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Physiological importance and control of non-shivering facultative thermogenesis

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1. ABSTRACT

This review examines general and evolutionary aspects of temperature homeostasis, focusing on mammalian facultative or adaptive thermogenesis and its control by the sympathetic nervous system and hormones. Thyroid hormone acquired a new role with the advent of homeothermy enhancing facultative thermogenesis by interacting synergistically with the sympathetic nervous system, and directly increasing basal metabolic rate (obligatory thermogenesis). Facultative thermogenesis is triggered by cold. The major site of facultative thermogenesis in mammals is brown adipose tissue, endowed with abundant mitochondria rich in a protein called uncoupling protein-1. This protein can uncouple phosphorylation in a controlled manner, releasing the energy of the proton-motive force as heat. Its synthesis and function are regulated synergistically by the sympathetic nervous system and thyroid hormone and modulated by other hormones directly, or indirectly, modulating sympathetic activity as well as thyroid hormone secretion and activation in brown adipose tissue. Alternate, evolutionary older forms of facultative thermogenesis activated in transgenic mice with disabled brown adipose tissue thermogenesis reveal this latter as the culmination of energy-efficient facultative thermogenesis.

2. INTRODUCTION

Mammals and birds, among vertebrates, maintain their body temperature constant in spite of wide variations of ambient temperature, reflecting a tight and effective homeostatic control. From the temperature control standpoint, these two classes of vertebrates are indistinctly classified as endothermic or homeothermic, although these terms are not identical. Endothermy means the capacity of these species to generate sufficient endogenous heat to maintain a higher core body temperature, with *some* independence from ambient temperature, whereas homeothermy indicates a higher level of complexity, namely the capacity of *regulating* body temperature around a set value (1). While endothermy is necessary to facilitate the maintenance of a constant temperature, particularly in environments colder than the set point of control, it is not sufficient to maintain a constant core body temperature, and indeed not all endothermic species are homeothermic (2).

3. EVOLUTIONARY ASPECTS

There is an ongoing debate as to how endothermy developed during evolution (1). Studies in poikilothermic species show for example that speed and

endurance of motor activity improves as temperature approaches 37°C (3;4). Other authors believe endothermy resulted from a selection pressure to enhance reproductive function and extend the protection of the offspring (5); and yet others think endothermy allowed the production of additional energy to larger animals allowing them to displace faster (6). Indeed, these selection pressures are not mutually exclusive and all may have contributed to select mechanisms to generate more heat. When compared with exothermic species, endothermic species have increased aerobic capacity, increased number of mitochondria and larger mitochondrial membrane surface area (7). Since a large fraction of metabolic energy is spent maintaining ionic gradient across membranes, essential to cell functioning, some investigators have theorized on the bases of comparisons between poikilothermic and homeothermic species that there has been a progressive desaturation of the fatty acids in the cell membranes that has made them more leaky to ions, increasing the energy cost of maintaining such gradients and consequently the production of more heat (8;9). The factor or factors leading to membrane fatty acids desaturation and leakiness to ions remain a mystery.

Homeothermy, or the capacity of *maintaining* the body temperature constant in a broad range of ambient temperatures, has doubtless been a major evolutionary gain. Environmental temperature varies widely with seasons and latitude on the surface of the earth; therefore, the absence of mechanisms to keep body temperature constant constrains the niche of animal species. Obviously, homeothermy has expanded the niche of species (10); but why body temperature in all homeothermic species is regulated between approximately 37°C (mammals) and 42°C (birds), is yet another fundamental question without an answer. Several cellular events as well as whole body functions, such as neuro-muscular performance studied in poikilotherms at different temperatures are faster, more effective or efficient as the environment in which they have been studied approaches 37°C (4). Nonetheless, even though we can only hypothesize on the reasons for endothermy and then homeothermy, both have been leaps forward in evolution that have resulted, among other advantages, in an expansion of the niche of species; in faster and enduring physical activity; and in facilitating reproduction improving the survival of progenies.

The acquisition of endothermy, though, posed a new evolutionary challenge. Heat production is energy costly and consequently increased the energy cost of living substantially. On the other hand, throughout the history of homeothermic species, food has been scarce and hard to obtain. It has been only in recent millennia that food has become abundant and consistently available, and only to humans and domesticated animal species (11), while endothermy and homeothermy have evolved over millions of years. Thus, homeothermy imposed the selection pressure of maintaining body temperature at the lowest possible energy cost. These opposing evolutionary pressures, the limited food availability and the energy demand of homeothermy have lead to mechanisms resulting in thriftiness and fuel efficiency of our metabolism and physical activity. Such mechanisms explain many of the observations discussed in this review, as well as our incapacity to deal with the excess food. This, and the technological advances reducing the need of physical activity in our daily life are the core of the pandemic of obesity and diabetes [(10;12) and references therein].

It is reasonable to assume that endothermy created the pressure to evolve temperature homeostatic mechanisms to produce heat in the most efficient manner, only when necessary while minimizing heat dissipation in cold environments. Interestingly, homeothermic species are not as efficient in reducing heat production and we defend our body temperature largely by increasing heat dissipation, although recent observations discussed later show that we may reduce to some extent basal metabolic rate and the resulting heat production (*obligatory thermogenesis)* via a reduction in thyroid hormone secretion and increased inactivation (13-15).

Note that of the two opposing forces mentioned above, food availability is a dominant one. Thus, in starvation or food restriction, brown adipose tissue (BAT) *facultative thermogenesis* in response to cold is reduced or blunted, whereas it is increased if food is readily available (16). The interdependence of food availability and temperature homeostasis at the lowest energy cost is further supported by behaviors, such birds migrating to warmer latitudes during the winter, or by the need of several mammals to hibernate i.e. to reach a state of torpor during which they reduce to a minimum the energy cost of living and stay alive when food in the cold of winter becomes scarce; it they did not hibernate, the need to spend energy in thermogenesis would markedly increased in face of the reduced food availability of winter (2).

4. THE MAJOR MECHANISMS OF TEMPERATURE HOMEOSTASIS

Temperature homeostasis requires mechanism to save and dissipate heat as well as to produce heat on demand. Since most of the earth surface, except around the equator is substantially colder than the core temperature of 37°C, the capacity to produce enough heat is essential. In homeothermic species, increased heat production results to a great extent from a higher basal metabolic rate (BMR). A higher BMR reflects more energy transformations and, as a mere consequence of the laws of thermodynamics, more heat production. By definition, BMR represents the minimal cost of living because is measured at rest, in a post-fed state and at an ambient temperature called *thermoneutrality temperature* because the body is in thermal equilibrium with the environment, eliminating the energy cost of keeping core body temperature. Such ambient temperature is called zone of thermoneutrality or simply *thermoneutrality temperature* (10;17). If ambient temperature departs from the *thermoneutrality temperature*, homeostatic mechanisms are activated to either dissipate or save heat. When looked from the viewpoint of temperature homeostasis, the heat generated by BMR is called *obligatory thermogenesis* to indicate that is the minimal amount of heat produced as result of being alive. It is

evident that the higher is *obligatory thermogenesis* the lower will be *thermoneutrality temperature*, as schematically illustrated in Figure 2.

Chronic heat-saving-mechanisms include thermal insulation in the form of fat (exemplified by fat in mammals such as whales) or fur. Acutely, thermal insulation is also increased by cutaneous vasoconstriction, deviating blood from the surface in contact with the cold, and by piloerection in furred species to reduce cold air circulation close to the skin. Another way to reduce heat loss to the environment is curling the body, approaching the shape of a sphere, geometrically being the form with the lowest surface area-to-volume ratio. Yet another behavioral adaptive response is immobility because by remaining on the same site or micro niche the animal does not have to spend heat warming another site (17). Obviously, in colder environments, these mechanisms are limited and limiting, and additional heat generation is certainly needed.

The capacity to produce heat on demand is called f*acultative* or *adaptive thermogenesis*. *Facultative thermogenesis* is activated as homeothermic species move to environments below *thermoneutrality temperature* and ceases as soon the environment warms to return to the thermo neutral temperature. This is important, because the persistence of *facultative thermogenesis* over *thermoneutrality temperature* will over burden the heat-dissipating mechanisms resulting in hyperthermia. Severe hyperthyroidism and malignant hyperthermia are conditions of non-regulated thermogenesis where the capacity of dissipate heat is saturated, resulting, particularly in the latter condition, in hyperthermia that could be lethal (18). To have *facultative thermogenesis* rather than higher *obligatory thermogenesis*, by definition virtually fixed, represents also an adaptive advantage. Recent evidence suggests that thyroxine (T_4), the major product of thyroidal secretion and 3,5,3'-triodothyronine (T_3) are inactivated by being converted respectively into the inactive compounds, reverse T_3 (rT_3) and 3,3'-diiodothyronine. The increased inactivation of thyroid hormone is accomplished by the activation of the 5-iodothyronine deiodinase or type-3 deiodinase. In addition, in hot environments there is a mild decline in thyroid stimulation by thyrotropin (TSH) (13-15). These are relatively recent observations that indicate that there mechanisms to reduce *obligatory thermogenesis*, although not as effective as thermogenic mechanisms activated in response to cold. Thus, body temperature is tightly regulated. The interplay of these temperature homeostatic responses is illustrated in Fig.2, and discussed in the ensuing text.

5. COLD ADAPTATION

Acute exposure to cold triggers immediate responses with the dual purpose of minimizing heat loss and producing heat. There is vasoconstriction and furred mammals undergo piloerection. Heat production is initiated instantly by shivering, the immediate form of *facultative thermogenesis* (17). Muscle contraction increases heat production. However, facultative *shivering* thermogenesis is energy expensive (19) and disrupts activity, and it is hence of limited value and rapidly replaced by *non-shivering facultative thermogenesis* (20). For convenience, I will call *shivering facultative thermogenesis* simply "*shivering*", while in the rest of the text "*facultative thermogenesis*" will be specifically used for non-shivering facultative thermogenesis and will be italicized.

Most of our knowledge to adaptation to cold derives from studies in small rodents. In these species, as well as most altricial newborn mammals, *facultative thermogenesis* resides in yet another evolutionary homeostatic advancement, the brown adipose tissue (BAT). This tissue can produce large amounts of heat when fully activated, in the range of 400 W x kg^{-1}, or 80 times the basal metabolic rate of a rat (21;22). The key molecule in BAT *facultative thermogenesis* is uncoupling protein (UCP), now called uncoupling protein 1 (UCP1) due to the cloning in the late 90's of paralogs and orthologs (23). UCP1 is a *sine qua non* for BAT thermogenesis. The observation that paralogs, such as UCP2 and 3 can uncouple ADP phosphorylation from mitochondrial respiration, dissipating the energy as heat when over-expressed in yeast or other mitochondria (24-27), led to the idea that they could play the same role as BAT UCP1. However, even though at high density in mitochondria UCP2 and UCP3 can act as UCP1, collapsing the proton gradient across the internal mitochondrial membrane, at the densities normally found per mitochondria, they do not seem to act as UCP1, and probably play other roles in the cells, such as facilitating fat oxidation and reducing the formation of reactive oxygen species [(28-30) for recent reviews]. In contrast, UCP1 is present in high concentrations in the inner membrane of abundant BAT mitochondria and constitutes about 5% of total mitochondrial protein in rodents living at 20-22°C (20;30). UCPs exist even in plants, suggesting that controlled uncoupling of ADP phosphorylation from respiration may be an ancient thermogenic mechanism, but BAT is unique to mammals. As mentioned and further discussed latter, BAT represents the culmination of a thermogenic site, as it not only has the capacity to produce impressive amounts of heat, but it is strategically placed around blood vessels and close to vital organs as kidney and spinal cord (20). The activation of BAT starts almost instantly upon acute cold exposure and increased transcription of the UCP1 gene can be detected within minutes (31;32), so that within short time BAT thermogenesis starts to replace shivering and within 6-8 hours becomes unnecessary and virtually disappears (17).

In birds, *facultative thermogenesis* seems to take place largely in skeletal muscle where Ca^{2+} exchange between cytosol and sarcoplasmic reticulum appears to be the major mechanism (33;34). Recently, however, uncoupling proteins have been identified in muscles from birds (28;35), but its role in thermogenesis remains to be resolved. Furthermore, mesenchymal stem cells can be differentiated into brown adipocyte-like cells but their UCP diverged from the mammal gene and does not seem sufficiently active and abundant to participate in thermogenesis. Instead, a possible function in birds is the elimination reactive oxygen species (ROS) that are

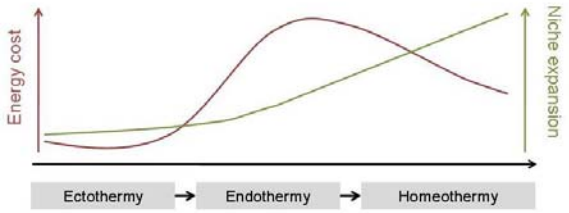


Figure 1. Schematic conception of the evolution of endothermy and homeothermy. In ectothermy or poikilothermy, body temperature and metabolic rate depend on ambient temperature. The cost of living is minimal even at ambient temperatures around 37°C. Endothermy, in response to several hypothetical evolutionary pressures and by as yet undefined mechanisms resulted in an increased metabolic rate, enhanced capacity to produce heat, with the benefit of an expansion of the niche of species into colder environments. With the advent of homeothermy, body temperature could be regulated. *Obligatory and facultative thermogenesis* (heat production) is sufficient to maintain body temperature in colder or warmer environments, but thermogenesis is subordinated to food availability. Heat production is increased above the heat produced by basal metabolic rate (*obligatory thermogenesis)* only when necessary (*facultative thermogenesis*), and heat-saving and heat-dissipating mechanisms appear including vasoconstriction, piloerection, behavioral responses and torpor, to allow life continuation in the cold at minimal energy cost. Mammals living constantly in cold areas develop more effective thermal insulation (thick fat subcutaneous layer, hairy fur, oily and more compact feathers, etc.), as result of which the energy cost of energy homeostasis is reduced, yet without constraining the size of the ecological niche.

generated at a much higher rate than in mammals (28;35;36), much as UCP2 in mammalian cells (37).

Although I will refer to bird temperature homeostasis for comparison, the focus of the subsequent discussion will be largely on mammals, on the interrelations between *obligatory thermogenesis* and *facultative thermogenesis*, and the role of the sympathetic nervous system and hormones on modulating and coordinating both forms of thermogenesis.

Figure 2 integrates schematically the concepts outlined so far. It represents the activation of various adaptive mechanisms as the environmental temperature changes. The rectangular box extending over the *x* axis represents *obligatory thermogenesis,* and is represented as not influenced by ambient temperature, to stress the concept that it largely represents the heat resulting from the energy sustaining the cost of living. As mentioned below, it is possible that in the individual animal, particularly in response to hot environments, there could adaptive reductions in thyroid hormone concentrations and activity that may reduce *obligatory thermogenesis*. If our energy expenditure were as low as that of ectotherms, the *thermoneutrality temperature* should be close to the set temperature of 37°C. If we were to live at the so-called room temperature, 20-22°C, which is the temperature at which we tend to live and keep most experimental animals in the laboratory, such low *obligatory thermogenesis* would be insufficient to maintain the core of the body at 37°C because the gradient between the body and the ambient would be too big. The problem will be worse in small animals, because they have a larger surface area-to-volume ratio. Thus, homeothermy set at 37-42°C requires more heat production. This has been accomplished during evolution by increasing BMR and hence *obligatory thermogenesis*. This is represented in Fig.2 by taller *obligatory thermogenesis* boxes. The figure illustrates that the higher the heat resulting from vital functions, i.e. *obligatory thermogenesis,* the lower *thermoneutrality temperature* can be. This is clinically evident in hyperthyroidism, where the excess of thyroid hormone increases BMR and with it *obligatory thermogenesis*. Accordingly, in hyperthyroidism *facultative thermogenesis* is triggered at lower ambient temperatures, that is, *thermoneutrality temperature* is reduced. The opposite occurs in hypothyroidism, which is translated clinically as cold intolerance and in animals by seeking higher ambient temperatures when placed a closed corridor with a gradient of temperature. Figure 2 shows that the *thermoneutrality temperature* is lower the higher is *obligatory thermogenesis.* As mentioned, and not illustrated in Fig.2 is the size of the animal, as advanced above. Thus, both BMR and body size are major determinants of *thermoneutrality temperature.*

Still, the environment will frequently be colder than *thermoneutrality temperature*, in winter and in latitudes distal from the equator. To maintain core body temperature, heat saving mechanisms, as those previously mentioned, vasoconstriction, piloerection, behavioral changes, etc. will be activated; and so will be heat production, first in the form of *shivering* but then gradually turning into non-shivering facultative thermogenesis (*facultative thermogenesis*, as defined). In most mammals, including humans, a major, if not the major, source of *facultative thermogenesis* is BAT.

5.1. Hormonal control of obligatory thermogenesis and basal metabolic rate

It is evident from Figure 2 that *thermoneutrality temperature* and the need of *facultative thermogenesis* are strongly influenced by *obligatory thermogenesis*. From the preceding discussion, it is evident that homeothermy requires mechanisms to generate heat, i.e. a robust *obligatory thermogenesis* and *facultative thermogenesis*. From a thermodynamically standpoint, homeothermy has forced the "biological machine" into being thermodynamically less efficient for the sake of keeping core temperature between 37 and 42°C. Essentially, this has been accomplished by increasing the number of energy transactions in homeothermic species, to some extent in a futile manner, and reducing the efficiency of generating ATP (10;38;39). In addition, the energy stored in ATP, which is transferred to molecules to elevate their energy level and accelerate biochemical reactions, may not be as efficient thermodynamically as previously thought (40). Thus, Meis postulates that the efficiency of ATP coupling

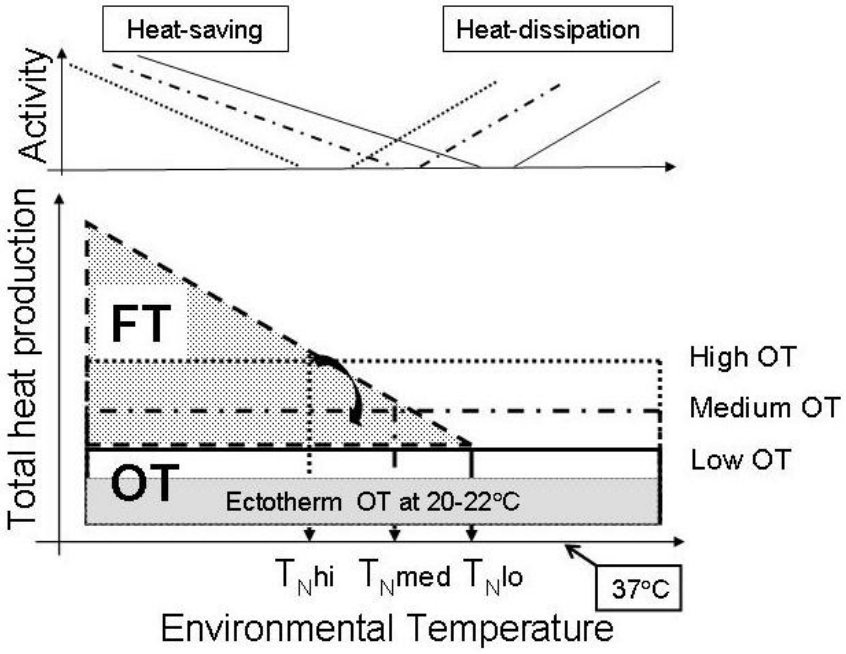


Figure 2. Schematic representation of interrelationships between the heat generation from vital functions (defined as basal metabolic rate and equated to *obligatory thermogenesis*), *facultative thermogenesis*, and *thermoneutrality temperature*. This later temperature represents the ambient temperature at which the animal is in thermal equilibrium with the environment, i.e. the heat from *obligatory thermogenesis* is sufficient to keep core body temperature at the set point of 37-42°C without the activation of heat saving or heat dissipating temperature and without *facultative thermogenesis*. No considered in this analysis is the size and geometric form of the animal. These are determinants of the body surface area-to-volume ratio that affects the loss of heat to the environment. Bigger animals generate more heat as the metabolic body mass is bigger and have a lower relative body surface area. This makes that metabolic rate is not linear with body mass but elevated to the 0.75 power (17). The higher is *obligatory thermogenesis* and the size of the animal, the lower is *thermoneutrality temperature*. To demonstrate the effect of *obligatory thermogenesis* on *thermoneutrality* temperature, in this Figure the *thermoneutrality temperatures* corresponding to a high, medium and low *obligatory thermogenesis* have been have been dubbed $T_N$hi, $T_N$med and$T_N$lo. The heat production of ectotherms has been arbitrarily represented as low to indicate they would need to live at a temperature very close to 37°C if they were to maintain their core body temperature at 37°C. Without extra thermogenesis they would not be able to live at the so-called room temperature, 20-22°C, in which we live and keep our laboratory and some domestic animals (pets). The arrow down to the right of $T_N$hi is to indicate that recent evidence (see text) suggests that in acclimation to warm environments *obligatory thermogenesis* is reduced, which is mediated by a reduction in thyroid hormone secretion and activation of thyroid hormone, as well as increased inactivation.

to pumping of cytosolic Ca^{2+} back into the sarcoplasmic reticulum in skeletal muscle could vary in a controlled manner and even that ATP could be split by the sarcoplasmic reticulum calcium ATPase (SERCA) totally uncoupled from the Ca^{2+} pumping by for sake of producing heat (41).

Since the pioneer work of Magnus-Levy it is known that a major controller of BMR is thyroid hormone (42). Thyroid hormone is present in all vertebrates, but only in homeotherms it increases oxidations and heat (43;43-45). The thermogenic effect of thyroid hormone is a newly acquired function in evolution. Accordingly, the lack of thyroid hormone is associated in homeothermic species with marked cold intolerance and quasi-poikilothermic status, whereas the administration of thyroid hormone to hypothyroid animals or humans recapitulates many of the differences between poikilothermic and homeothermic species [(10) and references therein]. In addition, thyroid hormone plays an important role in sustaining and stimulating *facultative thermogenesis*. How thyroid hormone has been acquiring these new functions is an evolutionary mystery. As discussed below, a whole body of evidence supports a central role for thyroid hormone in thermogenesis. For more than a century clinicians and lay people have recognized the cold intolerance of hypothyroidism and heat intolerance of hyperthyroidism. In supporting *facultative thermogenesis*, thyroid hormone acts coordinately with the sympathetic nervous system, so many aspects of this system's function are regulated or influenced by thyroid hormone, while the sympathetic nervous system in turn selectively activates thyroid hormone in BAT to provide enough amounts of the most active form of thyroid hormone, T_3, in quantities sufficient to virtually saturates BAT thyroid hormone receptors, which seems necessary

for the full realization of the BAT thermogenic potential (10;12;46-48).

5.2. BAT thermogenesis

Extensive reviews have been written on the physiology and biochemistry of BAT thermogenesis [see (30;49-53) among others]. The key molecule is UCP1, a protein located in the inner membrane of BAT mitochondria. In cold-stimulated small rodents UCP1 may constitute 5% or more of mitochondrial protein to the point of being readily identified as a 32,000 Dalton band in straight Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) of rat mitochondrial proteins (54;55).

Cells make ATP, the "energy currency of the cells", from capturing the energy released in a stepwise manner from the transfer of reducing equivalents to oxygen through the complexes of the mitochondrial respiratory chain. At each step, protons are expelled into the intermembrane space of the mitochondria, creating a proton gradient called the proton-motive force, as this gradient, a form of potential energy, is used by ATP synthase to phosphorylate ADP into ATP (40). Thus, the proton gradient created by such mechanism represents a transient accumulation of energy then used by the ATP synthase to produce ATP. UCP1 is believed to be a carrier protein that allows protons to rapidly flow from the intermembrane mitochondrial space into the mitochondrial matrix, dissipating the gradient created by the respiratory chain activity. By collapsing the proton gradient, UCP1 releases the potential energy of the gradient as heat (movement of protons in the direction of the gradient is an exergonic reaction) instead of being available to the ATP synthase i.e. it uncouples phosphorylation of ADP from mitochondrial respiration. The molecular mechanism whereby UCP1 reduces or collapses the proton gradient has not been defined. It is known that the collapsing of the gradient is intimately related to the release of fatty acids, but how these allow UCP1 to dissipate the gradient has not been resolved (52;56;57). I believe it is safe to say that UCP1 facilitates the passage of protons in the direction of the gradient in the presence of fatty acids, but whether are protonized fatty acids that carry the protons in the direction of the gradient, or fatty acids embedded into UCP1 that transport the protons across the "tunnel" formed by UCP1 is still matter of debate. Nucleotides, on the other hand block the dissipation of the proton gradient. They bind to UCP1 and somehow block the passage of protons. Among them, guanosyl diphosphate, GDP, has turned out useful to measure UCP1 as well as a tool to manipulate the uncoupling mechanism (58). In the resting state, BAT mitochondria produce comparatively little ATP, because of relatively low levels of ATP synthase, and consequently the proton flux into the matrix when UCP1 is not "activated" is low and in resting BAT there is a substantial proton gradient across the inner mitochondrial membrane (58). When BAT is stimulated, lipolysis occurs and the resulting fatty acids activate UCP1, and the protons move in the direction of the gradient, generating heat and accelerating respiration. Mitochondrial oxidations increase but without generating additional ATP. Because of the large size and number of BAT mitochondria and the abundance of UCP1, the potential to produce heat is as high as 400 $W \times kg^{-1}$ (21;22).

5.2.1. Role of adrenergic stimulation on BAT thermogenesis.

The uncoupling mediated by UCP1 in BAT mitochondria is regulated. Fatty acids cause a large flow of proton in the direction of the gradient, whereas nucleotides stop proton transport (56;58). The starter of BAT thermogenesis is the sympathetic nervous system. Regulatory centers are in the hypothalamus and brain stem (20;59). Afferent signals come largely from the skin temperature sensors. Skin becomes cold not only from the loss of heat to the environment, but very importantly from a rapid reflex causing vasoconstriction. This is the result of a reflex arc where afferent signals from skin temperature sensors reach the hypothalamic-diencephalic area that generates efferent signals that will cause vasoconstriction. Such rapid response will not only reduce the dissipation of heat but will further reduce skin temperature, amplifying afferent signals to thermoregulatory centers. The mounting efferent responses will cause now shivering and activation of BAT via sympathetic fibers. The neurotransmitter released by adrenergic end terminals on BAT is norepinephrine (NE) that acts on beta- and alpha-adrenergic receptors (α-AR, β-AR) and will start the thermogenic response. The role of each adrenergic receptor has not been clearly defined. It is believed that the initial response is mediated by β-AR, a class of Gs-protein coupled receptors. NE will release the alpha subunit of the G_s protein that will activate adenylyl cyclase to generate cAMP and the cascade of events leading, among other responses, to rapid lipolysis. In animals leaving below *thermoneutrality temperature*, which is the most current situation, there is enough UCP1 present in the mitochondria to be immediately activated by fatty acids, so heat production starts immediately. NE released by adrenergic terminals, will also cause vasodilatation, probably via β_2-AR. In addition, cAMP also activates the type-2 5'iodothyronine deiodinase (D2) interacting synergistically with α1-AR. The cAMP and T_3 will rapidly induce the transcription of the UCP1 gene, which is indeed detectable within minutes using intronic probes or run-on transcription assays (32;60).

All three β-AR are present in BAT, although some of them may be in the vascular stroma of the tissue, rather in brown adipocytes. It is possible that β1-AR and β2-AR are more important in the blood flow and initial response such as the activation of lipolysis (61). A third class of β-AR has been discovered later, the β3-AR (62). β3-AR is selectively expressed in white (WAT) and BAT, and parts of the gastrointestinal tract. Its exact role has not yet been clearly defined. This receptor has a lower affinity for NE, suggesting that requires higher degrees of adrenergic stimulation to be activated (62). The targeted disruption of this receptor is associated with increased adiposity, more in females than males, loss of the cAMP response of adipocytes membranes to the selective β3-AR agonist CL-316,243, but the reduction of the responses to NE and isoproterenol *in vivo* is small, which may be

explained by compensatory overexpression of β1-AR (63). The value of this compensation is supported by the deletion of *all* β-AR, which is associated with a higher susceptibility to cold and diet-induced obesity (63;64). On the other hand, the increased metabolism and leanness caused by CL-316,243 in intact rats supports a role for β3-AR in mediating BAT stimulation and WAT lipolysis (63).

5.2.2. Role of thyroid hormone on BAT thermogenesis

Since the stimulatory effect of thyroid hormone on thermogenesis was known long before the thermogenic function of BAT was discovered, it is not surprising that researchers promptly asked what the role of thyroid hormone in BAT thermogenesis could be. Mory *et al* observed that BAT of hypothyroid rats showed signs of adrenergic hyperstimulation, yet UCP1 levels and activity were not enhanced (65). Triandifillou *et al* (66) also observed that hypothyroid rats had a subnormal response of UCP1 to cold compared to euthyroid controls (as measured by GDP binding) while the response was readily normalized by thyroxine replacement. What it was intriguing in these studies, though, was that rats treated with *supraphysiological* doses of thyroxine did not have more, but less, GDP binding. In another study, Seydoux *et al* (67) showed that the thermogenic response of BAT the sympathetic nerve stimulation was reduced in hypothyroid rats. Lastly, Sundin and Cannon (68) showed that high doses of T_4 reduced in a dose-dependent manner the GDP binding increase with cold. These results led to the concept that thyroid hormone played just a permissive role in BAT function, being necessary for the normal response to the tissue to sympathetic stimulation, with inhibition at supraphysiological levels (66;69).

Thyroxine (T_4) is the major secretory product of the thyroid gland. T_4 has intrinsically very low hormonal activity, whereas 3,5,3'-triiodothyronine (T_3) is at least ten times more activity because it has that much higher affinity for the thyroid hormone receptors than T_4, while its concentration around the receptors is similar or even greater than that of T_4. The concentration of T_3 in the thyroglobulin as well as in the thyroidal secretion is low. In humans, not more than 20% of the extrathyroidal T_3 is directly secreted by the thyroid gland, but in rodents, the proportion of circulating T_3 that is secreted may approach 50%. The extrathyroidally-produced T_3 is produced by the enzyme-catalyzed removal of the 5'iodine from T_4 (5'deiodinadtion). In the early 80's only one iodothyronine-5'deiodinase was known. It was readily detected with high concentrations of the substrate T_4 in liver and kidney homogenates and was typically inhibited by the thiourylene propylthiouracil (PTU), a drug long used to treat hyperthyroidism because of its capacity of inhibiting thyroid hormone synthesis in the thyroid gland. Our laboratory characterized a new iodothyronine 5'deiodinase activity that we called Type-2 iodothyronine 5'-deiodinase (70;71), abbreviated D2, so that the previously known 5'deiodinase became the Type-1 iodothyronine 5'deiodinase and abbreviated nowadays D1(72). The first evidence of D2 existence came from the observation in the late 70s that TSH was inhibited by locally generated T_3 in the pituitary by a mechanism that was PTU-resistant. We then showed locally generated T_3 in cerebral cortex and cerebellum, also insensitive to PTU inhibition. D2, in contrast to D1, is stimulated by hypothyroidism and powerfully inhibited by its substrate T_4. The increased brain D2 activity protected the neonatal rat brain of hypothyroid rats from developing brain hypothyroidism with levels of serum T_4 <50% of that of euthyroid controls (73-75). It did not take long to find D2 in BAT, where we demonstrated it was markedly activated by NE and cold *in vivo,* via α1-AR receptors (76;77).

This constituted a major finding, a synergism between the sympathetic nervous system and thyroid hormone. As mentioned, it was early in the history of BAT that T_4 was found to be necessary for the thermogenic function of BAT (65-67), yet hyperthyroxinemia was associated with depression of GDP binding, i.e. with less UCP1. As discussed below, the nature and cellular localization of D2 is probably the explanation of these and several other findings.

We suspected from the beginning that D1 and D2 had a different cellular location in experiments injecting rats with ^{125}-I-T_4 and ^{131}-I-T_3 in which we found that in tissues containing solely D1, typically liver and kidney, the ratio of ^{125}-I-T_3 (derived from the injected ^{125}-I-T_4, T_3[T_4]) to ^{131}-I-T_3 (T_3[T_3]) was one (74;78;79), even though liver and kidney D1 produced over 60% of circulating T_3 made out of the thyroid and, as mentioned, contain large amounts of D1(80). In contrast, pituitary and brain, containing D2, such experiments show a large excess of T_3[T_4] over T_3 [T_3] specifically bound to nuclear thyroid hormone receptors. Thus, D2 generates T_3 in a cell compartment that does not exchange freely with plasma, while D2-generated T_3 remained in the cell and its passage to the nucleus was apparently facilitated, largely for local consumption. After the cloning of these enzymes, immunostaining showed indeed that D1 is located in the cell membrane, whereas D2 is located in the endoplasmic reticulum, in close relation to the nuclear envelope [see (81) for review and additional references]. The discovery that BAT D2 was stimulated by the sympathetic nervous system (77) by hypothyroidism; that its activity was rapidly reduced by T_4 (82;83); and that the increased activity in hypothyroidism could protect the cerebral cortex and cerebellum of significant drops in plasma T_4 (75;84;85), we set forth to test the hypothesis that D2 played a major role in regulating T_3 concentration in BAT, largely independently from plasma T_3.

We found that in hypothyroid rats the increased D2 in BAT protected hypothyroid rats from cold induced hypothermia. Injecting these rats with tiny doses of T_4 for just 48 hours while kept at 4°C resulted in protection of hypothermia and normalization of UCP1 concentration, *without* improving the thyroid status of the rest of the body or increasing significantly circulating T_3 levels (55). In contrast, T_3 given in replacement doses for 5 days did normalize the liver mitochondrial glycerol-3-phosphate dehydrogenase but did not improve BAT UCP1 levels (55). To normalize UCP with injected T_3 required doses nearly 100 times the physiological replacement doses and caused not surprisingly peripheral thyrotoxicosis (55;86). Testing increasing doses of T_3 to hypothyroid rats and measuring

UCP1 response to cold, we found that to achieve the euthyroid rats UCP1 response to cold we needed to fully occupy BAT nuclear thyroid hormone receptors, and obviously doses of T_3 needed to cause the maximal response caused peripheral thyrotoxicosis (86). This suggested that for a full response of UCP1 and protection from hypothermia to maximal endogenous adrenergic stimulation (48 hours at 4°C) thyroid hormone receptors should be nearly fully occupied, and that this would be accomplished by the adrenergic activation of D2 without causing systemic hyperthyroidism. So, we exposed euthyroid rats to cold and measured the contribution of D2-produced T_3 vis-à-vis plasma T_3 and found that within 4 hours of cold exposure, BAT thyroid hormone receptors were virtually saturated with T_3, and further, that most of the T_3 was being generated locally by the sympathetic nervous system-activated D2 (T3[T4]) while the contribution of plasma T_3 (T_3[T_3]) decreased with time (87). These results showing the importance of D2 for elevating locally T_3 in BAT have been later confirmed in D2 knockout mice, which are cold intolerant and have blunted responses to endogenous or exogenous adrenergic stimulation (88).

We later demonstrated that thyroid hormone synergistically stimulated transcription of the UCP gene with cAMP. Under adrenergic stimulation, such as in rats expose to cold, the stimulation of UCP1 by thyroid hormone was evident within minutes (32;60;89). To quantify the synergism between adrenergic and thyroid hormone stimulation of the UCP1 gene, we studied hypothyroid rats with unilateral denervation of BAT (each side of interscapular BAT receives separate innervation so the intact side served as control). The experiment was done at room temperature (20-22°C) that is associated with substantial sympathetic stimulation of the intact side, since this temperature is 8-10°C below *thermoneutrality temperature.* Accordingly, the injection of thyroid hormone significantly stimulated UCP1 gene (*Ucp1*) transcription and UCP1 mRNA accumulation on the intact side, but the stimulation in the denervated side by either NE or T_3 was minimal, whereas when given together the stimulation was 18-20 times, not different from the innervated side (60).

The role of BAT D2 is also evident in hyperthyroidism as advanced previously. Triandafillou *et al* (66) found that rats given T_4 in excess of the daily production rate was associated with a reduction of GDP binding and Sundin *et al.* reported that there was a T_4 dose-dependent reduction in GDP binding to progressive cold exposure, with abolition of the response at frankly thyrotoxic doses (68). This indicates that BAT D2 plays a gate-keeper role in this tissue, enhancing the fractional conversion T_4-to-T_3 at euthyroid, and particularly at reduced, levels of T_4, whereas its sensitivity to T_4 inhibition prevents the flooding of the tissue with T_3 in hyperthyroxinemic states. This will discussed further below, after considering other influences on D2.

5.3 Hormonal regulation of the UCP1 gene expression

A number of laboratories including ours have characterized the regulatory elements of the rat *Ucp1*. Both Ricquier's and Kozak's labs defined a 211-bp upstream enhancer, in rat and mouse respectively, about 2.25kb upstream of the gene transcription start site (90;91). This enhancer encompasses sequences conferring tissue-specificity and the major cAMP response element (CRE), responsible for the mediation of NE stimulation of the gene. We also studied this sequence in the rat gene and defined a thyroid hormone response sequence characterized by two thyroid hormone response elements (TRE) in tandem. These sequences were located about 2,300 base pairs upstream in the 5'flanking region of the rat gene (92). This whole 211 bp segment also contains sequences involved in the regulation of the gene by other hormones. We and others defined a retinoic acid response elementin the rat and the mouse *Ucp1* (93;94. Interestingly, Kozak's group described 4 possible CRE, none as strong as the one found in the 211 bp upstream enhancer (91;95) when studied with adrenergic agonists. We identified a 40 bp sequence downstream of the TREs that was responsible for activating the most downstream CRE (92;96), quite weak in the absence of T_3,and in addition found a complex sequence of about 90 bp in the upstream part of the 211 enhancer containing a sequence that could mediate the stimulation of *Ucp1* by thiazolidinediones, a PPARγ-response element [see (96) and references therein].

5.4. Other effects of thyroid hormone on responsiveness to sympathetic stimulation

Thus, there is no question that thyroid hormone plays an essential role on the expression of *Ucp1* in rat BAT. But thyroid hormone is in addition needed for other responses of BAT essential to its thermogenic function. The adrenergic activation of D2 is necessary for the responses to cold of other enzymes (86). Sundin *et al.* showed that isolated brown adipocytes of hypothyroid rats had reduced lipolytic and oxygen consumption responses to isoproterenol or forskolin, whereas cells from hyperthyroid rats showed the opposite response. Furthermore, the oxygen consumption response to fatty acids was also reduced indicating defects downstream of the cAMP generation (97). Many of these findings were not surprising as thyroid hormone was already known to be necessary for normal adrenergic responses and signaling in other tissues [(47;48) and references therein], largely through effects of hyper- and hypothyroidism on heart and white adipose tissue [reviewed in (47;48)]. However, except for white adipose tissue, by the early 1980's most of the effect of thyroid hormone on adrenergic responsiveness was attributed to its increasing the expression of β-AR (98), a concept still found in current textbooks. This proved to be incorrect. Even though thyroid hormone can stimulate the expression of certain adrenergic receptors, particularly β1-AR and β2-AR, the effect is limited and demonstrable in extreme hypo- and hyperthyroidism, as discussed further below. In addition to the findings of Sundin *et al.* (97) supporting more distal site of action, Seydoux *et al.* had earlier reported (67) that BAT responses to sympathetic nerve stimulation in hypothyroid rats were reduced to a substantially greater extent than the comparatively small reduction in β-AR number.

We undertook the study of study of thyroid hormone on NE signaling in rat BAT, using freshly isolated

brown adipocytes and cell membranes of hypothyroid and thyroid hormone-treated hypothyroid animals. As others, we did find a reduction in β_1-AR and β_2-AR, but our evidence suggested that this was rather desensitization due to the chronic sympathetic hyperstimulation of the tissue in response to the reduction in *obligatory thermogenesis* characteristic of the hypothyroid state. Indeed, just housing hypothyroid rats at *thermoneutrality temperature* resulted in an increase in β_1-AR and β_2-AR, suggesting that the reduction in the number of these receptors in BAT was caused by desensitization due to the chronic compensatory adrenergic stimulation of BAT stimulation in the hypothyroid state and not the hypothyroidism *per se* (61). Moreover, we found that *β_3-ARs were upregulated in hypothyroidism* and that the administration of thyroid hormone to hypothyroid rats promptly reduced β_3-AR mRNA levels (99). Therefore, the effects of thyroid hormone on adrenergic receptors have been overrated, and such effects are by no means uniform across adrenergic receptors subtypes and tissues. In contrast, as in white adipose tissue, we found in BAT that the quantitatively most important synergism between thyroid hormone and the sympathetic nervous system occurred downstream of the receptors, as one would have anticipated from the observations by Sundin *et al.* and Seydoux *et al.* (67;97) mentioned above. The synergistic effect of thyroid hormone on *Ucp1* transcription is indeed not limited by the reduced β_1-AR and β_2-AR in BAT as the restoration of the response of *Ucp1* to adrenergic stimulation is evident promptly after giving thyroid hormone, long before the number of these receptors is normalized (61). Lastly, like Sundin *et al.* (97), and others later (100), we also found reduced cAMP generation in response to β-AR agonists and forskolin in freshly isolated brown adipocytes, but to our surprise, when adenylyl cyclase activity was investigated in brown adipocyte membranes from hypothyroid rats, in standard buffer, the enzyme was equal or more active than in membranes from euthyroid brown adipocytes (101). This suggested that in the milieu of the whole cell the adenylyl cyclase activation was reduced but not when assayed in membranes incubated in standard buffer, further suggesting that the adenylyl cyclase expressed in hypothyroid BAT was different from that expressed in euthyroid rats. Indeed, the analysis showed that hypothyroid BAT membrane adenylyl cyclase responded to Ca^{2+} and nucleotides as the type-VI isoform in the membranes of hypothyroid brown adipocytes (101). We therefore proposed that the reduced response in intact cells was due to the intracellular milieu in hypothyroid brown adipocytes, containing higher Ca^{2+} and nucleotide concentrations (101). The higher cytosolic calcium concentration probably resulted from the increase in α1-AR receptors in hypothyroid brown adipocytes (102;103), along with the increased sympathetic tone.

As mentioned earlier, BAT D2 is stimulated by α1-AR agonists *in vivo* (77). However, *in vitro* studies reveal that the primary control is by cAMP, while α1-AR agonists alone have little effect but potentiate the effect of cAMP in hypothyroid brown adipocytes. Interestingly, this synergism is more pronounced in brown adipocytes from hypothyroid rats and is rapidly reduced by the administration of thyroid hormone to the rats before isolating the cells (102). Indeed, following the cloning of D2, a distinct and functional cAMP-response element was identified in 5'untranslated region of *Dio2* of rodents and humans (104). How then explain the powerful stimulation of D2 of α1-AR agonists in euthyroid rats *in vivo* (77;83)? This is probably because BAT of euthyroid rats (77) is under significant sympathetic tone at the usual laboratory temperature (20-22°C), which is 8-10°C below the *thermoneutrality temperature* (17); thus, the ambient concentration of cAMP in the brown adipocytes is significant and there is no need to further stimulate cAMP production to see the effect of α1-AR agonists.. In agreement with this idea, isoproterenol, a pure β-AR agonist, is less powerful than NE in stimulating D2 activity in euthyroid rats (82;83).

6. ROLE OF D2 AS MASTER SWITCH FOR BAT THERMOGENESIS IN RESPONSE TO ENERGY AVAILABILITY, OVERALL TEMPERATURE HOMEOSTASIS AND OTHER PHYSIOLOGICAL NEEDS

The above studies and others compiled in a recent review (46) demonstrate complex interactions between thyroid hormone and the sympathetic nervous system with BAT being no exception. In rats, D2 plays a key role in regulating the expression *Ucp1* and UCP1 activity, in addition to the activity of other enzymes, indicating that the regulation of the local T_3 concentration is critical for the full expression of the thermogenic potential of BAT (55;86), but D2 is also involved in curbing BAT thermogenesis in states of hyperthyroxinemia (66;68), indicating this enzyme activity is regulated negatively by the excess of thyroid hormone. Fig.2 shows that the higher *obligatory thermogenesis* is, the lower is *thermoneutrality temperature* and hence the ambient temperature at which *facultative thermogenesis* and heat-saving mechanisms are activated. Thyroid hormone increases oxygen consumption in many tissues, but notably in those that contribute more to *obligatory thermogenesis*, such as heart, liver, kidney and skeletal muscle (105). It makes physiological sense that in hyperthyroidism and thyrotoxicosis BAT thermogenesis and its responses to sympathetic stimulation be reduced. The importance of D2 is strongly supported by the cold intolerance and poor response to sympathetic stimulation of brown adipocytes of D2-knockout mice (88) as well as for the inhibition caused by the increased *obligatory thermogenesis* of thyrotoxicosis. In addition to the sympathetic nervous system and thyroid hormone itself, D2 activity is physiologically *regulated, modulated* or at least significantly *affected* by other hormones. This complex regulation makes of D2 the master switch for BAT thermogenesis, adjusting it to the physiological conditions, most notably the level of *obligatory thermogenesis* and food availability, will be examined in more detail in forthcoming paragraphs.

As mentioned, however necessary is T_3 for a full BAT thermogenic response, the *excess* of thyroid hormone in hyperthyroidism or thyrotoxicosis is associated with

reduced responses of BAT to adrenergic stimulation, and D2 also plays in this biphasic response an important role. Thus, D2 activity is sensitive to inhibition by excess its physiological substrate, T_4. One μg/100 of T_4 per 100 g injected to rats accelerates the disappearance of D2 activity by a factor of 3-4. This is not unique to BAT D2 but to brain D2 and probably other tissues as well. The effect is maximal within six hours of giving T_4 to hypothyroid rats as an intravenous bolus with an ED_{50} was 0.2 μg/100 g (83), which is about 20-25% the daily production rate of T_4 in this species. Such reaction limits the amounts of the more active T_3 formed in response to surges of T_4 or sustained hyperthyroxinemia. For tissues like brain, particularly during development, and BAT, the excess of T_3 is as harmful as it is the deficit (106).

The inhibition of D2 by T_4 is posttranslational, and indeed, it can be accomplished by other substrates of the enzyme like reverse-T_3 and iopanoic acid, an iodinated contrast medium of aromatic structure used for cholecystography. The binding of the substrate to the enzyme makes it more susceptible to ubiquitination and more rapidly cleared from cell by proteasomes (107). Therefore, in hyperthyroxinemia and for the matter in hyperthyroidism, D2 activity is reduced. In addition, there is evidence that thyroid hormone inhibits sympathetic output from the hypothalamus to several tissues, including BAT [see (47) for review and references]. These effects explain the observations by Sundin *et al* (68) and Triandafillou *et al* (66) that the injection of supraphysiological doses of T_4 is associated decreased levels of UCP1 at room temperature and reduced responses to cold.

The adaptive value of reducing *facultative thermogenesis* when *obligatory thermogenesis* is increased protects the animal from hyperthermia. It is conceivable that the hyperthermia of the thyrotoxic storm, which is frequently triggered by a stressful situation, results from a de-repression of the sympathetic nervous system. Recall that peripherally thyroid hormone increases the peripheral responsiveness to adrenergic stimulation, so a small increase in the availability of epinephrine or norepinephrine will be amplified substantially in tissues exposed to an excess thyroid hormone. In hypothyroidism, we see the opposite situation: both central sympathetic output and D2 activity are increased [(48) and references therein] maintaining BAT thyroid hormone receptor T_3 occupancy in spite of reductions of circulating T_4 down to 60% of normal (108).

D2 is also highly stimulated by insulin. One unit of insulin given intraperitoneally to euthyroid rats increases BAT D2 activity 8-10 fold in about 2 hours (82). The stimulation by insulin is fast and marked, and this hormone is probably one of the signals in the stimulation of BAT D2 and thermogenesis following a meal (109). Furthermore, insulin and carbohydrates are necessary for a maximal D2 response to adrenergic stimulation, whereas diabetes and fasting nearly abolish the response to cold or exogenous NE or isoproterenol, and these effects are reversed by the administration of carbohydrates or of insulin to streptozotocin-induced diabetes in rats (82). Fasting induces a rapid drop in UCP1 which is reversible upon refeeding (110;111). These observations indicates that BAT thermogenesis is subordinated to the availability of energy and is consistent with the observation that central sympathetic stimulation is reduced by starvation and increased by food, specifically carbohydrates (16;112). The stimulation by food of D2 is probably relevant because UCP1 or GDP binding move in parallel to D2 in fasting and overfeeding.

Hypophysectomized rats have higher levels of D2 than hypothyroid rats, suggesting that there could be a pituitary hormone inhibiting the enzyme. Of all hormones tested, growth hormone had an inhibitory effect. This inhibition, however, required repeated administration of the growth hormone, whereas D2 activity increase following the induction of hypothyroidism underwent an additional increase when the body growth of young rats ceased. Since in rats growth hormone synthesis and secretion strictly requires the presence of thyroid hormone and growth hormone levels rapidly respond to changes in the thyroid status (113;114), this body of data indicates that the inhibitory effect of growth hormone on D2 is not direct, but mediated by IGF-I (83).

This complex regulation of D2 by signals that alter *obligatory thermogenesis,* food and energy needs of the body, along with the biphasic effect of thyroid hormone on UCP1 expression in BAT make of D2 an important switch to coordinate BAT *facultative thermogenesis* with the overall needs of the body. It appears as though evolutionary pressures subordinated BAT thermogenesis to energy availability, growth and other functions such as lactation, when UCP and BAT activity are also reduced by mechanisms to be defined (115;116). Figure 3 summarizes the control of BAT *facultative thermogenesis* by the sympathetic nervous system, thyroid and other hormones, highlighting the role of D2 as a distal switch in the system to ultimately modulate the responses to sympathetic stimulation, nutritional needs and status by locally controlling the BAT availability of T_3.

It is very important to be cautious and keep in mind that most of the observations and studies presented have been done in rodents, and predominantly rats. Even though BAT function and responses to cold and food availability are virtually the same, the regulation and the relative importance of the signals and their underlying mechanisms may somewhat differ among species. For example, in the mouse, the regulation of *Ucp1* by the sympathetic nervous system and thyroid hormone seems quantitatively and perhaps qualitatively different. The mouse *Ucp1* transcription and mRNA levels appear less dependent on thyroid hormone than the rat counterpart. Thus, mUCP1 mRNA levels are *2-3 fold higher in hypothyroid than in euthyroid mice*, as we have observed in CD1 and C57Bl mice (unpublished observations; see Fig.4.), whereas in hypothyroid rats UCP1 mRNA level is lower than in euthyroid rats, although not as much as the protein (60;89). Moreover, the injection of T_4, T_3 or thyroid hormone beta-selective agonists such as GC1

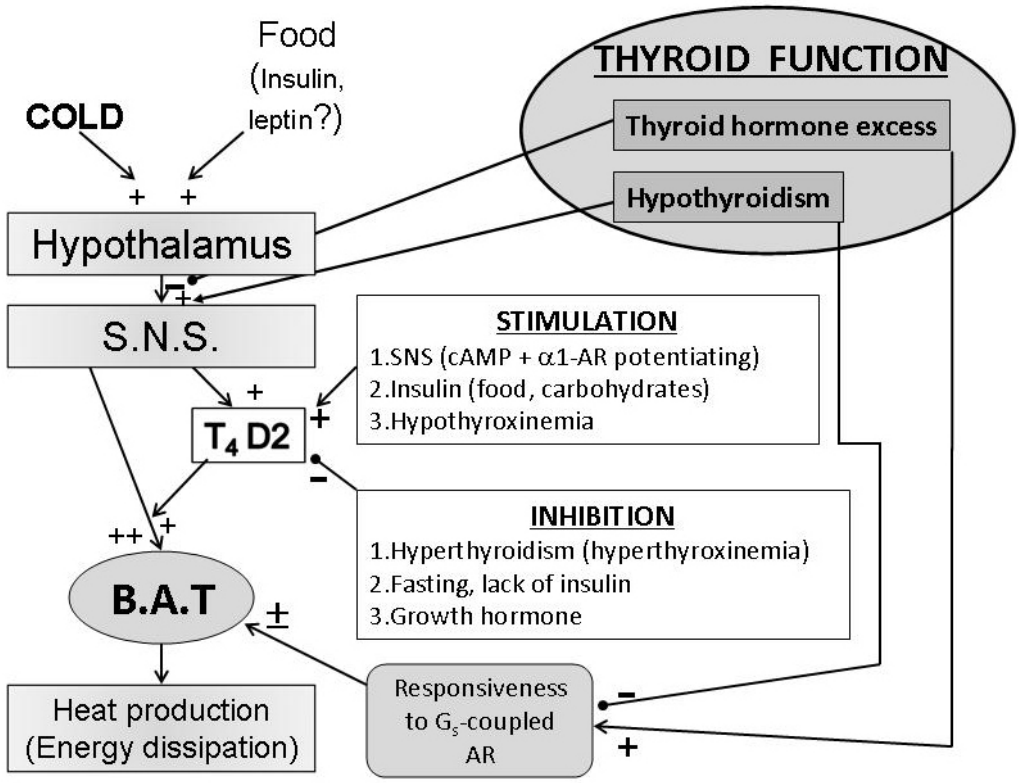
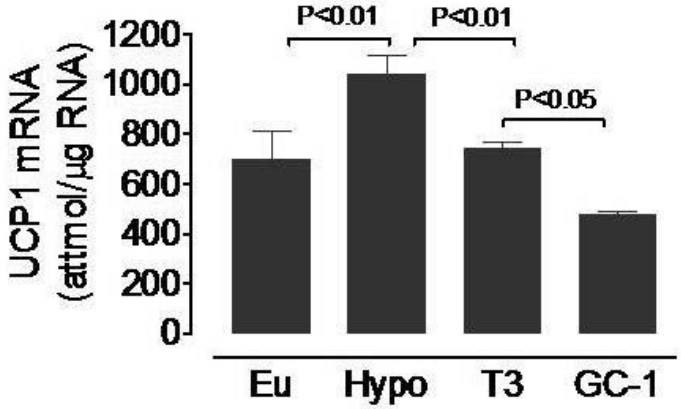


Figure 3. Schematic representation of the relationships between the various signals modifying BAT *facultative thermogenesis*. The left side of the figure shows the stimulation of BAT by the sympathetic nervous system, the primary activating signal. As a site of *facultative thermogenesis*, BAT activity is turned on by cold. The afferent signals travel from the skin, which senses ambient temperature, to the hypothalamus. The signal is modulated by other signals, importantly those that indicate food availability, such as insulin and leptin, and probably glycemia itself. The integration of these signals will determine the intensity of the sympathetic signal emerging from the hypothalamus to BAT, as described in the text. As shown by the extensive bibliography, the availability of T_3 is important for BAT to realize all its thermogenic potential. The relevance of this has been proven experimentally and in mice lacking D2 (55;86;88). Note the complicated relation with thyroid function. In hypothyroidism, probably as a result of cold stress and reduced *obligatory thermogenesis,* sympathetic stimulation is increased, but the lack of substrate, T_4, will result in reduced T_3 and lower responsiveness of BAT to the stimulation, both at the signaling pathway, particularly that of Gs-protein coupled β-AR, and at the level of distal responses such as the control of *Ucp1* transcription and lipolysis. The opposite occurs in hyperthyroidism, and here D2 plays an important role being so powerfully inhibited by T_4 and probably by a reduction in BAT sympathetic stimulation. D2 provides another, distal level for regulation by signals like insulin and carbohydrate availability. The specifics of all these regulatory signals is not yet fully understood, nor has the physiologically relevance of all been defined. Diabetes and starvation are negative signals that could override the stimulation of cold. Lastly, as mentioned in the text, most of this knowledge emanates from studies done in rats, and there is evidence of interspecies differences.

(Figure 4) *reduces* the levels of UCP1 mRNA in hypothyroid mice. This indicates that adrenergic-induced cAMP is sufficient to increase UCP1 mRNA in mice, while clearly its effect is minimal in hypothyroid rats, where there is 5-fold amplification of the effect of cAMP on *Ucp1* transcription or UCP1 mRNA levels by T_3 (60). However, Ribeiro *et al* (100) report reduced UCP1 (protein) in hypothyroid C57Bl mice which is corrected by T_3 but not by thyroid hormone receptor beta-selective analog GC1. Furthermore, as mentioned before, D2 knockout mice are cold intolerant and their brown adipocytes behave as those of hypothyroid mice and the deficiencies are all corrected by the presence of T_3 in a dose dependent manner (88). Altogether, these results suggest that in both species, rats and mice, thyroid hormone is necessary for the full thermogenic effect of BAT but the mechanisms are different. As shown in Figure 4, the differences in the *Ucp1* regulation may be caused by variations in the thyroid hormone response element (TRE) sequence. While rats have two thyroid hormone-response elements (TREs) in tandem, mice have only the downstream TRE. Besides, the rat *Ucp1* 211 upstream enhancer contains a sequence that mediates the synergism between cAMP and T_3, which seemingly activates an otherwise weak downstream cAMP-response element, and this cis-acting effect seems to need the concurrence of both TREs (92;96;117). These observations suggest the NE is the predominant stimulation of *Ucp1* in mice and the inhibitory effect of T_3 or GC1 is



RAT CGCGCCTCACCCCTACTGAGGCAAACTTTCTCCCACTTCTCAGAGGCTCTGAGGGCAGCAAGGTCAGCCCTTTC

MOUSE ACTGCTCTCTCCATTATGAGGCAAACTTTCTTTCACTTC-CAGAGGCTCTGGGGGCAGCAAGGTCAACCCTTTC

Figure 4. Differences between mouse and rat of the relative importance of thyroid hormone for the expression of UCP1. The upper panel shows mRNA in mouse interscapular BAT. Mouse BAT hypothyroidism is associated with an increase in UCP1 mRNA, whereas in the rat is reduced ((32;60;89) [rat data have been omitted for simplicity]). In an apparent paradox, the administration of T_3 or the thyroid hormone receptor-beta-selective analog, GC1, are associated with normalization or reduction of UCP1 mRNA. The experiment shown was done in CD1 mice, but we have observed the same, repeatedly in C57Bl mice. The lower panel shows the sequence of the upstream enhancer of UCP1 of both species containing the thyroid response sequences. In the rat we identified 2 TREs (92;117), whereas only the lower one is present in the mouse.

indirect, derived probably from the reduction in sympathetic tone resulting from the T_3 or GC1 stimulation of *obligatory thermogenesis* and/or a direct action in the hypothalamus. It is evident that further comparative studies are necessary to understand the regulation of the amount and activity of UCP1 in BAT by the sympathetic nervous system and thyroid hormone in different species.

7. INTERRELATION BETWEEN OBLIGATORY AND FACULTATIVE THERMOGENSIS, AND ROLE OF NEURO-HORMONAL SIGNALS

The observation that both cold and overfeeding in the form a cafeteria diet (which is a way to induce non-forced overfeeding in rodents) stimulate BAT via the SNS (16;118), expectedly suggested the BAT *facultative thermogenesis* had a dual function, namely cold-adaptation and protection against obesity during overeating. Such concept created great excitement and was reinforced by phenotype observed in transgenic mice with selective ablation of BAT by expressing diphtheria toxin A from the UCP1 promoter (119). These mice were –intriguingly- mildly cold intolerant but markedly obese, making appear BAT as more important to regulate body weight than body temperature and boosting the interest in BAT as potential target to treat obesity. When results of the targeted deletion of the *Ucp1* (*Ucp1-/-*) were reported by Kozak's lab, it was not surprise that *Ucp1-/-* mice were cold intolerant, but in view of the findings mentioned above, it was a big surprise that these mice were not obese. Conversely, they ate more than the WT controls, yet they were leaner and gained less weight when challenged with a high fat diet (120;121). The *Ucp1-/-* mice had no increase of oxygen consumption but a detailed analysis of the respiratory exchange ratio (RER, also called RQ or respiratory quotient) suggested that the deletion of the *Ucp1*was associated with increased fat oxidation. Most importantly, moving *Ucp1-/-* and the WT control mice from a 21°C environment to one at 27°C resulted in a rapid acceleration in weight gain in the *Ucp1-/-* mice, promptly reaching that of the WT controls.

We subsequently had the opportunity of making similar but more dramatic observations. Mice lacking all products of the thyroid hormone receptor alpha gene [*Thra-0/0* mice, (122)] have lower core body temperature and are cold intolerant due to a failure of BAT (by mechanisms under investigation) to produce heat in response to NE. *Thra-0/0* mice have increased metabolic rate, as evidenced by increased oxygen consumption and food intake (123), and they are leaner than the cognate controls. Furthermore, temperature homeostasis in these mice is highly dependent on continued food intake; they burn more fat, and have elevated levels of lipoprotein lipase and D2 mRNA in muscle, but if they are studied at *thermoneutrality temperature* (30°C) these differences between both genotypes are completely negated (124). Thus, *Thra-0/*0 mice have qualitatively a similar but quantitatively more marked phenotype than that of *Ucp1 -/-* mice. The common deficiency in these two models, *Ucp1 -/-* and *Thra-0/0*, is the failure of BAT thermogenesis, and both develop a metabolic phenotype that is evidently a form of

alternate *facultative thermogenesis* in response to BAT deficiency since it disappears if the mice are reared in a warm environment close to or at *thermoneutrality temperature* (121;124). Such an alternate form of *facultative thermogenesis* is an adaptation in these two transgenic models to a life-long disabling of BAT thermogenesis. This alternative to BAT *facultative thermogenesis* is more energy expensive and less efficient than BAT thermogenesis, as these mice eat more, are less sensitive to diet-induced thermogenesis and, while sufficient to defend body temperature at cool (20-22°C) is insufficient to keep core body temperature in more severe cold (4-6°C). Note that in the wild, particularly in latitudes far from the equator, mice live at even colder temperatures, so these mice with disabled BAT would certainly died in the wild. The nature and site of the alternate BAT *facultative thermogenesis* has not been defined, but changes observed in skeletal muscle suggest that this is likely the site (124). Nedergaard *et al* have suggested that *Ucp1 -/-* mice substitute BAT non-shivering thermogenesis by living in "a state of chronic shivering (125)", whereas the group of L. P. Kozak defends the hypothesis of an alternative non-shivering thermogenesis that would require leptin and thyroid hormone (126;127), which our data support (124). *Thra-0/0* mice have increased D2 mRNA and activity in muscle, with the highest enzyme specific activity in slow muscle (128), that is rich in mitochondria and normally contributes more to thermogenesis than fast twitch or glycolytic muscle(129;130). Preliminary observations in D2 knockout mouse that also have a disabled BAT (88) show that they are not resistant to diet-induced obesity, but they are instead more sensitive than the appropriate controls (Marsili *et al.* work in progress), suggesting that D2 activation is probably a factor in the hypermetabolism of *Thra-0/0* mice and their resistance to diet-induced obesity.

There are situations in which both *facultative thermogenesis* and *obligatory thermogenesis* are defective. One of them is fasting. The sympathetic stimulation of the BAT is reduced in fasting (16) probably in response to decreased insulin, leptin and blood glucose concentrations. Leptin has a complex action on BAT function. Injections of leptin in cerebral ventricles stimulate BAT by a mechanism independent on the sympathetic nervous system and only in fasted rats (131), but leptin-induced increase in BAT oxygen consumption and UCP1 expression is reduced in mice lacking all three β-AR (132). Leptin also increases centrally the set point of regulation of thyroid function (133). Indeed, the reduction of oxygen consumption and colonic temperature associated with fasting in rats is improved by about 50% when fasted rats are given T_4 (134). In pure hypothyroidism, also both *facultative thermogenesis* and *obligatory thermogenesis* are reduced. As mentioned, BAT sympathetic stimulation is increased, but BAT thermogenic responses is drastically reduced due to the lack of local T_3 generation causing a defective NE signaling and reduced production and activity of UCP1. These animals are extremely sensitive to cold, but our experiments providing acute, minute doses of T_4 that do not improve the systemic hypothyroidism but do normalize BAT UCP1 levels via the activated D2 do as well restore cold tolerance (55). In hypothyroidism, core body temperature is defended, but at a lower level, by heat saving mechanisms (intense vasoconstriction) and eventually torpor (10;135). These experiments show the conflict between energy availability and thermogenesis, and how nature sacrifices thermogenesis during food restriction.

An example of primary reduction of *obligatory thermogenesis* and a compensatory response of BAT *facultative thermogenesis* is our studies on mitochondrial glycerol-P-dehydrogenase knockout mouse (*mGpd -/-*). This enzyme is rate limiting in the G3P-NADH shuttle, which is very important in muscle, where the alternate malate-aspartate shuttle is virtually non-existing (40). The importance of the G3P-NADH shuttle in muscle is revealed by the increased lactate-pyruvate ratio and G3P in this, but not other tissues of *mGpd -/-* mice (136). In addition, G3P increases oxygen consumption 4-5-fold in skeletal muscle slices or shredded muscle fibers and this effect is absent in *mGpd -/-* mice, whereas G3P does not increase oxygen consumption in liver slices (unpublished observations). These transgenic mice have a slight but significant reduction of oxygen consumption in spite of a significant increase of T_4 and T_3 concentrations in circulation and the activation of BAT. Moreover, if *mGpd -/-* mice were kept at *thermoneutrality temperature*, BAT looked quiescent and T_4 and T_3 levels were reduced to the same level in WT and *mGpd -/-* mice (137). The *mGpd -/-* mice are also more sensitive to diet-induced obesity, and such sensitivity is clearly greater in females than in males. This gender difference may be due to a higher elevation in circulating T_3 levels and UCP3 expression at mRNA and protein level in males than in females, suggesting that males are better defended than females from the reduced *obligatory thermogenesis* derived from the lack of mGPD (136). Altogether, these observations demonstrate the complex relationships between *obligatory thermogenesis, facultative thermogenesis* and *thermoneutrality temperature* depicted in Fig.2, showing, for example, how the magnitude of *obligatory thermogenesis* increases *thermoneutrality temperature,* moving the activation of *facultative thermogenesis* and heat-saving mechanisms to higher ambient temperature. These results also show that the well-known activation of mGPD by thyroid hormone (138) is one of the mechanisms whereby this hormone increases *obligatory thermogenesis*, for oxygen consumption was reduced in *mGpd -/-* mice in spite of a significant increase in circulating levels of T_4 and T_3 (137).

8. CONCLUDING REMARKS

Homeothermy, being an advantage by constantly providing a temperature more appropriate for vital functions such locomotion, reproduction, neuromuscular function and by expanding the niche of species, increased energy demands and created the need to provide mechanisms to maintain body temperature at the least energy cost. Increased thermogenesis is an absolute need for homeothermy, and this has been accomplished by increasing the rate of metabolism, reducing the thermodynamic efficiency of some functions, and by the

selection of mechanisms that would further increase heat production (*facultative thermogenesis*) when the environment becomes cold enough to overcome the heat produced by BMR (*obligatory thermogenesis*) and the action of heat-saving mechanisms. Such a need is particularly important in smaller animals because of their higher surface area-to-volume ratio makes them more susceptible to lose heat to a colder environment.

The energy cost of thermogenesis is high. The availability of food has always been limiting except in recent times that represent a minimal fraction of the millions of years that took endothermy and homeothermy to evolve. Limiting food and the added energy cost of producing heat became selection pressures to produce heat at the minimal energy cost and to the selection of behavioral mechanisms, hibernation and torpor to survive.

In birds, most of FT seems to reside in skeletal muscle, and calcium recycling between cytosol and sarcoplasmic reticulum appears to be a major thermogenic mechanism. Although uncoupling proteins have been cloned in birds, they seem to be paralogs of the mammal UCP1 and it is to be demonstrated whether they play a major temperature homeostatic role. In mammals, UCP1 and BAT appear to be an energy efficient mechanism to produce heat on demand. In the absence of this mechanism, mammals resort to evolutionary older mechanisms, which are less efficient and more energy demanding. Recent studies manipulating genetically the ability of BAT to produce heat have made evident the effectiveness of BAT *facultative thermogenesis.* The disruption of BAT thermogenesis is associated with increased energy demands and the need of continued food supply to maintain body temperature in cold environments.

The signals controlling temperature homeostasis include: the SNS; thyroid hormone, which in homeothermic species acquires a role of controller of *obligatory thermogenesis* and a modulator *facultative thermogenesis*; and other hormones such as leptin and insulin that subordinate thermogenic responses to food and energy availability. Being dependent on food availability, the levels of these hormones allow or limit the magnitude of thermogenic responses. Starvation and anorexia nervosa are good examples of clinical relevance. The role of these hormones in modulating thermogenesis via setting the level of function of the thyroid gland is nicely illustrated by the depression of thyroidal axis in fasted animals which was corrected, but partially, by restoring leptin levels with exogenous leptin (133). Since animals were given leptin but not food, it is reasonable to assume that the persistent low insulin was responsible for the incomplete recovery of the thyroidal axis.

The thermogenic effect of thyroid hormone was recognized over a century ago (42), yet we still have a fragmented understanding as to how thyroid hormone acquired the role of stimulating thermogenesis. D2, being strategically located and sensitive to cAMP stimulation as well as T_4 itself, appears to be a critical factor in regulating the concentration of T_3, at least 10 times more active than T_4, in sites of thermogenic potential such as BAT and, according to our recent findings, in skeletal muscle. Recent studies in experimental animals adapted to heat show that *obligatory thermogenesis* can be reduced as well and the changes in circulating iodothyronines, increase in rT_3 and decrease in T_3, suggest that yet another deiodinase, an inner ring, 5-iodothyronine deiodinase, called type-3 deiodinase or D3, may play a critical role.

Most importantly, this review provides evidence of the energy cost of homeothermy is a major factor in our energy balance. Several transgenic mice models of BAT dysfunction show that the "strategies" utilized for temperature homeostasis have a readily evident impact on energy balance. Because of heat saving and heat dissipating mechanisms, we may maintain our temperature homeostasis with different levels of thermogenesis within narrow range of ambient temperatures, but the differences in energy spend in thermogenesis probably have a significant impact our total energy demands and our risk to become obese. The study of the relations between the cost of temperature homeostasis and energy balance is of inescapable importance.

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