 3,4-methylenedioxymetamphetamine (MDMA, known as "ecstasy"), and d-lysergic acid diethylamide (Bunzow et al., 2001), suggesting a role in neuropsychiatric disorders. Interestingly, human TAAR genes are clustered on the long arm of chromosome 6, in a region which is consistently associated with schizophrenia or bipolar affective disorder in linkage studies (reviewed by Zucchi et al., 2006).

Using cell cultures expressing heterologous TAAR1, T1AM was found to activate rat and mouse TAAR1, inducing cAMP production with EC_{50} of 14 and 112 nM, respectively (Scanlan et al., 2004). In these models T1AM was more potent than all other trace amines. Preliminary evidence that T1AM interacts with TAAR5 has been reported (Mühlhaus et al., 2013), and TAAR8 has also been suggested as a potential target, on the basis of the pharmacological effects produced in the isolated rat heart (Frascarelli et al., 2008).

TAAR1 and possibly other TAARs represent the most likely endogenous receptor(s) for T1AM. However, receptor antagonists and knockout models are available only for TAAR1, and they have not been extensively used in experimental investigations. Therefore, the role of specific TAARs in the response to T1AM remains largely speculative, and the underlying transduction pathways also require further investigations. Apart from TAARs, additional binding sites for T1AM may exist, since the comparison between tissue T1AM concentration and tissue TAAR expression revealed a clear mismatch (Chiellini et al., 2012). As a matter of fact, several candidates have been identified and are listed below. In general, the functional consequences of T1AM binding to these targets, if any, are still uncertain, and will be discussed more extensively in the subsequent sections.

Other G protein-coupled receptors might bind T1AM, although with a lower affinity than TAAR1. In particular, in COS7 cells transfected with human or mouse alpha 2A adrenergic receptor (α_{2A}), Ki values in the low micromolar range were obtained (Regard et al., 2007).

The plasma protein apo-B100, a component of VLDL and LDL lipoproteins, binds T1AM with a K_D of 17 nM (Roy et al., 2012). This is the likely reason for the difficulty in extracting T1AM from blood samples, and for the discrepancies reported with serum T1AM assays, as discussed above. The functional implications of this interaction are uncertain, since no evidence that T1AM may modify lipoprotein function has been reported so far.

Another molecular target appears to exist in mitochondria, since T1AM modulated the activity of sub-mitochondrial particles and soluble F_1-ATPase (Cumero et al., 2012). Functional and biochemical data suggested the existence of a high affinity

binding site (affinity on the order of 50 nM), which prevents the interaction between the ATP synthase and its physiological inhibitor IF1, and a low affinity binding site ($IC_{50} = 28\,\mu M$), through which T1AM reduces enzyme activity. Consistent with these findings, T1AM has been reported to reduce oxygen consumption and increase hydrogen peroxide release in rat liver mitochondria (Venditti et al., 2011).

At concentrations in the low micromolar range, T1AM interfered with monoamine transporters, namely norpinephrine transporter, dopamine transporter and vesicular monoamine transporter 2 (Snead et al., 2007). Both competitive and non-competitive mechanisms contributed to the inhibition since T1AM increased the K_m and decreased the V_{max} of each transporter. Interaction with these targets might inhibit dopamine and norepinephrine reuptake as well as their transport into synaptic vesicles. It has also been reported that micromolar T1AM displaced T3 and thyroxine (T4) from their membrane transporters, namely monocarboxylate transporter 8 (MCT8), organic anion transporting polypeptide 1A2 (OATP1A2), and organic anion transporting polypeptide 1C1 (OATP1C1) (Ianculescu et al., 2009).

METABOLIC EFFECTS OF T1AM

The effects that were originally reported after the administration of exogenous T1AM to rodents (50 mg/Kg i.e., 128 μmol/Kg i.p.) included transient decrease in body temperature and reduction of cardiac inotropic and chronotropic state (Scanlan et al., 2004; Chiellini et al., 2007). The former effect is not mediated by TAAR1, since it was reproduced in TAAR1 knockout mice (Panas et al., 2010), and it may be related to the inhibitory effect of mitochondrial function, which has been described above. Cardiac effects have been attributed to the modulation of ionic homeostasis, namely to a reduction of sarcoplasmic reticulum calcium release and an inhibition of potassium currents, particularly transient outward current and I_{k1} background current (Ghelardoni et al., 2009). The transduction pathway probably involves specific tyrosine kinases and/or phosphatases (Chiellini et al., 2007) and pharmacological evidence is consistent with the hypothesis that the receptor triggering this pathway belongs to the TAAR family, whose major component in rat heart appears to be TAAR8 (Frascarelli et al., 2008).

In the isolated rat heart preparation, dose-response curves were obtained, and the IC_{50} for T1AM was found to be on the order of 20–40 μM, i.e., substantially higher than average tissue levels, which are in the range of a few picomoles per g. Thus, these actions are unlikely to be physiological, although they might be exploited pharmacologically, since exogenous T1AM protected the myocardium from ischemia-reperfusion injury (Frascarelli et al., 2011). Central nervous system protection was also reported in a stroke model, and attributed to hypothermia (Doyle et al., 2007).

On the other hand, recent investigations have described metabolic and neurological effects of T1AM, occurring at relatively low dosages. Therefore, regulation of metabolic homeostasis and of central nervous system function appear to be the best candidates in the search for the physiological effects of T1AM, and they will be reviewed more extensively.

Acute metabolic responses to systemic (intraperitoneal) administration of T1AM in Siberian hamster or mouse (50 mg/Kg i.e., 128 μmol/Kg) included a reduction in respiratory quotient from ∼0.90 to ∼0.70 (Braulke et al., 2008), indicating a shift in metabolic pathways from carbohydrate to lipid oxidation. Consistent with these observations, T1AM treatment caused ketonuria and a significant loss of body fat. Both in mouse and in rat, the same dose of T1AM also increased plasma glucose, and this effect was attributed to hormonal changes, since inhibition of insulin secretion and stimulation of glucagon secretion were detected (Regard et al., 2007; Klieverik et al., 2009). The use of transgenic mouse lacking $\alpha_{2A,}$ and experiments performed in pancreatic islets with Gi protein modulators, showed a biphasic effect of T1AM on insulin secretion, namely stimulation through TAAR1 and inhibition through α_{2A}, the latter prevailing under physiological conditions (Regard et al., 2007).

The results discussed above were obtained using pharmacological dosages of T1AM, namely 128 μmol/Kg *in vivo* and 10 μM *in vitro*. However, metabolic responses, particularly increased plasma glucose and increased plasma glucagon, were also elicited by i.c.v. infusion with much lower dosages (0.5 mg/Kg i.e., 1.28 μmol/Kg), suggesting a neuroendocrine action on the hypothalamic-pituitary-adrenal axis, which is a recently identified target for several hormones, including insulin, glucocorticoids and thyroid hormone (Fliers et al., 2010). The potency of T1AM turned out to be even higher than initially thought, since in subsequent investigations plasma glucose was increased after i.c.v. injection of doses as low as 3.3 nmol/Kg (Manni et al., 2012) or 0.3 nmol/Kg (Manni et al., 2013). In the latter study brain T1AM was determined, and it was observed that effective doses increased endogenous T1AM concentration by about one order of magnitude (34-fold with 3.3 nmol/Kg T1AM).

However, the possibility of a peripheral, hormone-independent action should not be ruled out, since T1AM administration (at 0.5–1 μM concentration) was able to increase ketone body production and to stimulate gluconeogenesis in hepatic cell lines and in perfused liver preparations (Ghelardoni et al., 2014).

Recently, Haviland et al. (2013) used a combination of analytical techniques to explore the metabolic effect of prolonged treatment with T1AM (10 mg/Kg i.e., 26 μmol/Kg i.p., for 8 days) in a spontaneously obese mouse model. Breath carbon isotope ratio ($^{13}CO_2/^{12}CO_2$, or $\delta^{13}C$ value) was monitored continuously by cavity ring down spectroscopy (CRDS), and plasma samples were collected and analyzed by nuclear magnetic resonance (NMR). CRDS is a non-invasive technique that can be used to assess lipid vs. carbohydrate/protein oxidation in real-time: lipids are enriched in the lighter isotope (probably because of isotopic fractionation during the pyruvate dehydrogenase reaction), so during lipolysis more $^{12}CO_2$ is generated, resulting in a lower $\delta^{13}C$ value (De Niro and Epstein, 1977; Schöller et al., 1984). Breath $\delta^{13}C$ declined shortly after T1AM injection, and NMR metabolomics confirmed the increase in lipid utilization, as revealed by elevation in plasma 3-hydroxybutyrate concentration. Increased lipolysis was independent of food consumption and it was associated with weight loss (−8.2% of initial body weight after 8 days of treatment). The effect was persistent,

since 2 weeks after discontinuation of T1AM treatment mice regained only 1.8% of the lost weight. In an ongoing investigation (Chiellini et al., 2013) it has been ascertained that lipolysis and weight loss induced by 26 μmol/Kg T1AM in mice are not associated with increased plasma glucose, suggesting that lipid metabolism is a more sensitive target than carbohydrate metabolism.

These long-term effects are likely related to modulation of gene expression, since T1AM significantly modified the expression of over 350 genes in adipose tissue and over 100 genes in liver, in a direction consistent with the observed metabolic changes (Mariotti et al., 2014). Modulated genes included several members of the sirtuin family and genes playing established roles in lipolysis, beta-oxidation, adipogenesis and lipoprotein metabolism.

The molecular mechanisms underlying acute and chronic metabolic effects remain to be determined, but T1AM-triggered pathway(s) may be clinically relevant. In fact, in a small clinical series serum T1AM concentration was found to be significantly increased in type II diabetes, and T1AM levels were significantly correlated with glycated hemoglobin (Galli et al., 2012).

In general, the effects which were originally reported for T1AM, namely hypothermia and cardiac depression, were opposite to those produced by thyroid hormone, so T1AM was initially viewed as a sort of feedback effector of thyroid signaling (Liggett, 2004). However, its metabolic effects (summarized in **Table 1**) are to a large extent synergic with the response to thyroid hormone, which is also known to induce a lipolytic effect (Mullur et al., 2014).

Specific comparison between the transcriptional response to T1AM and T3 revealed that the former produced significant genomic effects, which were not reproduced by the latter (Mariotti et al., 2014). Since T1AM is possibly synthetized from T3, and T1AM may affect T3 transport and availability, it would be interesting to evaluate if some effects traditionally attributed to T3 may be directly or indirectly mediated by T1AM.

Table 1 | Metabolic and endocrine effects of T1AM.

• Increase in plasma glucose
• Reduced carbohydrate oxidation
• Increased gluconeogenesis
• Increased lipid oxidation
• Increased ketogenesis
• Decreased body weight in obese mice
Inhibition of insulin secretion*
Stimulation of glucagon secretion

The table summarizes the metabolic effects of T1AM. The reported effects have been produced with acute or chronic administration of different concentrations of T1AM (see text for further details). The effects are due to central effects on the hypothalamic-pituitary-adrenal axis, but a peripheral component may also exist.

**In pancreatic islets T1AM inhibited insulin secretion through α_{2A} adrenoceptor (α_{2A}R) and stimulated insulin secretion throughtrace amine-associated receptor 1(TAAR1), the former effect prevailing under physiological conditions.*

Similar considerations apply to the neurological effects of T1AM, which will be discussed in the more general context of central nervous system modulation by the thyroid hormone signaling system.

THYROID HORMONE AND THE CENTRAL NERVOUS SYSTEM: THE CO-TRANSMITTER HYPOTHESIS

It is well known that thyroid hormone is necessary for normal brain development, and limited thyroid hormone availability throughout fetal and neonatal periods results in mental retardation, deafness and ataxia (Schwartz, 1983). Its regulatory role in the maintenance of adult brain function has not been completely understood yet. Although it has long been known that mental retardation is a result of hypothyroidism (Jackson, 1998), different lines of research proved that significant reduction or increase in T3 levels jeopardize both cognitive and mnemonic processes when they are assessed in a clinical setting (Dugbartey, 1998) or evaluated in animal models (Alzoubi et al., 2009; Taskin et al., 2011).

Thyroid hormone can reach brain interstitial spaces through two pathways, since it can either be transported across the brain-blood barrier (BBB) or into the cerebral-spinal fluid (CSF) by specific carriers (Cheng et al., 1994). In rodents these carriers are mainly represented by MCT8, which transports both T3 and T4, and OATP1C1, which shows a higher affinity for T4 and reverse T3 (Sugiyama et al., 2003). MCT8 is expressed in choroid plexus cells, brain capillaries, and neurons but it is not present in astrocytes. It mediates T4 and T3 passage through the BBB and into the CSF (Heuer et al., 2005). OATP1C1 is present at the abluminal side of brain micro-capillary endothelium and is localized in BBB areas where acquaporin 4, a marker of astrocytes' end-feet, is also expressed. OATP1C1 is poorly expressed in adult primate BBB, suggesting a primary role for MCT8 in humans (Ito et al., 2011). Consistently, mutations in the MCT8 gene are associated with the Allan-Herndon-Dudley syndrome, an X-linked disease characterized by severe neurological involvement and reduced CSF T4 concentration (Kakinuma et al., 2005).

Even though blood-borne T3 can reach the brain tissue, nearly 80% of intracerebral T3 is produced locally from T4. Astrocytes throughout the brain and tanycytes in the third ventricle are the main site of conversion of T4 into T3, which is catalyzed by type II iodothyronine 5-deiodinase (D2) (Guadaño-Ferraz et al., 1999). At neuronal level, T3 can either cross the plasma membrane through MCT8 or OATP1C1 to interact with nuclear receptors, or modulate neuronal functions through non-genomic mechanisms. T3 is then catabolized into 3,5-T2 by type III deiodinase 3 (D3), which is specifically expressed in neurons and is also involved in the conversion of T4 into its inactive metabolite rT3 (Alkemade et al., 2005).

It has been speculated that in the brain iodothyronines may act as co-transmitters and in particular that they may modulate the response to the noradrenergic system (Gompf et al., 2010). This hypothesis is based on different lines of evidence. First of all, iodothyronines share considerable similarities with other neurotransmitters in their effects and mechanisms of action during brain development. Central neurotransmitters usually produce appreciable effects on brain even before the full development

of their specific circuitry. During this early phase they act as growth factors and mitogens, stimulating cell growth and directing their migration (Lauder, 1983). Then, when neuronal processes and synapses become widely available, they bind to their specific receptors and behave as "conventional" neurotransmitters. Likewise, iodothyronines show a differential neuronal distribution in early and late development (Crutchfield and Dratman, 1983). During the first week of rat postnatal life, iodothyronines accumulate in nuclear and cytosolic fractions and affect neuronal maturation. For instance both hyper- and hypothyroidism have been demonstrated to alter dendritic arborization of granule cells in the hypothalamus of adult Wistar rats (Martí-Carbonell et al., 2012), while T3 exerts a critical role in hippocampal cell proliferation and granule cell precursor commitment (Remaud et al., 2014). At a later stage the picture changes completely: there is a geometrical growth in iodothyronine concentration at synapses and nerve terminals, whereas the presence of iodothyronines in the nucleus drops steadily, reaching a plateau 10 days after birth.

In addition, while T3 has been located in almost all regions in adult brain, reaching concentrations on the pmol/g order (Dratman et al., 1983; Morreale de Escobar et al., 1985; Pinna et al., 2002), iodothyronines concentrate specifically in the *locus coeruleus* (LC) and in collateral centers involved in noradrenergic signaling (Dratman and Crutchfield, 1978; Dratman et al., 1982). This is consistent with the peculiar location of LC, which lies on the lateral floor of the fourth ventricle, a strategic site favoring the uptake of T3 and T4, which are translocated through the choroid plexus to enter the CSF. Furthermore, noradrenaline was demonstrated to enhance the expression of D2 gene in adult rat brain (Greer et al., 1991), providing a specific mechanism through which LC could concentrate T3 in its terminals. Immunoistochemical studies confirmed that T3 is concentrated in the regions that are integrated in the noradrenergic system, where subcellular T3 distribution is quite peculiar: while in other areas T3 is mainly identified in cell nuclei, in adrenergic neurons it is located in the cell perikaria and in cell processes (Rozanov and Dratman, 1996). The latter result is consistent with the hypothesis that T3 is transported anterogradely through axonal processes in LC neurons (Gordon et al., 1999). Treatment of rat LC terminals with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), a neurotoxin which specifically damages noradrenergic neurons, produced the expected selective degeneration in noradrenergic neurons, but also the loss of T3-immunoreactive cells in LC target regions.

As a matter of fact, thyroid hormone can acutely affect neuron firing. In Cornu Ammonis area 1 (CA1) T3 increased unit firing rates and magnified neuronal firing induced by norepinephrine stimulation (Caria et al., 2009). Furthermore, T3 injection in the preoptic area of hypo- and eu-thyroid rats produced significant changes in EEG patterns. In hypothyroid animals, administration of 3 μM of T3 reduced REM sleep, whereas higher doses (10 μM) induced an increase in the same parameter (Moffett et al., 2013). In euthyroid rats, 1–3 μM T3 induced an increase in REM sleep and a significant decrease in slow-wave sleep duration (Martin et al., 2013). The timing of these effects suggests the involvement of non-genomic mechanisms.

Besides catecholamines, other neurotransmitters might be affected by thyroid hormone. T3, T4, and rT3 decreased GABA uptake in synaptosomes derived from rat brain (Mason et al., 1987), while T3 inhibited $GABA_A$-gated chloride currents in several experimental models, namely *Xenopus laevis* oocytes (Chapell et al., 1998), synaptosomes derived from rat hippocampal neurons (Martin et al., 2004), and rat cultured hippocampal cells (Puia and Losi, 2011). Inhibition of glutamate binding to type NMDA receptor has been observed in the presence of thyroid hormone (Oguro et al., 1989), although these effects occurred at micromolar (i.e., non-physiological) concentrations. In rodent models both hypo-and hyperthyroidism increased serotonin turnover in the brainstem (Ito et al., 1977), which might result in a robust activation of $5HT_{1A}$ inhibitory autoreceptor in the raphe nuclei (Bauer et al., 2002), in turn leading to reduced levels of serotonin in the frontal cortex and decreased $5HT_{2A}$ receptor density in this area (Kulikov et al., 1999; Kulikov and Jeanningro, 2001). In this investigations only the frontal cortex was evaluated, therefore it cannot be excluded that serotoninergic transmission may be affected by thyroid hormone levels also in other neocortical areas. Indirect evidence of an action on the cholinergic system is based on the observation that in a rodent model sub-chronic and chronic T4 administration increased cholinergic activity in the frontal cortex and hippocampus, which was associated with improved spatial memory abilities, as assessed through the watermaze test (Smith et al., 2002).

NEUROLOGICAL EFFECTS OF T1AM

As detailed above, an intricate interplay appears to occur between neuronal function, neurotransmitter signaling pathways and specific genomic or non-genomic actions of thyroid hormone. Since 1995, it has been hypothesized that a crucial modulatory role could be played by thyroid hormone derivatives (Gordon et al., 1995), and T1AM is now emerging as a possible modulator of monoaminergic transmission and specifically of noradrenergic, dopaminergic and histaminergic circuitries.

Electrophysiological recordings performed in LC showed that the rate of discharge of adrenergic neurons was modified by local application of T1AM (10 μM) (Gompf et al., 2010). As discussed above, there are reasons to believe that TAAR1, now considered as a specific T1AM receptor, can interact with the adrenergic system. Apart from TAAR1, additional T1AM targets might be involved in neuromodulation. As already mentioned, in synaptosomal fractions T1AM inihibited dopamine and norepinephrine transporters (Snead et al., 2007). Since these two transporters are responsible for dopamine and norepinephrine reuptake into the presynaptic terminal, the expected downstream effects of T1AM are represented at first by accumulation of extracellular monoamines, and afterwards by depletion of neurotransmitter stores and reduction of neurotransmission. Vesicular monoamine transporter 2 (VMAT2) is also subjected to inhibition. This transporter is instrumental in the translocation of neurotransmitters (i.e., dopamine, norepinephrine, serotonin, and histamine) from cytosol to synaptic vesicles, and so T1AM could deplete neurotransmitters available for synaptic transmission, although this hypothesis still needs experimental validation.

Another potential target is represented by α_{2A}(Regard et al., 2007). α_{2A} plays a peculiar role in central neurotransmission, since along with α_{2D}it is expressed as an inhibiting autoreceptor on noradrenergic presynaptic terminals and as a modulating heteroreceptor in serotonergic, dopaminergic, and glutamatergic neurons (Gilsbach and Hein, 2012). Cortical activation might be another consequence of α_{2A} activation: while it is widely known that LC exerts an excitatory influence on the cerebral cortex through α_1-receptor activation (Papay et al., 2006), α_{2A} is also expressed at the cortical level (Blake et al., 1998), mainly in inhibitory interneurons, so α_{2A} stimulation induces disinhibition of the cerebral cortex (Andrews and Lavin, 2006).

In line with these results, microinjections of T1AM in the preoptic region induced a significant reduction in non-REM sleep (at doses of 1 and 3 μg = 2.5 and 7.5 nmoles) and an increase in low and theta frequencies in the power spectrum of EEG-defined wakefulness (at a dose of 3 μg = 7.5 nmoles) (James et al., 2013). Notably, these effects closely mirrored the effects of thyroid hormone administration. Consistent with these observations, i.c.v. injection of T1AM in a mouse model (at doses of 1.32 and 4 μg/Kg = 3.3–10.2 nmol/Kg) produced a significant increase in exploratory activity assessed through the hole-board test (Manni et al., 2013). As it could be expected, the same results on wakefulness and motor activity were produced by the injection of norepinephrine in the preoptic region of adult rat brain (Emlen et al., 1972).

In addition to these effects, T1AM (1.32–4 μg/Kg = 3.3–10.2 nmol/Kg) induced pro-learning and anti-amnestic responses when administered i.c.v. (Manni et al., 2013). In the object recognition task mice showed significantly enhanced exploratory preference and curiosity for the novel object, which was retained after 24 h. The passive avoidance test confirmed that T1AM favored learning both at 1 h and at 24 h after i.c.v. injection, and it counteracted the amnestic effect of scopolamine. The response to T1AM was antagonized by the monoamine oxidase inhibitor chlorgyline, which is consistent with the hypothesis of an interaction with the noradrenergic system. In fact, it is well known that LC projections to the hippocampus are involved in both formation (Sullivan et al., 1994) and retrieval (Sara and Devauges, 1988) of memories in rat models.

A recent study by Musilli et al. (2014) has suggested that T1AM main oxidative metabolite, 3-iodothyroacetic acid (TA1), may also play a role in the stimulation of memory acquisition, possibly by activating a histaminergic system, since its effect was prevented by H1 receptor antagonists. Interestingly, histamine is known to modulate the synchronization of neuron burst in CA3, which is an area of the hippocampus playing a central role in synaptic plasticity and in the formation of memory traces (Buzsaki and Draguhn, 2004).

Other interactions between T1AM signaling and histamine circuitry have been identified. Histamine has been demonstrated to modulate pain at the cortical and subcortical level, inducing hyperalgesia at low doses through H1 receptors (Malmberg-Aiello et al., 1994; Galeotti et al., 2004). In line with these observations, i.c.v. injection of TA1 (0.4 μg/Kg) reduced the threshold to painful stimuli in mice subjected to the hot plate test, and the effect was completely abolished when TA1 was co-administered with histamine receptor antagonists and in mice lacking histidine decarboxylase, the enzyme responsible for histamine synthesis (Musilli et al., 2014).

T1AM has also important effects on the regulation of food intake. Intracerebral T1AM injection induced significant alteration in feeding behavior in fasting mice and in mice fed *ad libitum*. In the latter group, when T1AM was administered either in the arcuate nucleus (at doses of 0.12–1.2 nmol/Kg) or in cerebral ventricles (at the dose of 1.2 nmol/kg), an orexigenic effect was induced (Dhillo et al., 2009). It was also observed that exposure of hypothalamic slices to T1AM *in vitro* induced neuropeptide Y (NPY) release, suggesting that this potent orexigenic peptide is involved in the hyperphagic effect. However, in fasting mice a biphasic response was elicited by i.c.v. T1AM administration, since low dosages (1.32 μg/Kg = 3.3 nmol/Kg) produced an anorexic effect while higher dosages (20 μg/Kg = 51 nmol/Kg) turned out to be orexigenic (Manni et al., 2012).

Effects of chronic treatment have also been reported. In a study that was published only in abstract form, Hettinger et al. (2010) observed that chronic systemic (i.p.) administration of T1AM (31 mg/Kg per day = 79 μmol/Kg per day for 14 days) reduced food intake in mice, while no change in food assumption was observed at a lower dosage (10 mg/kg per day = 26 μmol/Kg per day for 8 days) by Haviland et al. (2013).

The mechanism of feeding modulation by T1AM is not known, but a role for histamine cannot be excluded. In fact, histaminergic neurons form a network that is involved in the balance of neuroendocrine and feeding inputs within the hypothalamus. Histamine induces suppression of food intake when interacting with the satiety center in the ventromedial hypothalamus (Ookuma et al., 1993). On the other hand, i.c.v. injection of H3 histamine receptor antagonists induce suppression of food intake (Cohn et al., 1973). Therefore, this dual action on feeding behavior might be involved in the effects of T1AM and/or its catabolite TA1 on food intake.

Most interestingly in the few investigations in which brain tissue could be assayed to determine thyroid hormone and T1AM, it was observed that after administration of effective T1AM dosages (1.3 μg/Kg = 3.3 nmol/Kg), average brain T1AM concentration increased by about one order of magnitude over the baseline, while brain T3 and T4 concentrations were unchanged (Manni et al., 2013). So, functional effects occurred at tissue concentrations close to the physiological range. A summary of the neurological effects which have been described after T1AM administration is reported in **Table 2**.

EXPLOITING T1AM SIGNALING

The different functional effects of T1AM and the widespread distribution of TAARs raise the hope that this novel signaling system may be exploited in human therapeutics. A large number of T1AM derivatives have already been synthesized and evaluated as TAAR1 agonists. As shown in **Figure 1**, the first generation of T1AM analogs (Hart et al., 2006) featured removal of the phenol hydroxyl, an increase in the distance between the two aryl rings, a change in the electronic and steric requirements on the aryl ring distal to the amine functionality, alkylation or modification of the amine, and replacement of the 3-iodo substituent with an alkyl group. These analogs were evaluated using the cAMP accumulation assay in cells stably expressing either rat or mouse

Table 2 | Neurological effects of T1AM.

Electrophysiological effects on the *Locus Coeruleus:*
• Increased neuronal firing
EEG patterns (microinjection in the preoptic region):
• Reduction in nREM sleep
Behavior (i.c.v. administration):
• Increase in exploratory activity
Memory (i.c.v. administration):
• Prolearning and antiamnestic effect
Pain (i.c.v. administration):
• Decreased pain threshold to hot stimuli
Food intake (acute central administration):
• In *ad libitum* fed mice: orexigenic effect
• In fasting mice: biphasic effect, with anorexic properties at low doses and orexigenic effects at higher doses
Food intake (chronic peripheral administration, i.p.):
• Anorexic effect

The table summarizes the neurological effects of T1AM. The reported effects have been produced with acute or chronic administration of different concentrations of T1AM (see text for further details). Abbreviations: EEG, electroencephalography; i.c.v., intracerebroventricular; i.p., intraperitoneal.

TAAR1 (rTAAR1 and mTAAR1). Analysis of the results summarized in **Table 3** suggests the following requirements for TAAR activation:

(i) A basic amino group at Cα is required. In the case of compound **72,** where the amine was replaced with a hydroxyl, no TAAR1 activation was observed.
(ii) Monomethylation of the amine can be beneficial (compounds **62** and **85**) although larger alkyl groups (compounds **64-66**) and bis-alkylation (compound **67**) are deleterious.
(iii) mTAAR1 and rTAAR1 differ with respect to their tolerance of changes in the diaryl linker, both in length and in functionality. In the case of rTAAR1 activation, derivatives **77**, **91**, and **92** were the most potent, while in the case of mTAAR1, the most effective compounds were derivatives **85**, **91**, and **92**.
(iv) Within the thyronamine scaffold, an iodine or methyl substituent at the 3-position is optimal. Derivative **77** with a methyl group at the 3-position of the thyronamine scaffold was nearly as potent as T1AM against both rTAAR1 and mTAAR1.
(v) An H at the 4′-position vs OH is optimal (compound **91** was the most potent agonist of rTAAR1 and one of the most potent at mTAAR1) and substituents larger than OH are deleterious (compounds **93** and **97**).

The most potent derivatives reported by Hart et al. (2006), namely compounds **77**, **85**, **91**, and **92**, were also examined for hypothermia induction in mice. When administered to mice at a 50 mg/kg dose, all derivatives induced significant hypothermia within 60 min and exhibited a hypothermic induction profile analogous to T1AM. Derivative **91** was the most potent, and an ED50 of 30 μmol/kg was calculated.

On the basis of the report that TAAR1 can be activated by phenethylamine analogs like amphetamines and ergolines (Bunzow et al., 2001), an additional group of phenyltyramine derivatives was devised and analyzed by Tan et al. (2007). This investigation showed that the potency of thyronamines for both rTAAR1 and mTAAR1 can be enhanced by incorporating appropriate functionalities in the ethylamine portion of the phenoxyphenylethylamine scaffold. Even though rTAAR1 and mTAAR1 are 93% similar, the two rodent receptors have different structural preferences for this region of the scaffold, with rTAAR1 favoring unsaturated hydrocarbon groups and mTAAR1 preferring functional groups that are polar and hydrogen-bond acceptors. Analysis of single and double mutants of rat and mouse TAAR1 (Tan et al., 2009) showed that key, non-conserved specificity determinant residues in transmembranes helices 4 and 7 within the ligand binding site appear to be the primary source of the observed ligand preferences. In particular, residue 7.39 appears to dictate the specificity for a β-phenyl ring: a bulky tyrosine residue at 7.39 in mTAAR1 sterically clashed with the β-phenyl ring, whereas a smaller asparagine residue at the same location in rTAAR1 was able to accommodate a β-phenyl moiety. The lower potency of T1AM in mTAAR1 (EC_{50} = 112 nM) vs. rTAAR1 (EC_{50} = 14 nM) appeared to be caused by the presence of a tyrosine instead of a phenylalanine at residue 4.56. Despite this species variability, transforming the inner ring of the phenoxyphenethylamine scaffold into a naphthyl group, as in compound **24**, was equally beneficial to both receptors, most likely acting as an excellent isosteric replacement for the iodophenyl inner ring of T1AM (**Table 4**).

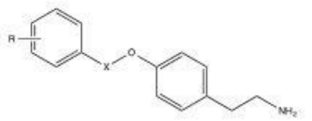
A different approach has been followed by Hoffmann-La Roche chemists, who have developed a novel series of imidazole compounds that are potent and selective partial and full agonists of the TAAR1 receptor (Galley et al., 2012; Revel et al., 2013). In rodents, activation of TAAR1 by the full agonist RO5256390 and the partial agonist RO5263397 (**Figure 1**), blocked psychostimulant induced hyperactivity and produced a similar activation pattern as the antipsychotic drug olanzapine. Notably, TAAR1 agonists do not induce catalepsy or weight gain; RO5263397 even reduced haloperidol-induced catalepsy and prevented olanzapine from increasing body weight and fat accumulation. In addition, TAAR1 activation promoted vigilance in rats and showed pro-cognitive and antidepressant-like properties in rodent and primate models (Revel et al., 2013).

Quite recently a preliminary report has described the synthesis of T1AM analogs in which the two aromatic rings are linked by a methylene group, the hydroxyl group (OH) at position 4′ has been replaced by an amino group (NH2), and the ethylamine side chain at position 1 has been replaced by an oxy-ethylamine side chain (Chiellini et al., 2014). Interestingly, these compounds were effective on mouse TAAR1 but in functional experiments they increased plasma glycemia and reduced cardiac output, i.e., they produced effects which are known not to be mediated by TAAR1 (Regard et al., 2007; Frascarelli et al., 2008). These observations raise the possibility that different receptors systems may be targeted by T1AM analogs and/or putative TAAR1 agonists.

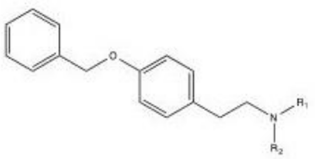
CONCLUSIONS

In conclusion, T1AM is a novel chemical messenger, that interacts with a specific G protein-coupled receptor, TAAR1, and

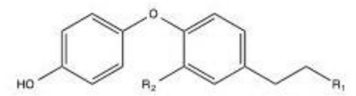
Table 3 | Activity of first generation analogs of thyronamines on rTAAR1 and mTAAR1 (as reported by Hart et al., 2006).



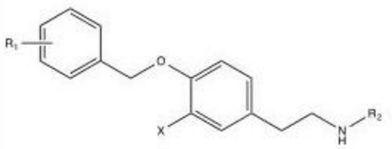
#	X	R	EC_{50} rTAAR1	EC_{50} mTAAR1
32	-	H	38	296
38	CH_2	H	209	168
39	$(CH_2)_2$	H	203	207
40	$(CH_2)_3$	H	89	102
41	$(CH_2)_4$	H	43	80
42	$(CO)CH_2$	H	309	83
50	CH_2	*m*,*m*-dimethyl	137	98
51	CH_2	*m*-methoxy	141	194
52	CH_2	*m*,*m*-dimethoxy	333	123
53	CH_2	*p*-fluoro	72	38.5
54	CH_2	*p*-nitro	64	21.5
55	CH_2	*p*-trifluoromethyl	55	20.2
56	CH_2	2,3,4,5,6-pentafluoro	>1000	>1000



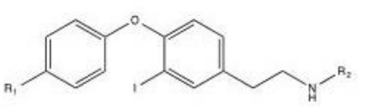
#	R_1	R_2	EC_{50} rTAAR1	EC_{50} mTAAR1
62	Me	H	119	54
63	Et	H	>1000	71
64	Pr	H	>1000	>1000
65	Bu	H	>1000	>1000
66	Bn	H	>1000	>1000
67	Me	Me	>1000	192



#	R_1	R_2	EC_{50} rTAAR1	EC_{50} mTAAR1
72	OH	I	>1000	>1000
77	NH_2	CH_3	**33**	**116**



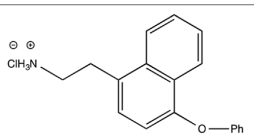
#	X	R_1	R_2	EC_{50} rTAAR1	EC_{50} mTAAR1
82	I	H	H	77	221
83	I	CF_3	H	>1000	284
84	I	H	Me	68	106
85	H	CF_3	Me	**61**	**12**



#	R_1	R_2	EC_{50} rTAAR1	EC_{50} mTAAR1
91	H	H	**2.4**	**35**
92	F	Me	**2.7**	**32.5**
93	CF_3	Me	>1000	>1000
96	OH	Me	18	221
97	Ome	Me	107	>1000

Concentrations are expressed as nM.

Table 4 | Activity of naphethylamine (derivative 24) on rTAAR1 and mTAAR1 (derived by Tan et al., 2007).



24

rTAAR1		mTAAR1	
EC_{50}± s.e.m. (nM)	**E_{max}± s.e.m. (%)**	**EC_{50}± s.e.m. (nM)**	**E_{max} ± s.e.m. (%)**
26 ± 1	113 ± 5	101 ± 22	104 ± 3

possibly with other molecular targets. At tissue concentrations close to the physiological range it produces significant metabolic and neurological effects. From the metabolic side, it stimulates lipid catabolism and induces in general anti-insulin responses; from the neurological side, it has been reported to favor learning and memory, modulate sleep and feeding, and decrease the pain threshold. While available evidence suggests that T1AM should be regarded as a neuromodulator, the molecular details of its actions, and the underlying transduction pathways, remain to be determined.

It has been suggested, although not yet formally demonstrated, that T1AM is synthetized from T3, and some of its actions are partly synergic with, but not identical to, the known metabolic and neurological responses to thyroid hormone. So T1AM might be responsible for some effects traditionally attributed to thyroid hormone itself, and should be viewed as a component of thyroid hormone signaling. Exploring this novel aminergic system might open new perspectives in the analysis of hormonal and neuroendocrine regulation of energy balance and behavior, and provide new targets for potential therapeutic interventions in metabolic, endocrine and neurological disease.

Critical research issues for the near future include clarifying the role of TAAR1 vs other receptors in the response to T1AM and dissecting the underlying transduction pathways. In order to

confirm the physiological actions of T1AM, it is also essential to reduce or abolish endogenous T1AM production by appropriate experimental interventions. The latter would require a better understanding of the biochemical pathway(s) responsible for T1AM synthesis.

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Metabolic and hormonal signatures in pre-manifest and manifest Huntington's disease patients

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Huntington's disease (HD) is an inherited neurodegenerative disorder typified by involuntary body movements, and psychiatric and cognitive abnormalities. Many HD patients also exhibit metabolic changes including progressive weight loss and appetite dysfunction. Here we have investigated metabolic function in pre-manifest and manifest HD subjects to establish an HD subject metabolic hormonal plasma signature. Individuals at risk for HD who have had predictive genetic testing showing the cytosine-adenine-guanine (CAG) expansion causative of HD, but who do not yet present signs and symptoms sufficient for the diagnosis of manifest HD are said to be "pre-manifest." Pre-manifest and manifest HD patients, as well as both familial and non-familial controls, were evaluated for multiple peripheral metabolism signals including circulating levels of hormones, growth factors, lipids, and cytokines. Both pre-manifest and manifest HD subjects exhibited significantly reduced levels of circulating growth factors, including growth hormone and prolactin. HD-related changes in the levels of metabolic hormones such as ghrelin, glucagon, and amylin were also observed. Total cholesterol, HDL-C, and LDL-C were significantly decreased in HD subjects. C-reactive protein was significantly elevated in pre-manifest HD subjects. The observation of metabolic alterations, even in subjects considered to be in the pre-manifest stage of HD, suggests that in addition, and prior, to overt neuronal damage, HD affects metabolic hormone secretion and energy regulation, which may shed light on pathogenesis, and provide opportunities for biomarker development.

Keywords: Huntington's disease, pre-manifest, metabolic hormones, lipids, peripheral energy metabolism

INTRODUCTION

Huntington's disease (HD) is a neurodegenerative disorder involving the extrapyramidal motor system and is characterized by chorea, progressive dementia, and other psychiatric symptoms (Walker, 2007; Ross and Tabrizi, 2011; Weir et al., 2011). The incidence of HD is approximately 5–10 cases per 100,000 worldwide, making it one of the most common inherited neurodegenerative disorders. HD is caused by a dominant genetic mutation in the *huntingtin* (*HTT*) gene that results in an expanded trinucleotide repeat of cytosine-adenine-guanine (CAG). This CAG repeat codes for an expanded polyglutamine repeat near the N-terminus of the HTT protein, which undergoes a conformational change, and causes cellular damage (Walker, 2007). The areas of the brain most affected by mutant HTT are the striatum, followed by the cerebral cortex, and then other brain regions. The onset of HD typically occurs around the age of 30–40, and as the disease develops patients progressively lose independence and, eventually, die.

Many HD patients suffer from weight loss and as a result it is believed that metabolic dysfunction contributes to HD pathogenesis (Martin et al., 2008; Van Der Burg et al., 2009; Cai et al., 2012). Aziz et al. (2010a) reported that in HD patients, energy expenditure in a fasted state is increased compared to healthy control subjects, suggesting that a hypermetabolic state could contribute to the observed increase in energy expenditure in HD (Aziz et al., 2010a). Further reinforcing the presence of somatic metabolic dysfunction in HD, abnormal insulin, and leptin secretion rates have been shown to be positively correlated with higher CAG repeat number (Aziz et al., 2010b), which may contribute to the weight loss that is evident in many HD patients. The R6/2 transgenic mouse, which express exon 1 of human HD gene with around 150 CAG repeats (Mangiarini et al., 1996), is the most widely used mouse model to study HD pathology. Blood glucose levels in R6/2 HD mice are significantly higher than in wild type controls. Reduced insulin production is also evident in this model (Andreassen et al., 2002). In addition, the N171-82Q

murine HD model expressing a 171 amino acid- N-terminal human HTT cDNA insertion giving rise to an 82 CAG repeat expansion, displays multiple aspects of diabetic-like pathophysiology (Martin et al., 2009, 2012). It has been demonstrated that anti-diabetic therapeutics can ameliorate the metabolic dysfunction present in this model (Martin et al., 2012). Deletion of huntingtin-associated protein 1 (HAP1), in pancreatic beta cells has also been shown to result in impaired glucose tolerance by reducing glucose-mediated insulin release in these mice (Kaushik et al., 2011).

In the current study, we recruited both pre-manifest and manifest HD subjects in order to conduct an investigation of the metabolic profile in HD, through the measurement of brain and metabolic hormone levels; lipid profiling and cytokine levels. We recruited familial control groups, to primarily control for lifestyle variables, and Baltimore Longitudinal Study of Aging (BLSA) control groups to effectively control for age, gender composition and BMI influences. We found that growth hormone (GH) and prolactin were significantly decreased in the manifest HD subjects. In the fasted state, HD subjects presented similar blood sugar, insulin, and adiponectin levels compared to all control groups, whereas plasma ghrelin, glucagon, and amylin levels were significantly altered. Total cholesterol (CH) levels as well as the high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C) levels in HD subjects were decreased. Our findings indicate that HD patients possess impaired energy homeostasis and abnormal hormone levels. This state can be detected as distinctive metabolic plasma "signature," even in the early pre-manifest stage of HD. These findings suggest that, in addition to neuronal damage, HD pathogenesis could involve widespread metabolic dysfunction.

MATERIALS AND METHODS

RESEARCH SUBJECTS

Institutional Review Board approval was obtained from the National Institute on Aging, and informed written consent was obtained from all participants. Fifteen pre-manifest and eight manifest HD subjects, as well as 16 control subjects from the patient's families were recruited for this study (Johns Hopkins familial cohort). Due to the difficulty of matching for age, body mass index (BMI), and gender in familial controls, we employed additional control subjects from the BLSA. The BLSA subjects are healthy people without diagnosed metabolic or neuronal diseases. With the BLSA control subjects we were able to avoid confounding factors such as body weight, age, and gender. Thus, BLSA control groups adequately control for age, gender, and BMI, while the Johns Hopkins familial control group control for the socioeconomic status and prevalent stress levels in the HD subject groups, as familial caregivers experience similar stressors/home environment as the HD subjects. Hence the BLSA pre-manifest control group matched pre-manifest subjects and the BLSA manifest control group matched the manifest subjects. Characteristics of all the subject groups are listed in **Table 1**. Blood samples were collected separately during each subject's visit to the hospital. Blood samples of subjects from Johns Hopkins hospital were taken in the morning after an over-night fast. Plasma samples were centrifuged at 3000 rpm at 4°C and were subsequently stored at −80°C until processed. BLSA plasma samples were taken in the morning after an over-night fast and prepared using the same methodology, and as described previously (Driscoll et al., 2012). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as follows: insulin (mU/L) × glucose (mg/dL)/405 (Matthews et al., 1985). BMI was calculated as: body weight (kg)/height (m^2) according to previously established standards (Golden et al., 2010).

PLASMA HORMONE MEASUREMENTS

Insulin, leptin, GIP (Gastric inhibitory peptide), amylin, PYY (Peptide YY), and PP (Pancreatic polypeptide) were assayed using a human gut hormone multiplex kit according to the manufacturer's instructions (Millipore, Billerica, MA). Intra-assay variation was lower than 11%. Adiponectin levels were measured by radioimmunoassay (Millipore, Billerica, MA) and the intra-assay variation for this was from 6.90 to 9.25%. Total plasma ghrelin levels were measured using an ELISA assay kit from Millipore according to the manufacturer's instructions. Glucagon levels were assayed using a Millipore RIA kit. Brain-derived neurotrophic factor (BDNF), GH, Agouti-related protein (AgRP), and prolactin were measured with a Human Brain-Derived/Pituitary Protein Multiplex Panel assay kit according to the manufacturer's instructions (Millipore): intra-assay variation was lower than 10%. Cytokines were assayed using a human cytokine/chemokine assay kit according to the manufacturer's instructions (Millipore): intra-assay variation was lower than 10.5%. C-reactive protein (CRP) was measured using an ALPCO ELISA kit according to the manufacturer's instructions (ALPCO Diagnostics, Salem NH), and the intra-assay variation was 5.5–6.0%.

Table 1 | Subject characteristics.

Subjects	Age	Gender (number of male/female)	Body mass index	Number of CAG repeats
Familial control	48.2 ± 1.9	8/8	26.2 ± 1.4	20.6 ± 0.14
BLSA pre-manifest control	46 ± 2.0	10/5	26.5 ± 1.0	–
BLSA manifest control	57 ± 3.7	5/3	25.4 ± 1.2	–
Pre-manifest subjects	46.8 ± 2.1	10/5	27.4 ± 1.5	42.3 ± 0.09
Manifest subjects	57.6 ± 4.1*	5/3	24.9 ± 1.4	42.5 ± 0.12

*$p \leq 0.01$, Significant difference was observed when compared with the familial control group.

Values are mean ± s.e.m. for age, body mass index, and number of CAG repeats.

GLUCOSE AND LIPID MEASUREMENTS

Plasma glucose levels were measured using a glucose assay kit (Cayman Chemical Company, Ann Arbor, MI). CH, HDL-C, LDL-C, triacylglycerols, and free fatty acid levels were measured using enzymatic assay kits according to the manufacturer's instructions (Wako Pure Chemical Industries, Ltd. Japan).

STATISTICAL ANALYSES

One-Way ANOVA was used for the statistical analysis, and Bonferroni's multiple-comparison test was used for specific comparisons within the five groups using the R software package. Probability (p) values of < 0.05 were considered statistically significant for one-to-one comparisons. We further performed linear regression analysis of hormones levels of individual subjects by age or BMI, respectively. Standard linear regression analyses were performed using GraphPad Prism v5.0. Probability (p) values < 0.05 were considered to have a slope statistically different from zero.

RESULTS

ALTERED GROWTH FACTORS IN HD SUBJECT PLASMA

As HD pathophysiology is tightly linked to neurodegenerative processes, and potentially peripheral metabolic dysfunction, we assessed the circulating levels of multiple factors associated with both of these functions (**Figure 1**). No difference in BDNF levels was observed among all five groups (**Figure 1A**). Compared to the BLSA control group (396.97 ± 84.67 pg/mL), manifest HD subjects showed significantly decreased levels (57.94 ± 14.28 pg/mL) of circulating GH (**Figure 1B**, $p = 0.001$). A similar, non-significant, trend for manifest HD reduced GH (57.94 ± 14. 28 pg/mL), was also observed compared to the familial control group (419.98 ± 169.96 pg/mL, $p = 0.08$). No difference was observed in the pre-manifest group. Compared to the BLSA control group (21.48 ± 1.18 pg/mL), pre-manifest HD subjects showed a significant increase in circulating AgRP (33.28 ± 3.05 pg/mL, $p = 0.007$) (**Figure 1C**), and the same trend was also observed when compared to the familial controls (25.14 ± 1.60 pg/mL, $p = 0.056$). This distinction for AgRP was not repeated in the manifest HD subjects. Between the two HD groups, we also observed that there was a reduction in manifest HD AgRP levels when compared with the pre-manifest HD. With respect to PRL levels we found a consistent effect, i.e., reduced PRL levels, in both pre-manifest and manifest HD subjects when compared to the respective BLSA control groups (**Figure 1D**). However, this effect was not observed when compared with the familial control group.

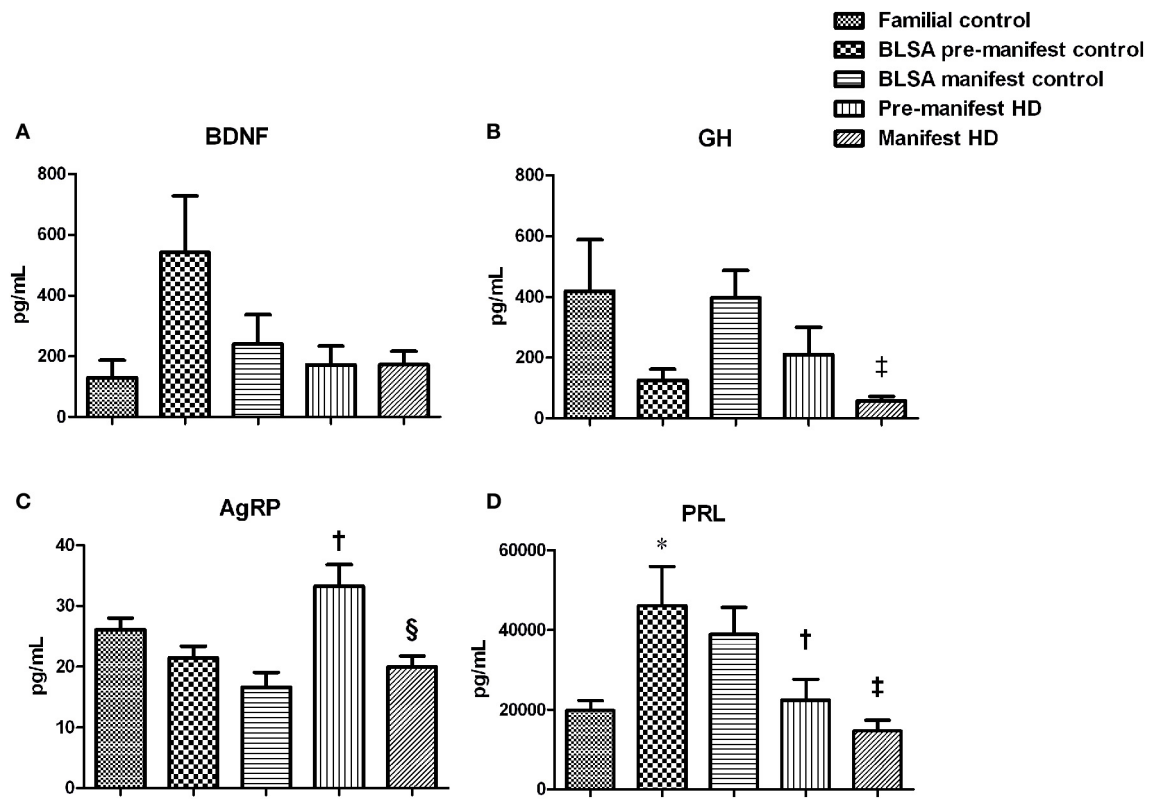

FIGURE 1 | Growth hormone changes in HD subjects. Plasma BDNF, growth hormone (GH), Agouti-related protein (AgRP), and prolactin (PRL) were measured using the Millipore brain-derived protein panel. Data are mean ± s.e.m., $n = 8$–16. Statistical analysis was performed using One-Way ANOVA followed by Bonferroni's multiple-comparison test, $p \leq 0.05$ (*, †, ‡, §) and $p \leq 0.01$ (**, ††, ‡‡, §§) were considered statistically significant. * Represents significant difference against familial control; † represents significant difference against BLSA pre-manifest control; ‡ represents significant difference against BLSA manifest control and § represent significant difference against pre-manifest HD subjects. No change was found in BDNF levels **(A)**. Reduction in GH was observed in manifest HD subjects **(B)**. Elevation of AgRP was found in pre-manifest group **(C)**. Reduction of PRL was observed in both pre- and manifest subjects **(D)**.

METABOLIC HORMONE CHANGES IN HD SUBJECT PLASMA

To further investigate the potential metabolic aspects of HD in pre-manifest and manifest subjects, we measured the circulating concentrations of a series of energy-related hormones including amylin, leptin, ghrelin, adiponectin, glucagon, GIP, insulin, PYY, and PP in fasted-state plasma (**Figure 2**). Amylin levels were found to be significantly elevated in both pre-manifest and manifest HD subjects (**Figure 2A**). Leptin levels were not different in pre-manifest HD subjects, however in the manifest subjects there was a trend for decreased leptin levels (**Figure 2B**, $p = 0.13$ for familial controls and $p = 0.06$ for BLSA controls). Compared with the BLSA control group (298.52 ± 38.26 pg/mL), a significant increase in ghrelin levels in pre-manifest HD subjects (554.26 ± 79.48 pg/mL) was observed (**Figure 2C**, $p = 0.009$). Adiponectin levels were comparable among all five groups (**Figure 2D**). We observed, only in the manifest HD subjects (39.21 ± 4.03 pg/mL) compared to the BLSA control (56.02 ± 5.75 pg/mL), a significant reduction in circulating glucagon levels (**Figure 2E**, $p = 0.045$). GIP, insulin, PYY, and PP levels were not different among all five groups (**Figures 2F–I**). In addition, we assessed fasting glucose levels: as with insulin levels, we found no difference between any groups.

We also performed linear regression analysis using hormones and factors against age and BMI. With respect to HD-associated significant alterations in these regression relationships we found that leptin- and adiponectin-BMI interactions were affected. Leptin levels positively correlated with BMI in all three control groups (**Figures S1A–C**). Whereas in both pre-manifest HD and manifest HD subjects, no such correlation was observed (**Figures S1D,E**). Furthermore, we found in all control groups that the adiponectin levels were negatively correlated with BMI (**Figures S2A–C**), while in pre-manifest HD and manifest HD groups, no correlation was found (**Figures S2D,E**).

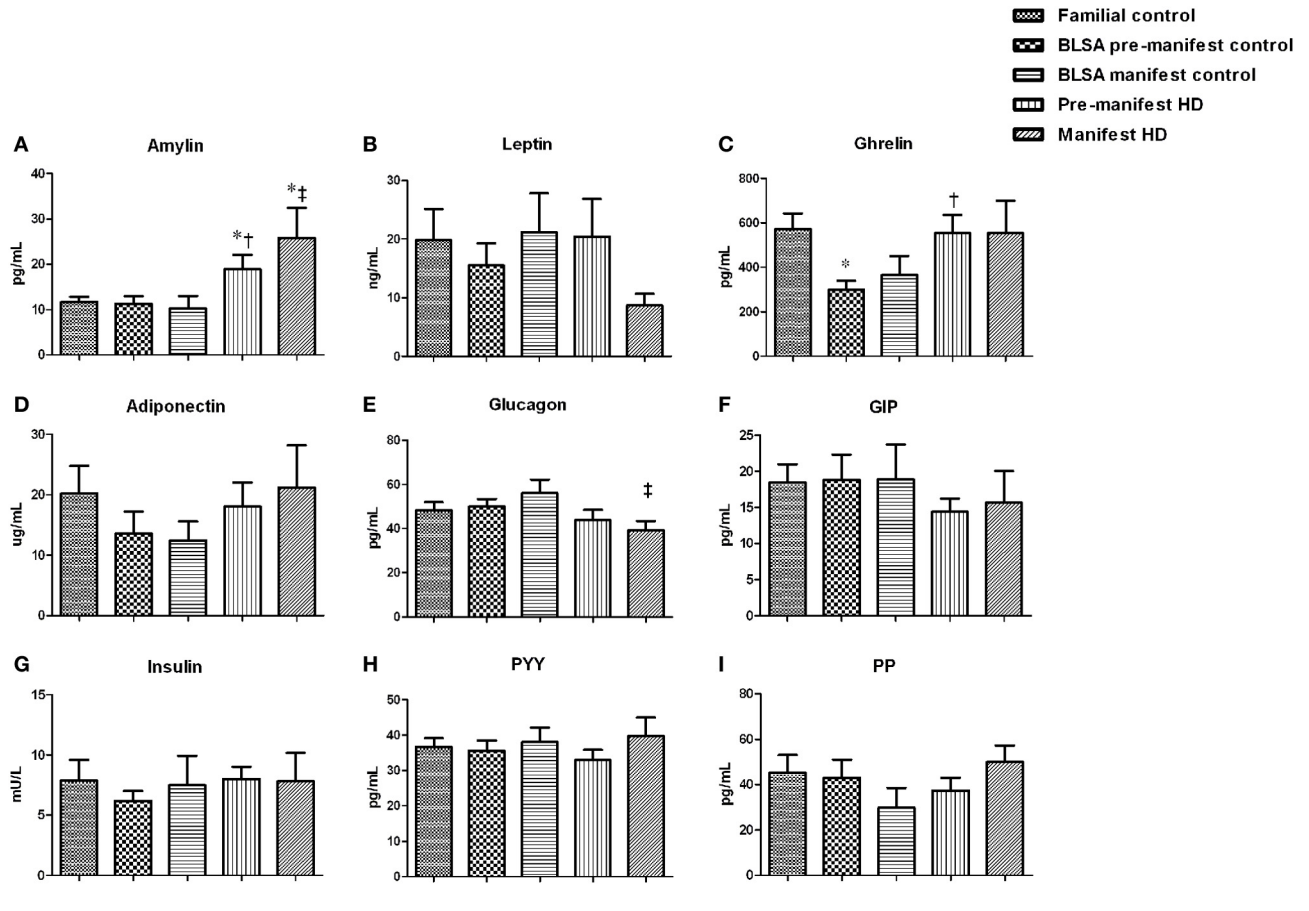

FIGURE 2 | Metabolic hormone levels in HD subjects. Plasma levels of amylin, leptin, ghrelin, adiponectin, glucagon, Gastric inhibitory peptide (GIP), insulin, peptide YY (PYY), and pancreatic polypeptide (PP) were measured. Data are means ± s.e.m., $n = 8$–16. Statistical analysis was performed using One-Way ANOVA followed by Bonferroni's multiple-comparison test, $p \leq 0.05$ (*, †, ‡, §) and $p \leq 0.01$ (**, ††, ‡‡, §§) were considered statistically significant. * Represents significant difference against familial control; † represents significant difference against BLSA pre-manifest control; ‡ represents significant difference against BLSA manifest control and § represent significant difference against pre-manifest HD subjects. Amylin levels increased in both pre- and manifest subjects **(A)**. HD manifest subjects have a trend of reduced leptin levels **(B)**. Both pre- and manifest subjects have increased ghrelin **(C)** with a significant difference in the pre-manifest group. No significant changes were observed for adiponectin **(D)**. Glucagon was significantly reduced in the manifest subjects **(E)**. No significant differences were found in the levels of GIP **(F)**, insulin **(G)**, PYY **(H)**, and PP **(I)**.

PLASMA LIPID PROFILE IN PRE-MANIFEST AND MANIFEST HD SUBJECTS

We next assessed the circulating lipid profiles of HD subjects to investigate how their metabolic status may affect lipid metabolism (**Figure 3**). Both pre-manifest and manifest HD subjects demonstrated significantly decreased CH levels compared to the BLSA control groups (**Figure 3A**). This significance was also seen in the manifest HD group when compared with the familial controls. With respect to the HDL-C (**Figure 3B**), the levels among the HD and familial control groups were comparable. Whereas decreased levels were observed in the pre- and manifest HD groups, compared with their BLSA control groups, LDL-C levels of pre-manifest HD were decreased relative to the BLSA pre-manifest controls. LDL-C levels of manifest HD subjects were less than both familial and BLSA control groups (**Figure 3C**). No differences in the LDL-C/HDL-C ratios were observed for pre-manifest or manifest HD subjects (**Figure 3D**). No differences in triacylglycerols (**Figure 3E**) or free fatty acids levels (**Figure 3F**) were observed.

CIRCULATING INFLAMMATORY FACTORS IN HD SUBJECTS

We have previously demonstrated a strong association between neurodegeneration, metabolic dysfunction, and circulating inflammatory factors (Johnson et al., 2007; Chadwick et al., 2008; Stranahan et al., 2012), therefore we assessed circulating inflammatory mediators including tumor necrosis factor alpha (TNF-α), CRP as well as interleukins 1, 6, and 10 (IL-1, IL-6, IL-10) in pre-manifest and manifest HD subjects. As shown in **Figure 4**, we found no differences in circulating TNF-α between any of the experimental groups (**Figure 4A**). With respect to CRP levels we found a significant elevation in CRP levels in pre-manifest HD subjects compared to the BLSA pre-manifest control group (**Figure 4B**). A similar trend was also observed when compared with the familial controls. However, this elevation was not evident in the manifest HD group. Circulating levels of the interleukins IL-6, IL-1α, IL-1β, and IL-10 were mostly below 2–3 pg/mL, such low and minimally-detectable levels are typical of human subjects that are not presenting excessive immune responses (Yamamura et al., 1998; Licastro et al., 2000).

HD SUBJECT PLASMA SIGNATURE

Based on the profiles we derived of plasma hormones, cytokines and lipids, we have generated a preliminary HD subject plasma signature, i.e., a series of factors the expression of which is indicative of the pre-manifest and manifest states in HD (**Figure 5**). Factors that were uniquely changed

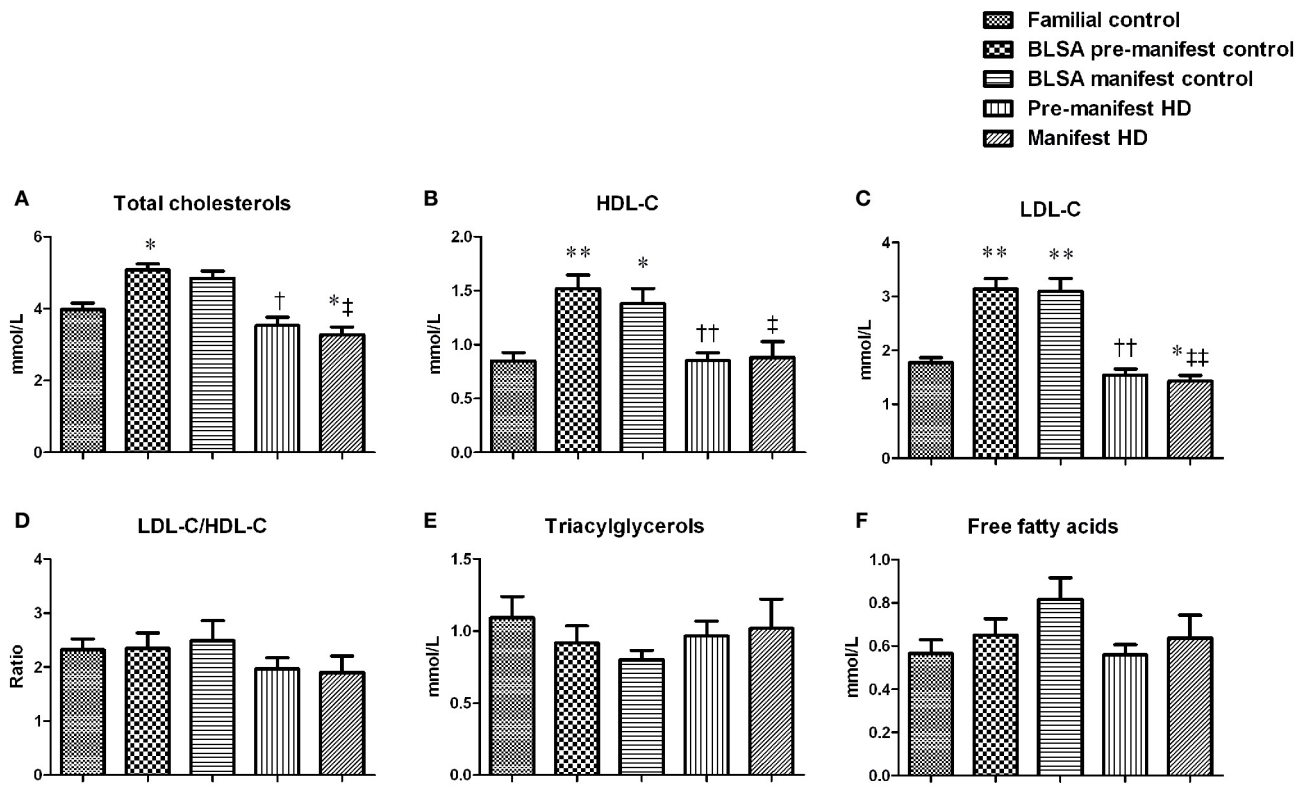

FIGURE 3 | HD subject plasma lipid profiles. Lipids were measured using enzymatic methods. Data are means ± s.e.m., $n = 8$–16. Statistical analysis was performed using One-Way ANOVA followed by Bonferroni's multiple-comparison test, $p \leq 0.05$ (*, †, ‡, §) and $p \leq 0.01$ (**, ††, ‡‡, §§) were considered statistically significant. * Represents significant difference against familial control; † represents significant difference against BLSA pre-manifest control; ‡ represents significant difference against BLSA manifest control and § represent significant difference against pre-manifest HD subjects. Both pre- and manifest HD subjects have reduced cholesterol, HDL and LDL levels **(A–C)** while no difference was found in the ratio of LDL/HDL **(D)**, nor the levels of triacylglycerols **(E)** and free fatty acids **(F)**.

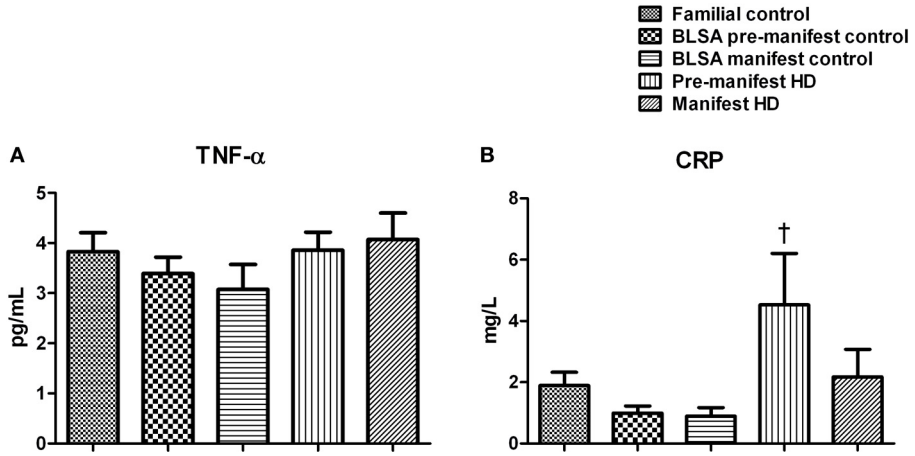

FIGURE 4 | Plasma levels of TNF-α and C-reactive protein in HD. Plasma levels of TNF-α and C-reactive protein (CRP) were measured. Data are means ± s.e.m., $n = 8$–16. Statistical analysis was performed using One-Way ANOVA followed by Bonferroni's multiple-comparison test, $p \leq 0.05$ (*, †, ‡, §) and $p \leq 0.01$ (**, ††, ‡‡, §§) were considered statistically significant. * Represents significant difference against familial control; † represents significant difference against BLSA pre-manifest control; ‡ represents significant difference against BLSA manifest control; and § represent significant difference against pre-manifest HD subjects. No difference was observed in the levels of TNF-α. There was a non-significant trend for increase in TNF-α in the manifest HD group. CRP was increased in the pre-manifest HD subject plasma compared to BLSA pre-manifest controls.

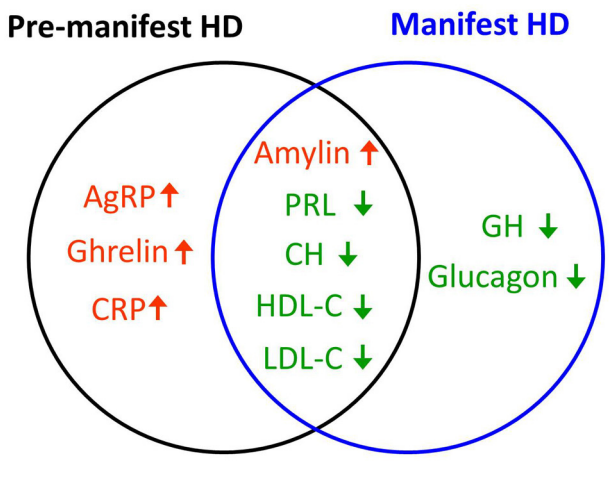

FIGURE 5 | HD plasma signature. Hormones and cytokines that are uniquely up- (red) or down-regulated (green) in pre-manifest (black circle) or manifest subjects (blue circle) as well as those that are commonly up- or down-regulated in both pre- and manifest HD groups are shown in the Venn diagram. Amylin is the only factor that was commonly up-regulated in both pre- and manifest HD group. Prolactin (PRL), total cholesterol (CH), HDL-C, and LDL-C were commonly down-regulated in both HD groups. Factors that were uniquely up-regulated in the pre-manifest HD group were AgRP, ghrelin, and C-reactive protein (CRP). Factors that were uniquely down-regulated in the manifest HD group were growth hormone (GH) and glucagon.

(up-regulated) in pre-manifest group were AgRP, ghrelin, and CRP while in the manifest HD group factors that were uniquely changed (down-regulated) were GH and glucagon. Factors that were commonly changed in both pre- and manifest groups were amylin, prolactin, CH, HDL-C, and LDL-C, among which amylin is the only factor that was elevated. The remaining four were decreased in both pre- and manifest HD groups.

DISCUSSION

In this study we investigated the alterations of circulating metabolic factors in HD subjects. We found that amylin levels were significantly increased in both pre-manifest and manifest HD subjects. GH and Glucagon levels were decreased in the manifest HD subjects compared to BLSA controls. We also found that levels of AgRP, Ghrelin, and CRP were increased in pre-manifest HD, PRL levels were reduced in both pre-manifest and manifest HD cases compared to the BLSA control group. CH as well as HDL-C and LDL-C were decreased in both HD subject groups. Pre-manifest HD subjects presented increased CRP levels. The correlation between leptin levels and BMI was diminished in both pre- and manifest HD groups, and the negative correlation between adiponectin and BMI was diminished in both pre and manifest HD groups.

Amylin is co-secreted with insulin from pancreatic islets in response to food ingestion (Butler et al., 1990; Pittner et al., 1994). Amylin is anti-orexigenic and suppresses gastric motility and glucose uptake (Macdonald, 1997; Gedulin et al., 2006; Potes et al., 2012). It is also known as islet amyloid peptide and is closely related to the beta-amyloid peptide that is associated with the cytotoxic and neurodegenerative aspects of Alzheimer's disease (Moreno-Gonzalez and Soto, 2011; Burke et al., 2013). Elevated fasting amylin levels observed in the current study raised an interesting speculation that in HD subjects (even in the pre-manifest subjects) abnormal control of gastrointestinal function may contribute to the metabolic aspect of the pathology in this disease. Amylin, like other amyloid molecules, often undergoes conformational changes to form ion channel-like structures that may destabilize cellular ionic homeostasis and thus induce cellular degeneration (Quist et al., 2005). Elevated amylin levels

are typically associated with dysglycemia and pancreatic beta-cell dysfunction in Type 2 diabetes (Koda et al., 1992; Lorenzo et al., 1994; Ye et al., 2001). It is therefore interesting, and potentially functionally-relevant, to speculate that the regulation of amylin levels could be a critical molecular connection between diverse forms of neurodegenerative diseases that also possess a metabolic deficit (Martin et al., 2008; Cai et al., 2012). With our identification of amylin as a potential therapeutic target of the peripheral metabolic dysfunction in HD, it is possible that pharmacological manipulation of such a factor could represent an important avenue for future anti-neurodegenerative therapies (Martin et al., 2005; Chapter et al., 2010). With respect to the relationship between HD pathology and somatic metabolism it is interesting to note that both amylin and ghrelin functionally target the gut (Butler et al., 1990). Our results therefore demonstrate an interesting phenomenon of a simultaneous increase of both an appetite stimulator, ghrelin, and an appetite suppressor, amylin. This suggests that HD patients possess multiple abnormalities in the hormone balance for the regulation of food intake and absorption. It has been demonstrated that in HD patients, energy expenditure increases with disease duration, but not with a greater degree of motor or functional impairment (Aziz et al., 2010a). Taken together these findings suggest that HD pathology and metabolic dysfunction may be initiated as a nutrient uptake and energy utilization deficiency, which then result in weight loss and dysglycemia.

Our observation of the relatively normal fasting glucose and insulin levels, as well as normal range HOMA-IR values in our subjects suggests that neither pre- or manifest HD subjects have impaired glucose tolerance or insulin resistance. Previous studies reported that there was no difference in glucose tolerance or insulin released during oral glucose tolerance test (OGTT) in early or middle stage HD patients (Kremer et al., 1989; Boesgaard et al., 2009). However, other studies, including those employing OGTTs, have demonstrated correlations between abnormal glycemic control and HD pathophysiology in patients (Podolsky et al., 1972; Podolsky and Leopold, 1977). These studies together with our findings suggest that the etiology of HD may be associated more with general impairment of hormone release and that glucose regulatory issues caused by this disruption might exist as an indirect effect.

Glucagon levels were decreased in the manifest HD subjects compared with BLSA manifest controls and the same trend was seen when compared with the familial controls. Our finding of reductions in plasma glucagon level is in accordance with previous studies in R6/2 transgenic mice (Hurlbert et al., 1999). In addition to its crucial role in maintaining effective energy metabolism glucagon can also act as a neuroprotective agent by reducing the neurotoxic glutamate (Fanne et al., 2011a,b), therefore reduction of this metabolic regulator may suggest an important role in mediating HD pathogenesis.

Leptin levels in plasma are usually acutely regulated by fasting and refeeding, and are also highly correlated to body fat mass in humans (Maffei et al., 1995; Kolaczynski et al., 1996). In our study we found a trend for reduced leptin levels in manifest HD subjects. As with the connection between HD pathophysiology and glycemic control, the specific relationship between leptin and HD appears highly nuanced. For example, a recent study has demonstrated that while circulating levels of leptin are similar between control and HD patients there is an increase in the rate of leptin secretion of HD patients (Aziz et al., 2010b). Another study has indicated that leptin levels can be doubled in pre-manifest HD patients compared to control, albeit in a non-significant manner (Goodman and Barker, 2011). Indicative of the complexity of energy balance networks, significant reductions in circulating leptin levels has also been reported (Popovic et al., 2004). In accordance with previous reports, our linear regression test showed that in both pre- and manifest HD groups the correlation between leptin and BMI was lost, also suggesting abnormal leptin secretion in the HD subjects. It should be noted that with respect to the disruption of leptin-secretion in HD, the small sample size and mixed gender may contribute to the degree of variability observed. With improvement in sample size and gender difference it is likely that some clinical consensus can be achieved to the important hormonal systems.

Adiponectin is secreted from adipose tissue and reported to be negatively correlated with body fat mass (Gavrila et al., 2003). We found that the adiponectin levels in all the three control groups showed a significant negative correlation with BMI. However, this correlation was attenuated in both pre- and manifest HD subjects. Adiponectin mediates insulin sensitization in adipose tissue via support of insulin signaling and kinase pathways (Ballantyne et al., 2005). Reductions in adiponectin levels have also been associated with obesity and insulin resistance (Kadowaki et al., 2006). In fact, in two HD animal models, reductions in adiponectin levels occur before body weight loss, suggesting that disruptions in adipocytokine secretion may be intrinsic to HD pathology (Phan et al., 2009). Together with our findings, the consistent changes of leptin and adiponectin in HD, suggests abnormal adipocyte function is linked to the metabolic alterations in HD pathophysiology.

In addition to the multiple altered appetite-associated hormones, we found that both GH and prolactin levels were decreased in manifest HD subjects. Not only is the signaling activity of prolactin associated with nervous system protective behavior (Torner et al., 2009), prolactin is also linked with diabetic pathology, metabolic syndrome and inflammatory conditions (Balbach et al., 2013; Chirico et al., 2013). Impaired prolactin responses have been reported in the HD patients (Hayden et al., 1977) as a result of abnormal dopaminergic activity in hypothalamus. Our results suggest that changes of basal levels of prolactin are detectable early in pre-manifest HD subjects. GH actions often synergize with those of prolactin as they are both situated in the hypothalamic-pituitary system that links both central and peripheral control of energy metabolism and cellular growth. In the periphery both prolactin and GH can interact to affect energy metabolism (Schäffler et al., 2005) and immune/inflammatory functions (Redelman et al., 2008). Reduced GH and prolactin levels suggest that the HD patients may possess a dysfunctional hypothalamic-pituitary system (Phelps, 1994; Phelps and Hurley, 1999), which potentially affects food intake and energy balance, leading to further weight loss and energy insufficiency (Gerardo-Gettens et al., 1989; Auffret et al., 2012).

We observed significantly decreased levels of cholesterol, HDL-C and LDL-C in both pre- and manifest HD subjects. For manifest HD subjects, the same reductions in CH and LDL-C were seen when compared with both familial and BLSA controls, in accordance with previous reports (Markianos et al., 2008). In addition to impaired cholesterol synthesis in HD patients (Valenza et al., 2005), cholesterol changes may also be the result of dysfunction related to food digestion and absorption, which correlates with our observation of elevated amylin levels. Cholesterol is important for adult brain neuron membrane formation, myelination and synaptic plasticity (Mauch et al., 2001; Hering et al., 2003; Quan et al., 2003). Studies have also demonstrated that circulating cholesterol concentration is related to dementia and emotion changes (Evans et al., 2000; Dietschy and Turley, 2004). Impaired cholesterol metabolism was also reported in HD patients recently (Leoni et al., 2011). In their study reduced plasma level of 24S-hydroxycholesterol (24OHC), the brain specific elimination product of cholesterol, and reduced levels of the cholesterol precursors lanosterol and lathosterol were observed, suggesting a critical role of cholesterol in HD pathology (Leoni et al., 2011).

We found that CRP was significantly elevated in the pre-manifest HD patients (**Figure 4B**), as has also been reported by Stoy et al. (2005). A CRP level of this nature is indicative of the inflammation of coronary vessels and is related to atherosclerotic process (Lagrand et al., 1999). It is also interesting to note that in a murine HD model the presence of a progressive cardiac dysfunction associated with impaired myocardial contractility, reduced left ventricular pressure and a significantly reduced coronary blood flow has been demonstrated (Wood et al., 2012). Therefore, it is possible that elevated CRP levels in HD could be strongly associated with potential cardiac and cardiovascular dysfunction, which may further exacerbate the disorder. As we have shown that amylin is elevated in HD patients, this may also cause cardiovascular issues as amylin is linked to the generation of stenosing deposits or plaques in the thoracic aorta (Westermark and Westermark, 2011). It is therefore likely that these two factors, amylin and CRP, could interact to disrupt both metabolic and cardiovascular function in HD. Therefore, pharmacotherapeutic targeting of both or either of these circulating hormone systems in patients could present an important new avenue for remedial research.

In conclusion, our study indicates that abnormal hormone levels in HD patients may contribute to the progression of energy imbalance. The hormones altered in HD affect not only food intake and absorption, potentially causing weight loss and malnutrition, but also impair interactions among metabolic systems, thereby inducing more global energy deficits that, ultimately, could adversely impact CNS function. Our study on both pre- and manifest HD subjects suggests that metabolic dysfunction occurs before the onset of diagnosable HD symptomology. It is possible that along with its deleterious actions on central nervous tissue, aggregated mutant HTT protein may also have direct effects on peripheral tissues such as the intestine, pancreas and adipose tissue. We have previously demonstrated in animal models of HD that therapeutic targeting of these peripheral energy-regulating organs can be beneficial for HD pathologies (Martin et al., 2012). Therefore, our current study further supports the idea that a combined therapeutic strategy, targeting both central and peripheral systems, may prove effective at ameliorating this devastating genetic disorder.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fphys.2014.00231/abstract

Figure S1 | Correlation between leptin levels and the body mass index (BMI). Linear regression test was performed using leptin levels against the BMI. $p \leq 0.05$ was considered statistically significant with respect to the linear correlations' deviation from a zero slope. r^2 correlation was calculated by GraphPad Prism v5.0. In familial, BLSA pre-manifest, and manifest control groups, leptin levels were positively correlated with the BMI **(A–C)**; in pre-manifest HD and manifest HD groups no correlation was observed **(D,E)**.

Figure S2 | Correlation between adiponectin levels and the body mass index (BMI). Linear regression test was performed using adiponectin levels against the BMI. $p \leq 0.05$ was considered statistically significant with respect to the linear correlations' deviation from a zero slope. r^2 correlation was calculated by GraphPad Prism v5.0. In familial, BLSA pre-manifest, and manifest control groups, adiponectin levels were negatively correlated with the BMI **(A–C)**; whereas in pre-manifest HD and manifest HD groups no such correlation was observed **(D,E)**.

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