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Norepinephrine as a morphogen?: its unique interaction with brown adipose tissue

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ABSTRACT Norepinephrine is normally considered a neurotransmitter mediating acute metabolic effects in target cells. However, analysis of the regulation of the recruitment process in brown adipose tissue has indicated that norepinephrine may interact with this tissue in such a way that it could be considered a morphogen for this tissue. Besides stimulating the acute thermogenic processes, norepinephrine can induce the expression of tissue-specific proteins such as the uncoupling protein, induce expression of non-tissue specific proteins necessary of the thermogenic process (e.g. lipoprotein lipase) and repress the expression of non-essential proteins (e.g. subunit c of the ATP-synthase). Upon chronic adrenergic stimulation, the general differentiation state of the tissue is advanced, indicating that the expression of factors with a more general effect on brown adipocyte differentiation is also under adrenergic control. It may even be discussed that norepinephrine may be involved early in the embryonal determination process directing cell clones into this line. The molecular basis for these effects of norepinephrine are only poorly known at present, but adrenergic effects on the expression level of many transcription factors, such as C/EBPα, C/EBPß and PPARγ2, have been noted. These collective recruitment effects of norepinephrine are well suited to allow the tissue to grow or atrophy in response to the physiological needs of the organism.

KEY WORDS: *norepinephrine, brown adipose tissue, cell proliferation, uncoupling protein, cell differentiation*

Introduction

Traditionally, it has been customary to divide the hormones and neurotransmitters that interact with cells into two groups, as illustrated in the sketch in Figure 1A.

In this classification, one group of hormones/neurotransmitters consists of the classical transmitters, such as acetylcholine, norepinephrine, etc. These transmitters are even chemically rather simple molecules, and they have most often been discussed as being regulators of the acute "rather simple" events of the cell: stimulation of the cellular metabolic activity, of the release of exocrine products, etc.; this regulation would mainly occur through phosphorylations of already existing enzymes.

The other group of hormones/neurotransmitters includes mainly factors which are often more complex even chemically (steroids, peptides etc.). These substances are most often discussed as being regulators of more chronic and fundamental processes: cell proliferation and cell differentiation; this regulation would mainly be expected to occur by interaction with the cell nucleus and through effects on gene expression.

Presently, this traditional classification can no longer be maintained. As indicated in Figure 1B, the effects of the classical transmitters extend to the cell nucleus and through this can have extensive effects on cell status.

This extension of the role of the classical transmitters is especially noteworthy in the case of the brown-fat cell. Recent developments in brown-adipose-tissue research clearly indicate that the neurotransmitter norepinephrine not only activates the acute metabolic response of the brown-fat cell, the thermogenic process but also has a dominant role in this tissue as the inducer and regulator of the recruitment process: the process which within a few weeks can transform a practically dormant tissue into a tissue which has a metabolic capacity that in itself can utilize as much energy as the rest of the entire organism. As detailed below, the recruitment process involves the concerted stimulation of cell proliferation and cell differentiation required for this increase in metabolic capacity.

The recruitment process

The recruitment of brown adipose tissue is the process which is evoked e.g. when a small mammal is exposed to cold. In order to maintain a body temperature of 37°C, the cold-exposed mammal must produce sufficient heat to compensate

Abbreviations used in this paper: UCP, uncoupling protein thermogenin; CREB, cyclic-AMP response element binding protein; NST, nonshivering thermogenesis.

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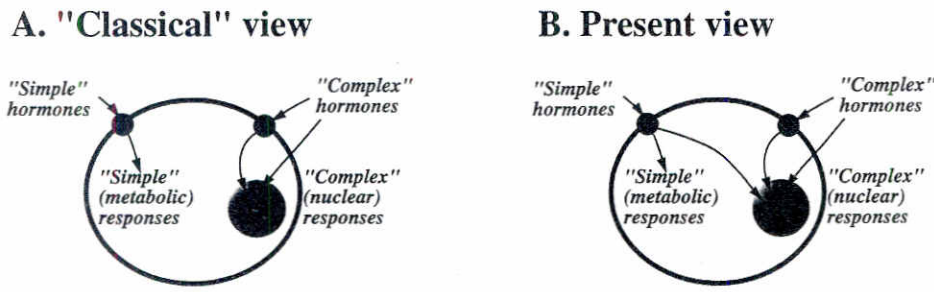

Fig. 1. The classical (A) versus the present (B) view of the interaction between different groups of hormones and neurotransmitters on cellular function. *An earlier view* **(A)** *that "classical" transmitters (epinephrine, acetylcholine etc.) regulate acute processes whereas more complex agents (growth factors etc.) regulate more fundamental processes (proliferation, differentiation) has been modified* **(B)** *to allow for more fundamental effects of the classical transmitters.*

for the heat loss occurring. For a small mammal with an unfavorable surface/volume ratio, more than a double of its metabolic rate may be necessary when the animal is experiencing 5°C rather than 25°C, and this increased metabolism has to be maintained as long as the animal remains in the cold (cf. e.g. Fig. 2A). Acutely, the extra heat needed comes from the uncomfortable process of shivering (Fig. 2B). However, during continued exposure to cold, brown-adipose-tissue is recruited, which leads to such an increase in metabolic capacity of the tissue that the need for shivering to produce heat is eliminated. Classical experiments demonstrate this recruitment process: with time in cold, shivering disappears but the high metabolic rate (heat production) is maintained (Fig. 2A,B). Thus, the capacity for non-shivering thermogenesis is recruited (Fig. 2C). It has been demonstrated that this is due to the recruitment of brown adipose tissue (Fig. 2D), the organ that is responsible for non-shivering thermogenesis. Thus, during this process, the brown adipose tissue grows and differentiates, physiologically induced by a simple external stimulus: cold. The recruitment process of brown adipose tissue represents one of few tissue development processes which can be easily elicited in the adult mammal. It therefore lends itself to analyses which may be significant also for the understanding of the development of tissues in general.

It is the present but novel understanding, that it is the classical neurotransmitter norepinephrine that directs this recruitment process. It will be illustrated below how this understanding has been achieved from a succession of observations on each of the phenomena characteristic for the recruited state. Generally, each recruitment phenomenon was first observed to occur in cold-acclimated animals; norepinephrine treatment of intact animals was then demonstrated to mimic this effect. That a given recruitment phenomenon is a genuine effect of norepinephrine directly on the brown-fat cells themselves has then been demonstrated by treating isolated brown-fat cells in culture with norepinephrine. Presently, we have arrived at the state where for each recruitment phenomenon it is the regulation at the level of gene expression that is under investigation. Thus, the analysis of the involvement of transcription factors (of different hierarchical orders) in the mediation of the norepinephrine effects is under discussion.

In the development of a tissue, the processes of determination, proliferation and differentiation are successively encountered in order to reach the final, fully differentiated state. However, we discuss these processes here in reversed order, i.e. in an order of increasing degree of complexity – and decreasing degree of knowledge. We especially address the question as to which extent norepinephrine is able to promote each of these steps (Fig. 3).

The fully differentiated state: molecular markers

It is the features of the fully differentiated state of the brown-fat cell that determine what properties that are truly characteristic of (and possibly unique for) a brown-fat cell. The brown-fat cell is both "fat" and "brown", i.e. it possesses properties related to each of these characteristics.

The brown-fat cell is a fat-cell because it possess a series of features which are fat-cell-specific, such as a high lipogenic potential, a high ability to accumulate fat in triglyceride droplets, etc.; we discuss further these general fat-cell characteristics below.

We will first concentrate on the features which are "brown"-adipose-tissue-specific, i.e. those that distinguish brown-fat cells from white-fat cells.

Molecular markers for the thermogenic function

The most conspicuous property of the brown-fat cell is its ability to exhibit norepinephrine-induced thermogenesis. As understood today, this ability of the mature brown-fat cell to produce heat when stimulated by norepinephrine is due to a combination of two properties: a pathway for degradation of triglycerides, which is well-developed but not principally different from what is seen in other fat cells, plus the presence of a unique thermogenic mechanism attributable to the presence of a unique protein: the uncoupling protein thermogenin.

Thus (as reviewed in detail in Nedergaard and Lindberg, 1982) the degradation of the stored triglycerides in the brown fat cell occurs when the cell is stimulated with norepinephrine released from the branches of the sympathetic nervous system when the animal experiences cold (Fig. 4). This can be mimicked by injection of norepinephrine into the intact animal (Fig. 2C). The norepinephrine released interacts primarily with β_3-receptors, coupled via G-proteins to adenylyl cyclase. The resulting increase in cAMP leads to stimulation of protein kinase A activity and stimulation of the hormone-sensitive lipase which also seems to be similar to that found in e.g. white adipose tissue (Holm *et al.*, 1987). Nothing in this scheme gives rise to greater heat release than similar processes in e.g. white adipose tissue. The further thermogenic processes occur in the mitochondria of the brown-fat cells.

The brown-fat mitochondria are very conspicuous. This is true both visually (they dominate the cytosol of the cell) and functionally. Thus, the recruitment process includes the stimulation of

mitochondrogenesis in general. However, there are 3 features of the mitochondria which are characteristic of brown-fat mitochondria: a high endowment with oxidative enzymes, a low complement of the ATP-synthase, and the unique presence of the uncoupling protein (reviewed in Nedergaard and Cannon, 1992) (Fig. 5).

It is illustrated in Figure 6 how these features lead to principally different oxidative behavior of brown-fat mitochondria and other mitochondria (here exemplified with liver mitochondria). It should be especially noted that not only is the effect of the presence of the unique uncoupling protein evident in the brown-fat mitochondria; it is equally evident that the ATP synthetic capacity is much reduced compared to "normal" mitochondria. We thus have here 2 distinct regulatory processes occurring in brown adipose tissue: the unique expression of the uncoupling protein, and the repression of ATP synthase expression.

We first examine to which extent these features of brown-fat mitochondria are due to a direct effect of norepinephrine.

Norepinephrine-induced expression of the uncoupling-protein gene

It can be demonstrated by different techniques that chronic exposure of animals to cold leads to an increase in the amount of the uncoupling protein. This increase can e.g. be followed immunologically (Fig. 2D). In animals which are chronically stimulated by norepinephrine with minipumps, the amount of UCP increases (Mory *et al.*, 1984), and injection of norepinephrine leads to an increase in the amount of UCP-mRNA (Jacobsson *et al.*, 1986). In untreated, cultured brown-fat cells, practically no UCP is found but treatment of brown-fat cells with norepinephrine leads to an increase in UCP mRNA and to an increase in the total amount of UCP (Herron *et al.*, 1990; Kopecky *et al.*, 1990; Rehnmark *et al.*, 1990). Thus, the expression of the uncoupling protein is under acute adrenergic control, and there is evidence that this occurs through an increase in the level of cAMP.

The most likely explanation for the stimulation of UCP expression via this pathway would be that the protein kinase A – which is stimulated when cAMP level is increased – phosphorylates the cAMP-response-element-binding-protein (CREB) and that this is sufficient to induce expression, provided that the gene is already

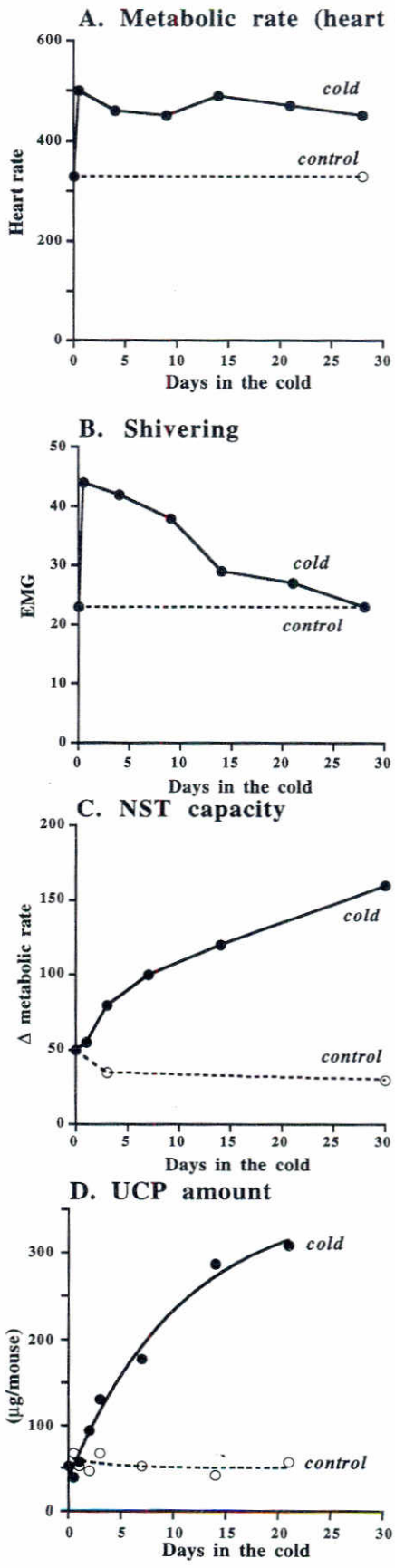

Fig. 2. The recruitment of the capacity for non-shivering thermogenesis during acclimation to cold. (A) *The exposure of rats to cold leads to an increase in metabolic rate (here measured as the heart rate; adapted from Hart et al., 1956.* **(B)** *The increase in metabolic rate is initially explainable by an increase in shivering (here measured electromyographically; adapted from Hart et al., 1956). However, surprisingly, as an effect of constant exposure to cold, shivering disappears but the high metabolic rate persists. As this represents an increased metabolism not emanating from shivering, it may be referred to as non-shivering thermogenesis.* **(C)** *The capacity for NST (non-shivering thermogenesis) can be estimated as the metabolic response to norepinephrine injection, and this capacity increases as a mirror image of the disappearance of shivering (adapted from Depocas, 1960).* **(D)** *The increase in NST capacity resulting from an increase in the thermogenic capacity of brown adipose tissue, as biochemically measurable e.g. as an increase in the amount of the uncoupling protein thermogenin (UCP) (adapted from Jacobsson et al., 1994). This thus represents the recruitment process of brown adipose tissue.*

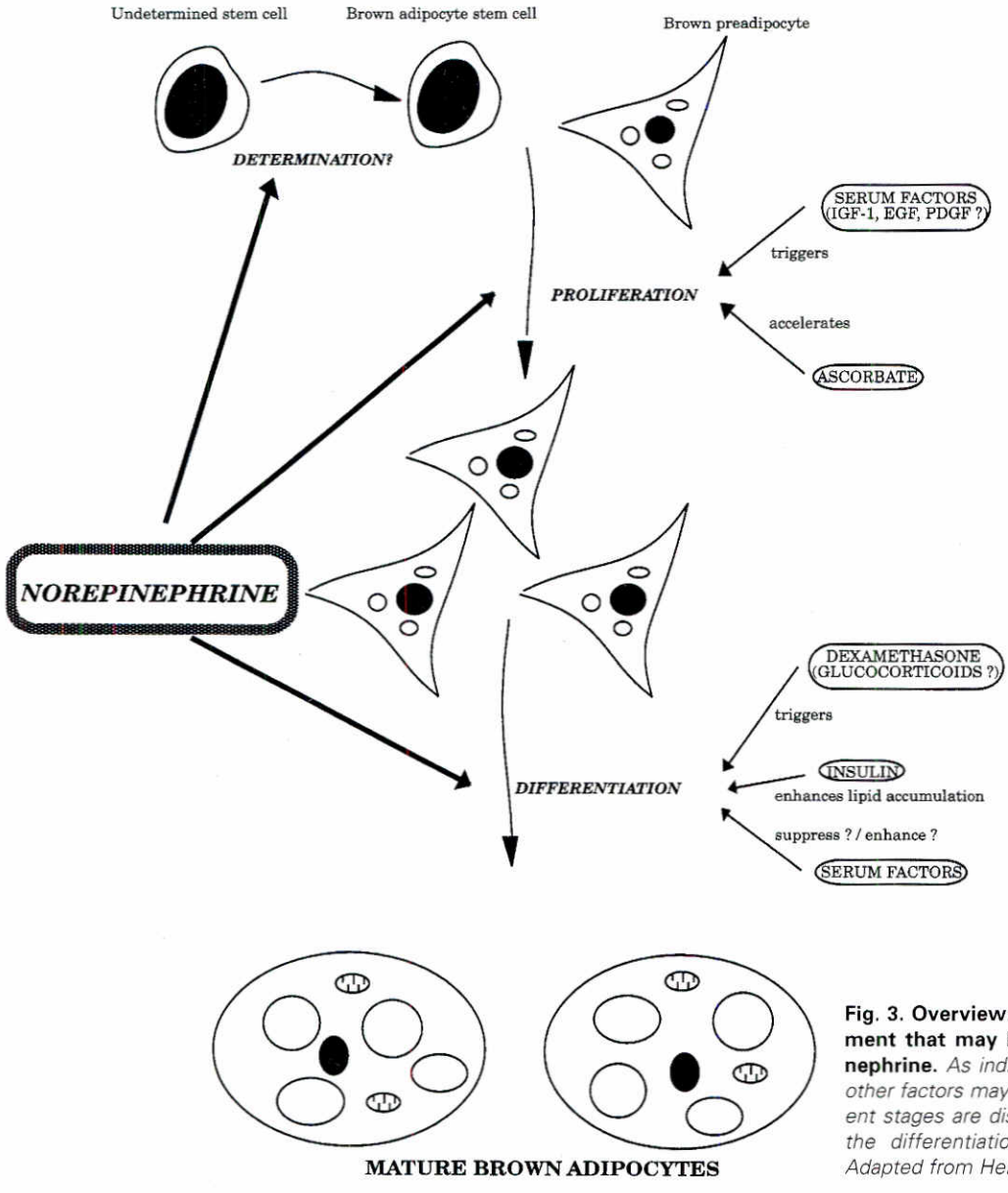

Fig. 3. Overview: the steps in brown-fat cell development that may be influenced or directed by norepinephrine. *As indicated (but not further discussed here), other factors may also be involved. In the text, the different stages are discussed in the reverse order, i.e., from the differentiation state to the determination state. Adapted from Herron (1992).*

"open". Not all steps in this likely chain of events have yet been directly demonstrated, but cAMP-response elements have been identified in the promoter of the uncoupling protein gene (see below).

Possible norepinephrine-induced suppression of the expression of the ATPsynthase subunit c gene

Concerning the ATP-synthase, a very interesting situation has been unravelled. As can be deduced from Figure 7A, a very low ATP-synthase activity is observable in the tissue (Cannon and Vogel, 1977; Houstek and Drahota, 1977); this is, of course, in accordance with the thermogenic task of the tissue. Despite the fact that the capacity for ATP synthesis is low and that very low amounts of the subunits of the ATP-synthase are found, a paradoxically high amount of mRNAs coding for these subunits is found in the tissue (Houstek *et al.*, 1991; Tvrdik *et al.*, 1992) (Fig. 7). However, there is a single exception to this rule: the mRNA level for subunit c of the F_0 part of the enzyme is extremely low (Houstek *et al.*, 1995) (Fig. 7). As this subunit may be essential for the assembly of the ATP synthase complex, the lack of subunit c could explain the phenotype, despite the high expression of the other subunits. It would seem that a specific regulation of subunit c must prevail and that the extreme repression should be a brown-fat-specific phenomenon. Whether the repression is a norepinephrine-induced state is presently under investigation in our laboratory.

Norepinephrine-induced expression of the lipoprotein lipase gene

During prolonged thermogenic activity, the lipid stored in the brown adipose tissue is not sufficient for maintained heat production, and extra lipid has to be imported into the tissue. This

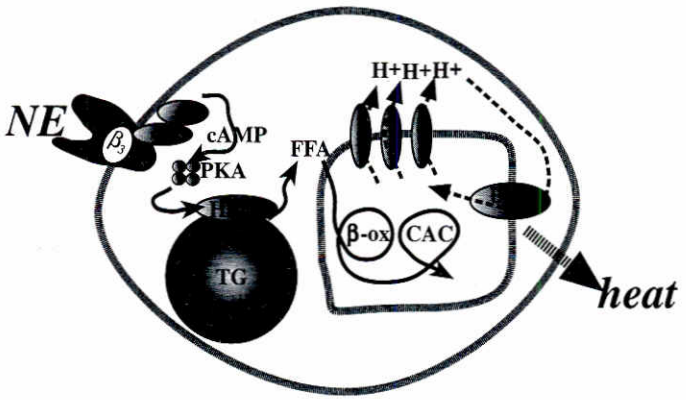

Fig. 4. The acute effects of norepinephrine on the brown-fat cell. *Norepinephrine (NE) interacts with the b_3 receptors, which leads to an increase in cyclic AMP levels (cAMP) that activates protein kinase A (PKA). The protein kinase phosphorylates the hormone-sensitive lipase (HSL) which becomes activated and releases free fatty acids (FFA) from the triglyceride droplets (TG). The fatty acids are combusted in the mitochondria through b-oxidation (b-ox) and the citric acid cycle (CAC). The reducing equivalents are transported through the respiratory chain, leading to the pumping out of protons (H^+) that return to the mitochondria through the uncoupling protein (UCP, thermogenin), leading to the release of heat.*

increased uptake of lipid from the circulation is accomplished by the enzyme lipoprotein lipase. In contrast to what is the case for the hormone-sensitive lipase of the lipolytic cascade (whose activity is altered by phosphorylation), the activity of lipoprotein lipase is largely regulated by alterations in the total amount of the enzyme itself.

In white adipose tissue, the activity of lipoprotein lipase is increased by insulin but is not influenced or is even reduced by norepinephrine. However, in brown adipose tissue, both the activity and the amount of mRNA coding for lipoprotein lipase is increased, not only by insulin, but also by norepinephrine (Carneheim *et al.*, 1984, 1988; Carneheim and Alexson, 1989; Mitchell *et al.*, 1992). A positive effect of norepinephrine on lipoprotein lipase gene expression is also observed in cultured brown-fat cells (Kuusela *et al.*, 1995). This positive effect of norepinephrine on lipoprotein lipase gene expression thus constitutes a clear distinction between white and brown adipose tissue. However, the ability of norepinephrine to promote lipoprotein lipase gene expression is not unique to brown-fat cells: a similar positive effect is seen in heart cells. At the genomic level, these observations have interesting implications. There is only one copy of the lipoprotein lipase gene, and as the expression of this gene is differently regulated in brown-fat cells than in white-fat cells, a dominant factor must alter the way the gene responds to the same norepinephrine stimulation, presumably mediated through CREB in both white and brown fat, but leading to divergent expression effects.

The carnitine palmitoyltransferase I-like protein

An interesting parallel to the situation encountered for lipoprotein lipase – that the expression pattern is more like that of heart than that of white adipose tissue – has recently been observed. In a search for further brown-fat-specific proteins, Yamazaki *et al.* (1995) isolated a cDNA with a sequence similar but not identical to that of carnitine palmitoyltransferase I. This mRNA species is only found in brown adipose tissue and in the heart, i.e. not in white adipose tissue. It would thus seem that a group of proteins may with time be encountered that shows this characteristic tissue distribution. Molecularly, this would imply that there are transcription factors that are common to brown adipose tissue and heart but that are not found in white adipose tissue. The nature of such factors is presently unknown.

Other brown-fat versus white-fat specific gene expression

It is still possible that further genes with an expression pattern selective for brown adipose tissue may be identified. Work in progress in our laboratory indicates that a protein exists which has a molecular weight of 34000, with no resemblance to any characterized protein and which seems to be virtually limited to brown adipose tissue; this gene also shows an adrenergically induced increase in expression when the tissue is recruited (Tvrdik *et al.*, unpublished). A large number of other genes are

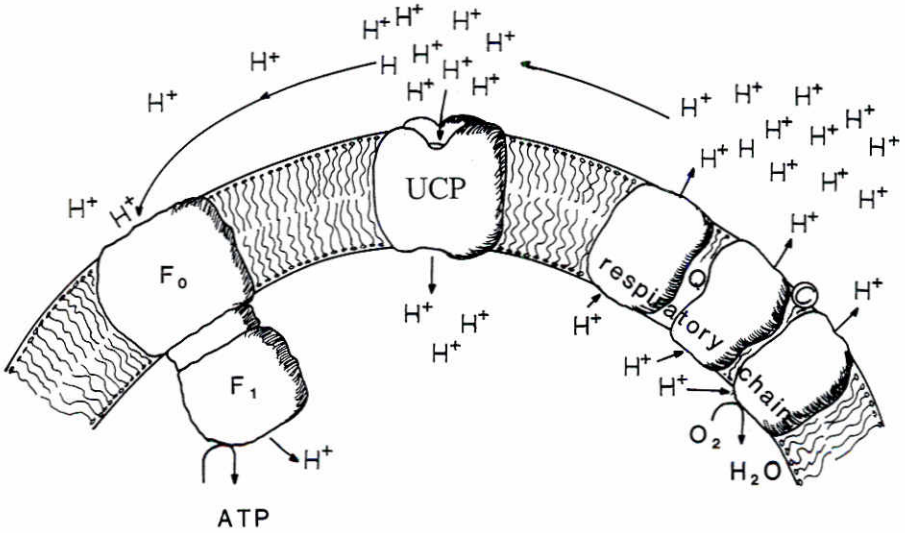

Fig. 5. The function of the uncoupling protein. *The protons that are pumped out from the mitochondria by the respiratory chain have a choice in brown-adipose-tissue mitochondria: to re-enter through the uncoupling protein (UCP) – in which case the energy stored in the proton electrochemical gradient is released as heat, or to re-enter through the ATP synthase (that consists of two subunits: F_o and F_1), in which case the energy is used for ATP production. As illustrated in Fig. 6, the choice is not equal, because very low amounts of ATP synthase are found in brown-fat mitochondria.*

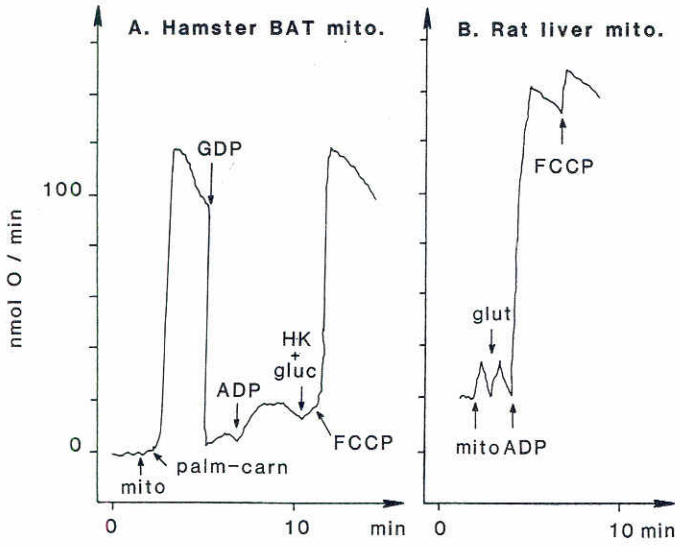

Fig. 6. Comparison between the oxidative behavior of liver and brown-fat mitochondria. *In liver mitochondria* **(B),** *the addition of oxidative substrate (here α-keto-glutarate=glut) does not lead to any increased rate of oxidation. However, after the addition of ADP, respiration is much stimulated, until all ADP is converted to ATP. The addition of an artificial uncoupler (here FCCP) that enables unlimited proton transport over the membrane can restimulate respiration. In brown-fat mitochondria* **(A),** *the addition of substrate in itself (here palmitoyl-carnitine) is sufficient for full respiratory stimulation. However, the addition of GDP (that binds to the uncoupling protein) inhibits respiration. Addition of ADP has, in comparison to what is the case in liver mitochondria, a very modest effect on respiration; this implies that the total amount of ATP synthase is very limited in these mitochondria. Even the addition of an ATP trap (in the form of glucose (=gluc) plus hexokinase (HK)) does not stimulate respiration. Only the addition of the artificial uncoupler FCCP restores full respiration. Modified from Lindberg et al. (1976).*

also highly expressed in the tissue; they include genes coding for enzymes involved in β-oxidation of fatty acids and in mitochondrial oxidative processes (Tvrdik *et al.*, unpublished) but they are not unique for the tissue. The leptin gene *(ob)* is an example of the opposite: a gene expressed in white adipose tissue but apparently not in brown (Trayhurn *et al.*, 1995).

The search for a brown-fat-specific key regulator

What we have described above is a number of molecular markers which show a very distinct pattern of expression in brown adipose tissue. For some of these, the expression has also been demonstrated to be under adrenergic control.

If the regulation of tissue-specific gene expression were simple, it could be suggested that these brown-fat-specific gene expression events occur because these genes were under the control of one specific transcription factor. We could, hypothetically, assume that such a factor exists and binds to specific elements in the promoters of the respective genes. We could ascribe the name BREB (brown-fat-specific response element binding protein) to this transcription factor. It should be noted that a specific sequence in the UCP promoter – termed BRE – has been suggested to confer tissue-specific expression to UCP (Kozak *et al.*, 1994). The generality of the occurrence of this element has not been investigated yet. Thus, in a simple system, the same BREB that would allow for the expression of the uncoupling protein, would suppress the expression of subunit c of the ATP-synthase etc. As several of the molecular markers mentioned above are also under the control of adrenergic stimulation, the formulation must be that the expression of each of these proteins must be under dual control: they must be "opened" by the BREB and, when opened, they would have the ability to respond to norepinephrine (Fig. 8).

A transcription factor with such a general role has not as yet been identified in brown adipose tissue. Most efforts have concentrated on the examination of the control of the uncoupling protein, based on the reasonable assumption that the promoter region of the gene for the uncoupling protein carries information that is brown-fat specific and restricts expression of the gene to the "genuine" brown-fat cell (truly, this is a circular argument: we argue that a true brown-fat cell is one that can express the gene for the uncoupling protein and simultaneously we point out that the uncoupling protein is only expressed in brown-fat cells; in the absence of any other definition we have to accept this).

According to the simplified model in Figure 8, we could hope that analysis of the promoter of the uncoupling protein would only reveal tentative BREs and CREs. However, as sketched in Figure 9, the reality is that, despite the fact that only a handful of studies have been published, there is already a plethora of transcription factors implied in the regulation of UCP gene expression.

Norepinephrine as a promoter of differentiation

It is important to distinguish between norepinephrine as inducer of the expression of a single gene (such as the uncoupling protein thermogenin or lipoprotein lipase), and as an inducer (or a stimulator) of differentiation as such. In the latter case that would mean that norepinephrine induces the expression of transcription factors which in turn activate the expression of genes that are not directly under adrenergic control. This possibility is illustrated in Figure 8.

There is evidence from the behavior of brown-fat cells in culture that differentiation needs a promoting factor in the form of norepinephrine. When some differentiation markers are investigated in cells maturing in culture, they show a bell-shaped pattern of expression with time in culture (e.g. Rehnmark *et al.*, 1990). Similar tendencies may be observed for other features of the cells; this could be called type II behavior (Fig. 10) and indicates that the cells may become spontaneously dedifferentiated with time. However, it would seem that the chronic presence of norepinephrine can make cells remain or advance in their differentiation so that type I behavior (Fig. 10) is observed (Puigserver *et al.*, 1992). This is an indication that also the differentiation process as such is under norepinephrine control.

The brown-fat cell as a fat cell

In the present article we do not discuss extensively the phenomena associated with adipocyte development in general; they are well discussed in other reviews (e.g. Ailhaud *et al.*, 1992). However, we would like to point to the behavior of a few transcription factors which may be associated with adipocyte conversion.

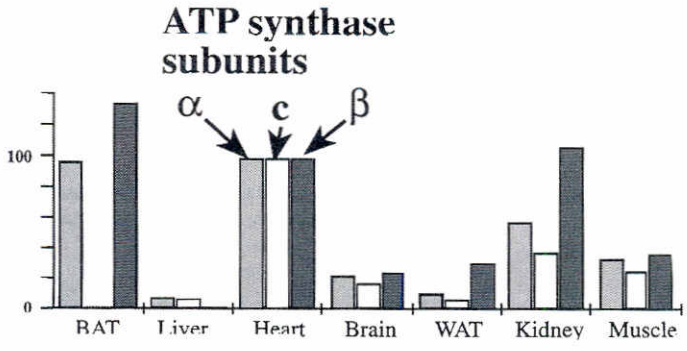

Fig. 7. Unique repression of subunit c of the ATP synthase in brown adipose tissue. *The levels of mRNA coding for the F_1 subunits a and b and for the F_o subunit c in different tissues are shown. The expression level in the heart is set to 100% for each subunit. It may be seen that the expression levels of the different subunit in general are altered in parallel in the different tissues; however, in brown adipose tissue, the levels of the α and the ß subunits are very high (at least equal to heart) but the level of the c subunit is very low (so low that it is invisible in this representation). As the c subunit forms part of the membrane-anchoring part of the ATP synthase, it is possible that it is the absence of this particular subunit that leads to the very low amount of ATPsynthase in the tissue, despite high expression of most subunits. Adapted from Houstek et al. (1995).*

The expression of the *c-fos* gene is under positive adrenergic control in the brown-fat cells (Thonberg *et al.*, 1994). However, *c-fos* expression is found in many cell types and the genes which are under control of c-Fos in the brown-fat cells are not identified.

The C/EBPα transcription factor was originally suspected to be a specific regulatory factor for liver differentiation but it was soon found to be well expressed both in white and brown adipose tissue. C/EBPα is well expressed already early during development of brown-fat cells in culture (Rehnmark *et al.*, 1993; Manchado *et al.*, 1994) and the expression of this factor is apparently essential for tissue function (Wang *et al.*, 1995). Also the sister transcription factor C/EBPβ is well expressed in the tissue (Rehnmark *et al.*, 1993). It has been discussed whether the ADD1 factor (Tontonoz *et al.*, 1993) may have a more pivotal role in adipocyte differentiation, but more detailed studies in brown adipose tissue have not been performed.

Recently, the so-called peroxisomal proliferator-activated receptor PPARγ2 – which is highly expressed in adipose tissue – has been suggested to be "a" or "the" pivotal transcription factor for adipocyte differentiation, perhaps in concert with C/EBPα (Tontonoz *et al.*, 1994). In preliminary studies with cultured brown-fat cells we found it to decrease when the proliferative activity was enhanced (and the degree of differentiation therefore decreased).

Norepinephrine-induced cell proliferation

The recruitment process *in vivo* is associated with growth of the tissue, not only in total mass but also in the number of cells, as witnessed by a several-fold increase in the total amount of DNA (Thomson *et al.*, 1969; Bukowiecki *et al.*, 1978; Rehnmark and Nedergaard, 1989). The increased rate of synthesis of DNA observed in the animals (Cameron and Smith, 1964; Hunt and Hunt, 1967) can be mimicked by treatment of the animals with norepinephrine (Géloén *et al.*, 1988; Rehnmark and Nedergaard, 1989). Norepinephrine has a similar stimulatory effect on DNA synthesis in cultured brown-fat cells, even in the absence of serum growth factors; this means that the cells must be directly responsive to norepinephrine, which thus apparently functions as a mitogen (Bronnikov *et al.*, 1992; Golozoubord *et al.*, unpublished). The finding that this norepinephrine effect is not mediated through the β_3 adrenergic receptors but rather through β_1 receptors (Bronnikov *et al.*, 1992) implies that those cells responding proliferatively to norepinephrine are not in the same state as those responding to norepinephrine by increased UCP gene expression or by advancing in the differentiation pathway.

Practically nothing is known about the molecular mediation of this mitogenic effect of norepinephrine. It is mediated, as indicated, via β_1 adrenoceptors and via an increase in cAMP, but the further steps have not been characterized.

In many other cell types it has been found that proliferative processes are regulated or accompanied by an increased expression of the proto-oncogene *c-myc*. However, this does not seem to be the case for adrenergic activation of cell proliferation. Neither *in vivo* during cold exposure, nor *in vitro* during norepinephrine treatment, is the stimulation of proliferation accompanied by an increase in *c-myc* expression (Tvrdik *et al.*, unpub-

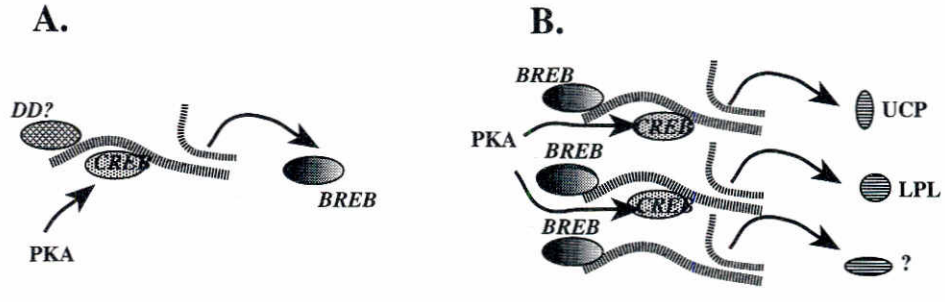

Fig. 8. A hypothetical scheme of the transcription factors involved in the control of the molecular markers for brown adipose tissue. *It may be suggested that brown-fat-specific transcription factors (BREB) may exist; however, they must, in turn, be under the control of a differentiation-dependent transcription factor (DD). Thus, first when a crucial switching point in the "spontaneous" development of the cell has been passed, the BREB is produced (or activated). The BREB is supposed to be able to influence the expression of several different genes, here illustrated by lipoprotein lipase (LPL) and the uncoupling protein (UCP), and others. Concerning several (but not all) of these genes, their expression is also under control of norepinephrine, probably working through CREB (the cyclic AMP response element binding protein), but this control is only allowed to be functional when the gene has become "opened" by the BREB. This illustration also suggests that the BREB itself is under positive adrenergic control, in agreement with suggestions that differentiation in general (and not only the expression of certain genes) is enhanced by adrenergic stimulation.*

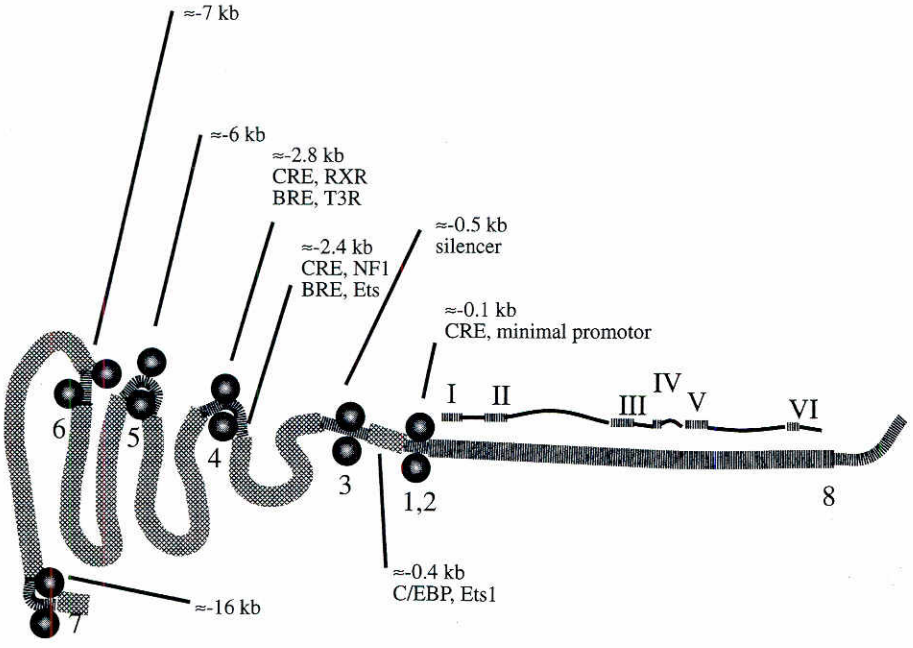

Fig. 9. The gene for the uncoupling protein: suggested regulatory promoter regions. *The figure is based on data combined from Boyer and Kozak, 1991; Cassard-Doulcier et al., 1993; Kozak et al., 1994; Yubero et al., 1994a,b; Alvarez et al., 1995; Rabelo et al., 1995. A detailed and comprehensive analysis is outside the scope of the present review but it is clear that response elements for a series of transcription factors have been identified in the promoter region. The numbers refer to DNase hypersensitive sites originally observed by Boyer and Kozak (1991). Most of the other letter combinations refer to suggested response elements earlier characterized elsewhere (CRE: cyclic AMP response element, etc.). However, the BRE elements are tentatively brown-fat specific (Kozak et al., 1994).*

lished); if anything, rather the reverse is seen: a decrease in *c-myc* expression in the tissue in the proliferative state. This absence of correlation between proliferation and increased *c-myc* expression may be in agreement with the present view that *c-myc* expression should rather be seen as response to PKC-activating systems (which may be related to cell division in many cell lines) than to cell division as such.

A better association to norepinephrine-induced cell proliferation is found for the transcription factor C/EBPα. In the same cell stages that respond proliferatively to norepinephrine stimulation, norepinephrine causes a decrease in the expression of C/EBPα (Rehnmark *et al.,* 1993). It is noteworthy that this decrease is only observed in cells in the proliferative state; in mature cells, C/EBPα gene expression is increased by norepinephrine. Furthermore, the effect is specific in as much as the transcription factor C/EBPβ does not show this norepinephrine-induced repression. As C/EBPα is discussed as a determinative factor involved in differentiation, a repressed expression would be in agreement with a promotion of proliferation at the expense of differentiation.

Cell division acceleration would probably also require increased capacities of enzymes directly involved in DNA synthesis, such as ribonucleotide reductase. In many tissues, such enzymes have been reported to be under negative control of the cAMP system; this should not be the case in brown-fat cells, and preliminary experiments do indicate positive effects of adrenergic stimulation on the expression of e.g. ribonucleotide reductase (Fredriksson *et al.,* unpublished results).

The developmental switch

Thus, from the studies referred to above it can be implied that the cells spontaneously progress from a state where an increase in cAMP is interpreted as a signal for proliferation to one in which it is interpreted as a signal for advanced differentiation (Fig. 11). This switch apparently occurs spontaneously, and it is difficult to avoid the feeling of using entelechy (the "internal goal-directed vital force" invoked by Driesch for the sea urchin development) in order to "explain" the phenomena of this apparently spontaneous differentiation pattern.

There is at the moment no clue as to what events control this switch. What is seen in cell culture is a rather concerted progression from the young to the mature state, and it is striking that practically all cells develop into brown-fat-like cells under these conditions. One may see the cells of the brown adipose tissue as being in a state akin to that of the cells in the embryo, already knowing their fate but waiting for the right cue to proceed. It could be suggested that it is "just" the age of the cells or the phenomenon of cell contact that activates the spontaneous differentiation (the DD in Fig. 8). This can, however, not be the situation in animals, where a stem-cell-like population seems to remain along the capillaries to allow for later proliferation.

The state of determination

The conclusion must therefore be that the cells are aware of their fate as potential brown-fat cells and already when they are still fibroblast-like, this makes them respond to stimuli in a particular way, different even from that of white-fat cells.

It is implicit in the above discussion of the "spontaneous" development and switch of the brown-fat cells in culture that the cells in question must already have been "determined" to become brown-fat cells – although their potential and determination to do so is only successively revealed, when the biochemical and morphological characteristics become evident. Furthermore, it is our impression that practically all cells in culture develop into true brown-fat cells. Indeed, when parallel cultures are grown with precursor cells isolated from white or brown adipose tissue depots, the cells in the two cultures develop differentially, despite the fact that the incubation conditions are identical (Néchad *et al.,* 1983). This means that all the precursor

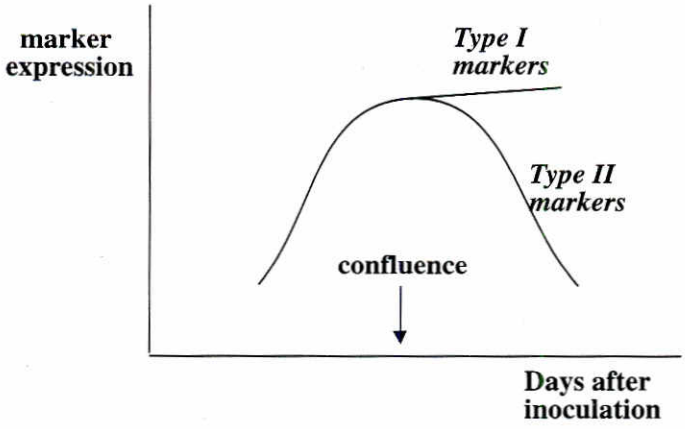

Fig. 10. Distinction between type I or type II behavior during maturation of brown-fat cells in culture. *It may be noted that chronic treatment with norepinephrine may be sufficient to alter the expression pattern for a given gene from type I to type II. (From Herron, 1992).*

cells must already be determined; there must be an early, embryonic determinatory event.

During normal development, the determination to become a brown-fat cell probably only occurs in a few cells rather late during embryonal development. This would be the most likely event, as the tissue is only found in mammals and even in these it only occurs very late during gestation (reviewed in Nedergaard *et al.*, 1986). However, serendipitous and unexpected information in this respect has been obtained in now classical studies by Svajger and collaborators (Skreb *et al.*, 1976) (recently repeated and advanced in our laboratory: Loncar, 1992). In these experiments, different germ layer combinations obtained from 9-day old rat embryos (where the three germ layers are distinguishable) were implanted under the kidney capsule of adult rats. From these implants, teratomas consisting of cell types corresponding to different organs of the body developed. What was particularly remarkable was that when only mesoderm was implanted under the kidney capsule, a significant fraction of the resulting teratoma cells were morphologically identifiable as brown-fat cells (Skreb *et al.*, 1976), and later studies confirmed that these cells were genuine brown-fat cells in as much as they were able to express the gene for the uncoupling protein (Loncar, 1992).

This development only took place when the mesodermal cells were explanted to the kidney capsule. If the mesodermal germ layer was grown *in vitro*, no such development took place. Thus, it would seem that there are environmental conditions in the kidney capsule localization which are sufficient to induce a determination of mesodermal cells into brown adipose tissue cells.

It is possible that this determination step may also be due to an effect of norepinephrine. When the mesoderm is implanted under the kidney capsule, the mesodermal cells do not receive the putative inductive factors from ectoderm or endoderm that would normally direct their further development towards muscle etc. The implant does, however, become vascularized (apparently the mesoderm releases some angiogenic factor/s), and e.g. through this, the tissue may come into contact with sympathetic innervation. If this is sufficient to initiate the differentiation process, it would quickly become a self-promoting process. This is because the tissue, already when only slightly differentiated, has the ability to attract sympathetic innervation; this has e.g. been observed in brown-adipose-tissue explants in the eye chamber (Néchad and Barnard, 1979). The background for this ability is that the tissue releases Nerve Growth Factor (Néchad *et al.*, 1994) and in this way directs sympathetic innervation towards it. As the kidney is under constant sympathetic control it may not be surprising that the mesoderm received a sufficient degree of sympathetic stimulation to promote its further differentiation. Thus, hypothetically, the development of the mesoderm is controlled during normal embryonal development by the inductive factors from the endo- and ectoderm and only late during embryonal development do these influences become sufficiently weak to allow for a determinatory influence of norepinephrine. In the kidney capsule, the norepinephrine effect would not have to compete with the other inductive factors, and determination would take place immediately. Further investigation into these questions should include studies of the ability of norepinephrine to promote mesodermal differentiation in tissue culture.

The central role of norepinephrine in the life of the brown fat cell

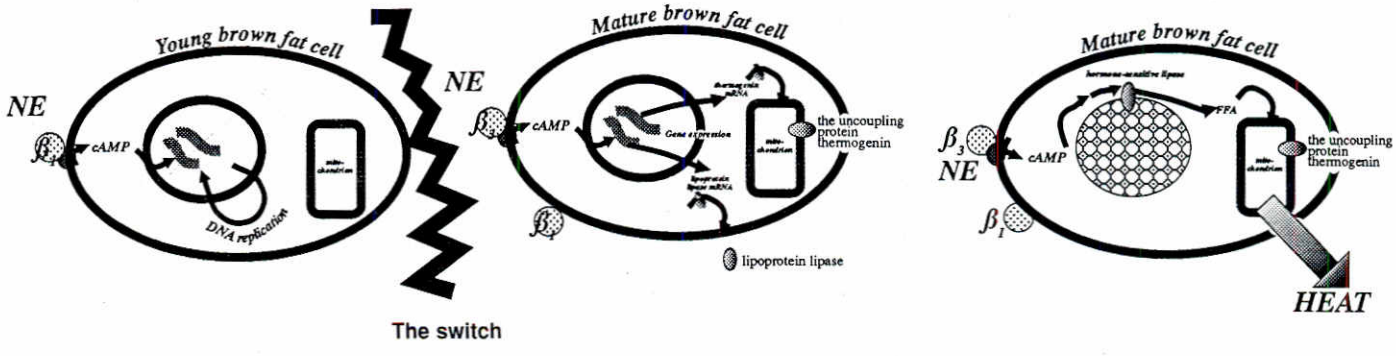

Fig. 11. The switch during the development of the brown-fat cell.

It is an experimental challenge – as brown adipose tissue is an exclusive mammalian tissue – that no other experimental system can be used to study the determination step of this tissue.

The evolution of a new tissue: a mammalian development

Brown adipose tissue is evolutionarily a new tissue. It is only found in mammals and no tissues can be described as forerunners. It is therefore open to speculation how mammals obtained this unique heat-producing tissue.

In this context it should be realized that the ability to regulate body temperature is not exclusive of mammals. Even e.g. reptiles show behaviorial body temperature regulation (and fever), and thus the need for extra heat production and the ability to induce this through shivering may have occurred rather early during pre-mammalian evolution. Extra heat production is, of course, not a useful occupation as long as the mammalian precursors were poorly isolated, but purportive heat production through shivering is found e.g. in birds (that lack brown adipose tissue but are well insulated). Thus, the mammalian forerunners may have become more insulated and may have started to develop the ability to shiver in order to keep warm.

A constant stimulation of muscular activity in the form of shivering would lead to a need for a constantly increased supply of substrate for combustion in the muscles. This, in turn, would lead to a constant norepinephrine-induced stimulation of (white) adipose tissue to deliver fatty acids for such combustion. There is evidence from present-day mammals that training (Stallknecht *et al.*, 1991) – or a cold exposure that induces shivering (Loncar *et al.*, 1988, 1989) – leads to morphological and enzymatic alterations in white-adipose-tissue mitochondria which gives them properties that in many ways resemble those of brown-fat mitochondria (except that they do not contain the uncoupling protein). It may be hypothesized that a similar situation occurred in the mammalian forerunners. Thus, we may have here the physiological background for the selection of an adipose tissue as the tissue that could be induced to provide an elevated metabolism when needed. However, these processes in themselves would not be sufficient. What was further needed and what apparently occurred was that one of the genes of the mitochondrial carrier proteins was duplicated and that this gene developed a new transport function: that of proton (equivalents), i.e. an uncoupling protein was invented, possibly through a minor alteration in the function of another mitochondrial carrier protein. From this point on, norepinephrine stimulation would lead not only to lipid mobilization in the adipose tissue and to induction of mitochondrogenesis – but also to an increased combustion of the released fatty acids. In this way, norepinephrine may have driven the evolution of this new tissue.

If we allow ourselves to extend this theme even further we may wonder about the significance of this step for the evolution of mammals. The properties normally pointed out as being the mammalian advantages are e.g. an improved insulation against thermal stress (hairs) – but a similar (or better) insulatory system was developed in the birds. The ability to develop the progeny internally has evolved on several occasions (e.g. in adders); the same may be said about the ability to produce bodily secretions which can feed the young. However, the ability to produce heat by an internal and nearly imperceptible method was truly new and unique. It may be argued that in combination with the improved insulation system, it was this mechanism for extra heat production that opened a new niche for the evolving mammalian animals: the night. Nights are cool, and animals without an ability for extra heat production will encounter a low body temperature and become inactive during the night due to this. The mammalian forerunners which started to develop a capacity for norepinephrine-induced heat production would therefore have been in a favorable situation when it was necessary to compete for prey. One may therefore wonder whether the development of a norepinephrine-induced capacity for heat production was not the crucial invention that made us – the mammals – successful.

Acknowledgments

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