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Research Article

Differential expression of genes for uncoupling proteins 1, 2 and 3 in brown and white adipose tissue depots during rat development

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Abstract. The different expression patterns of genes for uncoupling proteins (UCPs) 1, 2 and 3 (*ucp1*, *ucp2* and *ucp3*) were studied in interscapular brown adipose tissue (BAT) and in four white adipose tissue (WAT) depots (epididymal, inguinal, mesenteric and retroperitoneal) in male rats of different ages (18 days–12 months). UCP mRNA expression levels were determined by Northern blotting. In BAT, there were high levels of expression of UCP1 and UCP3 mRNA, but no detectable levels of UCP2 mRNA. Both *ucp1* and *ucp3* followed a similar expression pattern with age, with high levels in suckling rats which decreased to 50% or less in rats just under 2 months old, declining thereafter until 5 months and then recovering with age. However, an additional peak of expression was observed for *ucp3* at the age of 3 months. In WAT, *ucp1* expression was rare: occasional expression was found for UCP1 mRNA in the retroperitoneal depot in suckling rats and in the epididymal and inguinal depots in suckling and mature adult rats. *ucp2* and *ucp3* had different developmental expression patterns, but these were similar for each gene in the different depots studied. UCP3 mRNA was highly expressed in rats soon after birth, it decreased until 3 months, and increased thereafter, except for the mesenteric WAT where *ucp3* expression decreased until 7 months before recovering. The fact that changes with age of both *ucp1* and *ucp3* expression have a similar profile in BAT, which is also similar to the *ucp3* and also *ucp1* profiles in some WAT depots, might reflect a common regulatory pattern for the expression of these genes, and also a common function. In contrast to *ucp1* and *ucp3*, *ucp2* had a peak of expression at about 2 months, and lower expression at 3 months, suggesting different regulation and probably a different role for this UCP.

Key words. Uncoupling proteins; ageing; adipose tissue; development.

Uncoupling protein 1 (UCP1), a specific brown adipose tissue (BAT) protein of the inner mitochondrial membrane, is the major molecule involved in adaptive (non-shivering) thermogenesis in this tissue [1–4]. This protein acts by dissipating as heat the proton gradient generated by the respiratory chain, therefore uncoupling oxidative phosphorylation. Two main UCP1 homologues have been identified, UCP2 [5, 6] and UCP3 [7–9]. *ucp2* is expressed in white adipose tissue (WAT) and in a variety of other tissues, while *ucp3* is expressed predominantly in BAT and in skeletal muscle. Other homologues with abundant expression in brain, termed UCP4 and BMCP1 (brain mitochondrial carrier protein-1), have also been described [10, 11]. Several genes encoding proteins with a high degree of homology to UCP1 have also been identified in plants [12, 13].

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An increase in adaptive thermogenesis in BAT, including an increase in *ucp1* expression, is physiologically elicited by exposure to cold (cold-induced thermogenesis) or by excessive caloric intake (diet-induced thermogenesis) [2, 14]. The main physiological regulator of thermogenesis in BAT is noradrenaline, which acts through adrenergic receptors, especially through the β3 subtype [15, 16]. UCP1 is also induced by thyroid hormones (T3) [17], retinoid acid [18, 19] and naturally occurring carotenoids [20]. The regulation of the new members of the UCP family, UCP2 and UCP3, is still not well known [1, 4, 21, 22]. *ucp2* seems to be regulated by diet, with increased expression when overeating, not being affected by short-term cold exposure [5]. *ucp3* regulation appears more similar to that of UCP1, as both are upregulated by cold exposure and downregulated by fasting in BAT [23].

Unlike UCP1, the functions of its homologues are currently unknown [1, 21, 22]. UCP2 and UCP3 have been implicated in lipid utilisation [24] or antioxidant mechanisms [25, 26], but their relevance to body weight control has been argued. Both UCP2 and UCP3 possess an uncoupling action when ectopically expressed [5, 6, 27, 28]; however, in UCP1-ablated mice, the high ectopic expression of the novel UCPs in brown adipocytes has not been found associated with signs of mitochondrial uncoupling or thermogenesis [29]. Conversely, skeletal muscle mitochondria lacking UCP3 (from UCP3 knockout mice) are more coupled [26] and have less proton leak [30], indicating that UCP3 has uncoupling activity. Despite these effects on mitochondrial function, UCP3 does not seem to be required for body weight regulation, since UCP3 knockout mice do not develop obesity, are not cold sensitive and do not have upregulation of either UCP1 or UCP2 [26, 30]. Other observations, such as increased expression of UCP2 and UCP3 mRNA in skeletal muscle in response to food deprivation [31, 32], go against a putative thermogenic function of these new UCPs. However, direct evidence for a thermogenic role of skeletal muscle UCP3 has been recently provided [33], by showing that transgene mice overexpressing UCP3 in skeletal muscle are hyperphagic but weigh less than their wild littermates.

Earlier studies have suggested age-related decreases in brown fat thermogenesis, as indicated by measures of GDP binding to BAT mitochondria [34, 35], which was used as an index of UCP1 levels and the thermogenic capacity of brown fat. However, other studies, measuring UCP1 mRNA expression [36] or UCP1 levels [37], were not able to confirm lower levels in old compared with young rats. On the other hand, an inability to appropriately thermoregulate during cold exposure has been established in aged humans and in laboratory animals [34, 38], and has been related to a decreased response of UCP1 [see ref. 39] and UCP2 to cold [40]. Reduction in BAT thermogenic capacity with age seems to be caused, at least in part, by altered cellular signal transduction rather than by changes in neural and hormonal signalling. This is supported by the reduction in brown fat β3-adrenergic receptor observed with age [41, 42] in the presence of an increased activity of the sympathetic nervous system [43, 44]. Moreover, β3-mediated thermogenesis and β3-adrenoceptor signal transduction in BAT have been shown to be impaired with age [45]. A diminished response with age to food deprivation, in terms of decreasing UCP1 levels, has also been previously reported [46].

In addition to the UCP1 studies, changes in UCP2 and UCP3 mRNA expression in rats during development have been studied in BAT during the perinatal period [47] and different regulatory mechanisms for each UCP type determining their appearance have been suggested. However, as far as we know, a systematic study of changes in UCP mRNA expression in adipose tissues, from suckling to adult rats, has not been previously undertaken.

The aim of the present study was to characterise the ontogenic patterns, from suckling to 1-year-old rats, of *ucp1*, *ucp2* and *ucp3* gene expression in four different WAT depots (epididymal, retroperitoneal, mesenteric and inguinal) and in interscapular BAT.

Materials and methods

Chemicals. The RNA isolation reagent (Tripure), Hybond nylon membranes and most reagents for Northern blotting (digoxigenin-labelled probes, Dig-Easy Hyb, blocking reagent, anti-digoxigenin antibodies and CDP-Star) were from Boehringer Mannheim (Barcelona, Spain). Other reagents were purchased from Sigma (Madrid, Spain) and routine chemicals used were from Merck (Darmstard, Germany) and Panreac (Barcelona, Spain).

Animals. Male Wistar rats (CRIFFA, Barcelona, Spain), caged in groups of three animals, were acclimated to 22 °C with a 12 h light/12 h dark cycle and free access to a standard chow diet (Panlab, Barcelona, Spain). Male rats (n = 5) were weighed and then killed by decapitation at different ages: 18, 55, 93, 159, 212, 294 and 355 days, always at the same time in the morning. After killing the animals, the interscapular BAT and the epididymal, inguinal, retroperitoneal and mesenteric WAT depots were rapidly removed in their entirety, frozen in liquid nitrogen and stored at –70 °C until RNA analysis.

Northern blot analysis. Total RNA was extracted using Tripure reagent following the instructions provided by Boehringer Mannheim. Twenty micrograms of total RNA, denatured with formamide/formaldehyde, was fractionated by agarose gel electrophoresis as described

elsewhere [48]. The RNA was then transferred onto a Hybond Nylon membrane in 20 × saline sodium citrate (SSC) buffer 1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) by capillary blotting for 16 h [48], and fixed with UV light.

The mRNA for UCP1, UCP2, UCP3 and the 18S rRNA (used as a control to check the loading and transfer of RNA during blotting) were detected by a chemiluminescence-based procedure, using antisense oligonucleotide probes [48] which were synthesised commercially (Boehringer Mannheim), labelled at both ends with a single digoxigenin ligand. The probes were, for UCP1: 5′-GTTGGTTTTATTCGTGGTCTCCCAGCATAG-3′, for UCP2: 5′-GGCAGAGTTCATGTATCTCGTCTTGACCAC-3′, for UCP3: 5′-GACTCCTTCTTCCCTGGCGATGGTTCTGTAGG-3′ and for 18S: 5′-CGCCTGCTGCCTTCCTTGGATGTGGTAGCCG-3′.

Pre-hybridisation was at 42 °C for 15 min in DIG-Easy Hyb. Hybridisation was at 42 °C overnight in DIG-Easy Hyb containing the oligonucleotide probe (34 ng/ml for the UCPs and 70 pg/ml for 18S rRNA). Hybridised membranes were submitted to 2 × 15 min washes at room temperature with 2 × SSC/0.1% sodium dodecyl sulphate (SDS), followed by 2 × 15 min washes at 48 °C with 0.1 × SSC/0.1% SDS. After blocking, the membranes were incubated first with an anti-digoxigenin-alkaline phosphatase conjugate and then with the chemiluminescent substrate CDP-Star, essentially as in the protocols provided by Boehringer Mannheim. Finally, membranes were exposed to Hyperfilm ECL (Amersham, UK); bands in films were analysed by scanner photodensitometry and quantified using the BioImage program (Millipore, Bedford, Mass.). Blots were probed for UCP1, and then stripped by exposure to boiling 0.1% SDS and reprobed sequentially for UCP2 and UCP3 mRNA and for 18S rRNA.

Duplicates of RNA isolation and Northern blot analysis were performed for all samples.

Statistical analysis. All data are expressed as the means ± SE. The statistical significance of differences between UCP1 and UCP3 mRNA expression in BAT was assessed by one-way analysis of variance (ANOVA). Differences in UCP2 and UCP3 mRNA expression among the WAT depots for the different ages were assessed by two-way ANOVA. $P < 0.05$ was the threshold of significance.

Results

UCP1, UCP2 and UCP3 mRNA expression levels in brown and white adipose tissues. *ucp1* and *ucp3 w*ere highly expressed in BAT, while *ucp2* was not detectable. In WAT, in contrast, there were high expression levels of *ucp2*, low levels of *ucp3* and only occasional low expression of *ucp1* (see fig. 1, corresponding to 10-month-old animals).

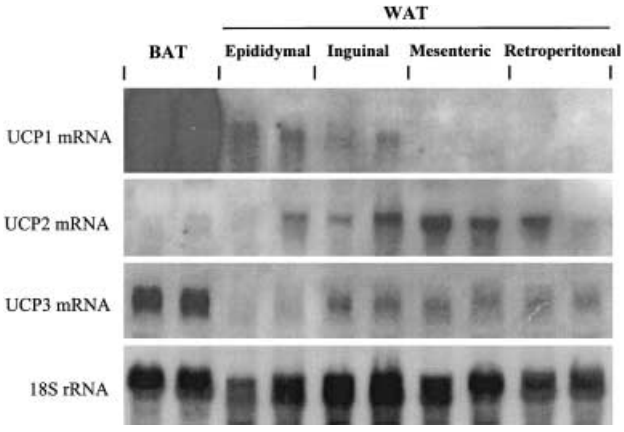

Figure 1. Representative Northern blots for UCP1, UCP2 and UCP3 mRNAs in interscapular BAT and in epididymal, inguinal, mesenteric and retroperitoneal WAT in 10-month-old rats. Twenty micrograms of total RNA was used for specific determination of the UCP mRNAs by Northern blotting, using 18S rRNA as a control. Exposure times were 5 min for *ucp1*, 15 min for *ucp2* and 15 min for *ucp3*.

The mesenteric WAT depot did not present any detectable *ucp1* expression at any time during the period studied. There was occasional (suckling rats) UCP1 mRNA expression in the retroperitoneal depot (results not shown), while in the epididymal and inguinal depots, there was also expression in mature adult rats (fig. 1). In adult rats, UCP1 mRNA expression in the inguinal depot appeared earlier than in the other depots, with increasing levels from 7 months on (fig. 2).

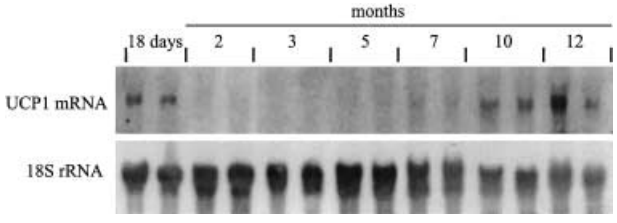

Figure 2. Representative Northern blot for UCP1 mRNA expression in inguinal WAT depot of rat at different ages. Twenty micrograms of total RNA was used for specific determination of the UCP1 mRNA, using 18S rRNA as a control. Exposure time was 15 min for *ucp1*.

Changes with age in UCP1 and UCP3 mRNA expression in BAT. As shown in figure 3, UCP1 and UCP3 mRNA expression in BAT followed a similar behaviour with age ($P > 0.05$). The expression of both mRNAs was high in suckling rats (18 days) and decreased to 50% or less in 2-month-old rats. A new peak was observed for *ucp3* at the age of 3 months, which was not evident for *ucp1;* the expression of both genes declined thereafter

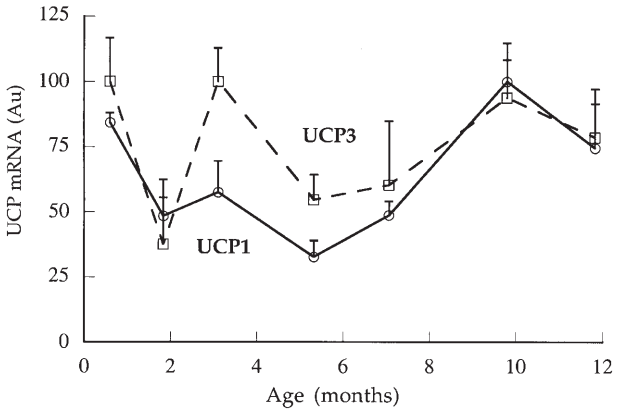

Figure 3. UCP1 and UCP3 mRNA expression levels in interscapular BAT in rats of different ages (18 days–12 months) measured by Northern blotting. Results represent means ± SE (n = 5) of ratios of specific mRNA levels to 18S rRNA (arbitrary units, Au). The maximum level of expression for each gene was set to 100% and the other values are relative to this. The expression pattern of both UCPs with age was not significantly different (P > 0.05, one-way ANOVA).

untill 5 months when they began to recover, reaching a peak of expression at the age of 10 months.

Changes with age in UCP2 and UCP3 mRNA expression in WAT depots. Figure 4 shows the pattern of mRNA expression for UCP2 in WAT depots during development. The expression of UCP2 mRNA followed a similar pattern in all WAT depots studied from 18-day- to 10-month-old rats. There was a transient peak of expression at the age of 2 months and then an increase with age. However, in the epididymal and retroperitoneal WAT depots, UCP2 mRNA decreased after the age of 10 months, both having a similar behaviour with age (P > 0.05).

The pattern of mRNA expression for UCP3 in WAT depots during development is shown in figure 5. In all the WAT depots studied there was a general trend for UCP3 mRNA expression to decrease with age until 3 months, with a recovery thereafter. In the epididymal WAT, the levels remained stable from 5 months on. In the retro-

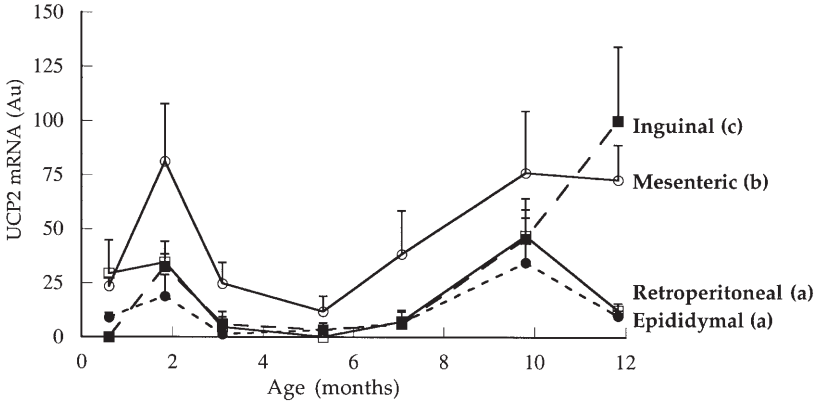

Figure 4. UCP2 mRNA expression levels in epididymal, inguinal, mesenteric and retroperitoneal WAT depots in rats of different ages (18 days–12 months) measured by Northern blotting. Results represent means ± SE (n = 5) of ratios of specific mRNA levels to 18S rRNA (arbitrary units, Au). The maximum level of expression was set to 100% and the other values are relative to this. Depots not sharing a common letter are significantly different (P < 0.05, two-way ANOVA).

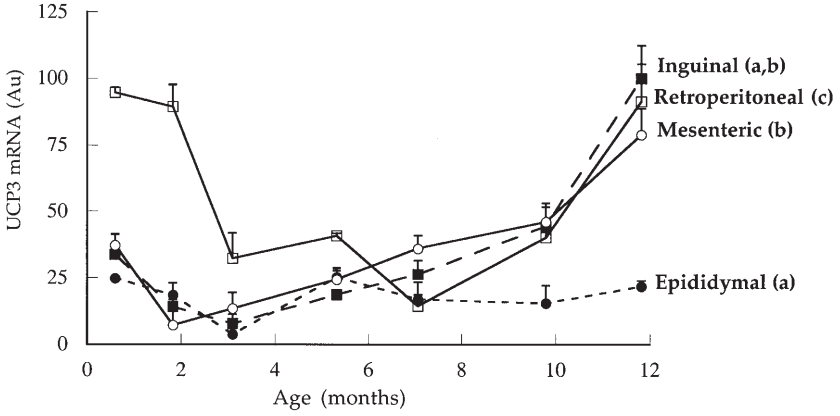

Figure 5. UCP3 mRNA expression levels in epididymal, inguinal, mesenteric and retroperitoneal WAT depots in rats of different ages (18 days–12 months) measured by northern blotting. Results represent means ± SE (n = 5) of ratios of specific mRNA levels to 18S rRNA (arbitrary units, Au). The maximum level of expression was set to 100% and the other values are relative to this. Depots not sharing a common letter are significantly different (P < 0.05, two-way ANOVA).

peritoneal WAT, low levels were maintained from 3 to 7 months, recovering thereafter. In inguinal and mesenteric WAT depots, the UCP3 mRNA levels reached, with age, much higher levels than in young rats, while in epididymal and retroperitoneal depots, the levels at the end of the period studied were not higher than the initial levels.

Discussion

BAT expresses high mRNA levels of UCP1 and UCP3 and the expression of both genes has a close ontogenic profile, with high levels in suckling and mature adult rats. Heat produced by BAT is required in special conditions such as birth and early development, at which time this tissue is well developed [1]. It is also known that there is an inability to appropriately thermoregulate with ageing due to a decrease in the number of β3-adrenergic receptors [39]. Taken together, these facts could explain the increased UCP1 mRNA expression in BAT in suckling animals, producing the required heat in newborn animals, and in our older rats, to compensate for the decrease in thermogenesis. Our results agree with the relatively high levels of UCP1 mRNA expression previously described in suckling [47, 49] and mature [36] rats.

It is noteworthy that UCP3 mRNA expression in BAT followed the same pattern as UCP1, supporting a similar thermogenic function for both genes in this tissue. Parallel regulation of *ucp1* and *ucp3* is not surprising, since *ucp3* has been reported to be highly responsive to the regulatory signals of energy balance that also modulate BAT *ucp1*; *ucp3* expression is upregulated by T3 in muscle and BAT [9, 50], by the β3-adrenergic receptor agonists CL316243 in WAT [9] and CGP-12177 in BAT [48] and by long-term cold exposure in BAT [50]. Similar regulation of *ucp1* and *ucp3* could explain the similar expression pattern of both genes with ageing, thus suggesting that the stimulating factors that increase UCP1 mRNA in conditions requiring extra heat production also stimulate UCP3 mRNA expression.

Nevertheless, despite the close behaviour in BAT of both *ucp1* and *ucp3*, a relatively transient peak for UCP3 mRNA expression levels was found at 3 months of age, but was not evident for UCP1. This difference suggests that specific factors could operate and cause differences in the relative expression of *ucp1* and *ucp3* at particular ages. Different regulatory mechanisms of *ucp1* and *ucp3* have also been indicated in other physiological situations, although involving different tissues. This is the case for food deprivation, which decreases UCP1 mRNA levels in BAT [23, 46] while increasing UCP3 mRNA expression in skeletal muscle [23, 32].

In WAT, the developmental pattern of UCP3 mRNA expression in the different depots studied was similar to that of BAT. *ucp3* expression levels were high early in development, decreased thereafter and increased again in our older rats. *ucp1* expression varied among the different WAT depots. In the epididymal and inguinal depots, UCP1 mRNA was found in suckling and mature adult rats, and increased with age in the inguinal depot. The retroperitoneal WAT depot also expressed UCP1 mRNA, but only in suckling rats (data not shown). This unusual expression of *ucp1* in WAT occurs at the same time as high expression of *ucp1* takes place in BAT, and this is also accompanied by high *ucp3* expression levels in both BAT and WAT. Thus, thermogenesis, which in rodents is mainly dependent on BAT, could also be dependent on WAT in special situations where higher levels of heat production are needed. *ucp1* expression in WAT has been previously shown under certain conditions, such as stimulation with β3-agonists [48, 51–53]. Immunocytochemical examinations [52, 53] have shown that fat pads, considered conventionally as white fat, contain multilocular and mitochondria-rich cells which are indistinguishable from typical brown adipocytes. Some of the multilocular cells present in WAT depots are the ones expressing UCP1, whereas the unilocular cells are negative for this protein [52]. Himms-Hagen et al. [53] have proposed two origins for these multilocular fat cells: the main source could be convertible unilocular white adipocytes, with many mitochondria containing UCP3, while a small proportion may have arisen from brown pre-adipocytes already present in the WAT and able to express UCP1. Thus, UCP1 expression in our WAT samples could be tentatively explained by the presence of a certain number of brown adipocyte-like cells in the fat pads. This seems more likely than UCP1 being expressed by unilocular white adipocytes.

The expression pattern of *ucp2* clearly differs from that of *ucp3* and *ucp1*. UCP2 mRNA had a very transient peak of expression at 2 months when the relative levels of the other *ucp* genes were low, then the levels of expression dropped rapidly, with practically no detectable levels at 6 months. The peak of *ucp2* expression could be related to the change in diet, from milk to chow, or to a compensatory response to the relatively low levels of expression of the other *ucp* genes which occur at this time. In fact, opposite expression of UCP1 and UCP2 has been found in several situations [40, 50], supporting the idea that UCP2 could be induced in BAT to compensate for the inability of UCP1 to maintain thermogenesis. Mice lacking UCP1 have a compensated induction of UCP2 in brown fat and they are cold-sensitive but not obese [54]. This suggested a greater role for UCP2 in diet-related regulation of energy balance than in cold-induced thermogenesis. However, the involvement of UCP2 in cold-induced thermogenesis cannot be ruled out, considering that UCP2 expression is upregulated in young rats at 72 h after cold exposure, when UCP1 expression is down-

regulated [40]. Interestingly, UCP2 induction was almost blunted in 2-year-old rats [40]. In fact, in addition to the UCP1 inactivation observed in old animals [39], a decrease in *ucp2* gene expression in some adipose tissue depots and a failure of UCP2 induction by the cold stimulus [40] have also been associated with the decline in thermogenic ability with ageing and with the increased susceptibility to obesity.

In summary, *ucp1* and *ucp3* expression have, in general terms, a similar developmental pattern with age in BAT and in some WAT depots, with higher levels of expression soon after birth and in mature adult rats. This similarity in *ucp3* and *ucp1* expression patterns could favour a putative thermogenic role of UCP3 not only in BAT but in WAT also, although this is not conclusive. Moreover, it could indicate that both genes are under common regulation. However, the influence of specific factors for *ucp1* and *ucp3*, which operate at specific stages of development, must also be taken into account. The different pattern of *ucp2* expression might suggest a different role for UCP2 compared with UCP1 and UCP3, not apparently related to any extra-heat need. However, further studies are required to elucidate the factors controlling the different regulation of UCP expression, particularly of UCP2 versus both UCP1 and UCP3 during development.

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