

UNCOUPLING PROTEINS AND SLEEP DEPRIVATION

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INTRODUCTION

Fifteen years ago Michel Jouvet showed that in pontile cats, who are poikilothermic, central temperature is the principal factor that regulates the quantity of paradoxical sleep (PS), its ultradian periodicity, as well as the duration of PS episodes (17). Ten years later, Jouvet and colleagues also showed that PS is exquisitely sensitive to conditions that impair oxidative metabolism, and linked the positive effect of hypothermia on PS to its ability to reduce metabolic rate, and to protect the brain against hypoxic alterations and oxidative stress (1). A few months ago, in occasion of the meeting held in Lyon in his honor, Michel Jouvet strongly maintained that the link between sleep regulation, energy metabolism, and thermoregulation should be further explored, because it may hold important clues relative to the functions of sleep. Below is the first study by our laboratory to address this issue, and we dedicate this work to Prof. Jouvet. The paper shows that sleep deprivation has profound effects on the expression of uncoupling proteins, which play a major role in thermoregulation, as well as in the response to oxidative stress.

Long-term sleep deprivation (SD) in rats causes an early increase in food intake and in energy expenditure (EE), followed by a complete loss of body fat, a decrease in body weight, and, ultimately, death (21, 22). In humans, EE is increased in insomniacs relative to normal sleepers (7), and in normal sleepers on nights of poor sleep relative to baseline nights (6). Also, humans affected by fatal familial insomnia, a prion disease characterized by an extreme and prolonged insomnia, show an early increase in EE followed by a decrease in body fat and body weight (20). Thus, SD appears to have significant and consistent metabolic effects in both humans and animals.

In long-term sleep deprived rats the rate of increase in EE correlates negatively with their survival time (21, 22). Most importantly, rats that show a similar increase in EE but are not sleep deprived do not die (21, 22). Thus, the increase in EE during SD is a core element of the syndrome developed when rats are prevented from sleeping, and clarifying its mechanisms may help understand the detrimental effects of prolonged sleep loss.

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The increase in metabolic rate in long-term sleep deprived rats cannot be accounted for by an increase in waking time per se or in gross motor activity (21, 22). Instead, Rechtschaffen and colleagues (21, 22) have suggested that the increase in EE during SD is most likely due to an increase in resting EE. This is because when long-term sleep deprived rats are allowed to enter NREM sleep for short periods of time, they maintain very high levels of heart rate, as high as during forced waking and higher than during normal spontaneous waking. How SD could affect resting EE, however, is not clear.

Resting EE is the largest component of daily metabolic rate and is required to maintain physiological tissue function in the resting state. A significant component of resting EE is accounted for by the mitochondrial proton leak, which uncouples oxygen consumption in the respiratory chain from ATP synthesis. The leak is mediated by the so called uncoupling proteins (UCPs, 9, 18). UCPs include UCP1, which has a well established thermogenic role in the brown adipose tissue, and four newly discovered proteins: UCP2 is ubiquitous, UCP3 is mainly expressed in the skeletal muscle, and UCP4 and UCP5 are preferentially expressed in the brain. The leak mediated by UCPs accounts for 20 to 30% of resting EE in liver, muscle, and brain. An increase in mitochondrial uncoupling during SD, therefore, could significantly affect resting EE.

The goal of this study was to determine whether SD in rats is associated with the induction of UCPs. To this aim, we measured mRNA levels of UCP2, UCP3 and UCP5 in cerebral cortex, skeletal muscle and liver during the spontaneous sleep/waking cycle and after short-term (8 hours) and long-term (7 days) SD. The results show that the expression of UCP2 is increased in the skeletal muscle and liver as a function of prior time awake. Thus, UCP2 may represent at least one of the mechanisms by which SD increases resting EE.

METHODS

Sleep recordings and sleep deprivation (SD).

Under pentobarbital anesthesia (75 mg/kg, ip), adult male WKY rats (300-450 g) were implanted with screw electrodes in the skull to record the electroencephalogram (EEG) and with silver electrodes in the nuchal and temporal muscles to record the electromyogram (EMG). Transmitters (Barrows, Inc., Sunnyvale, CA) were implanted in the peritoneum to record peritoneal temperature (T_{ip}). One week after surgery, all rats were recorded continuously for as many days (7-30 days) as required to satisfy the criteria for their respective groups. EEG signals were visually scored for 4-sec epochs (SleepSign™, Kissei Comtec America, Inc., Irvine, California). As shown in Table I, four experimental conditions were used: 1) spontaneous sleep, 2) short-term total SD, 3) spontaneous wakefulness, 4) long-term total SD. Rats for conditions 1-3 were kept in a 12:12 light:dark schedule (light on at 10:00, ~150 lux), while long-term sleep deprived rats were maintained in constant light to flatten their diurnal sleep and temperature rhythms. Sleeping rats (8hS group, $n = 6$) were killed during the light hours (at 18:00), at the end of a long period of sleep (> 45 min, interrupted by periods of wakefulness < 2 min), and after spending at least 75% of the previous 8 h asleep. Short-term total sleep deprived rats (8hSD group, $n = 6$) were killed at the same circadian time as the 8hS group, after being kept awake for 8 h by introducing novel objects in their recording cage. Every new object was delivered just following the first signs of synchronization in the frontal EEG signal. Spontaneously awake rats (8hW, $n = 6$) were killed 8 h after light off (at 6:00),

after a long period of continuous wakefulness (> 1.5 h, interrupted by periods of sleep < 5 min) and after spending at least 70% of the previous 8 h awake. Long-term SD was performed by the disk-over-water method essentially as in (4). This method can induce effective chronic sleep loss in one animal, while its yoked control has an acceptable amount of sleep in spite of receiving the same stimulation. Briefly, a rat to be sleep deprived (TSD) and its yoked control (TSC) were housed in rectangular Plexiglas cages. During baseline, the disk was rotated once per hour for 6 seconds to habituate the rats to rotation. The baseline period continued until sleep, food intake, body weight and temperature had stabilized (usually 3-7 days) in both rats. Cage air temperature was thermostatically maintained at 25 ± 1 °C. When total SD was initiated and sleep onset was detected in the sleep deprived rat, the disk was rotated slowly by a computerized monitoring system, forcing both rats to walk in a direction opposite to disk rotation to avoid the water. When the sleep deprived rat was spontaneously awake, the disk was stationary and the yoked control rat was able to sleep. Total SD lasted for 7 days. Animal protocols followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Wisconsin.

Ribonuclease protection assays (RPAs) and quantitative real time PCR (qPCR).

Rats were deeply anesthetized with isoflurane (within 2 min) and decapitated. The head was cooled in liquid nitrogen and the whole brain was removed. Cerebral cortex, hippocampus, and cerebellum were dissected, while the rest of the brain was left intact. Liver and skeletal muscles (hind limb) were also removed. Samples were immediately frozen on dry ice and stored at -80 °C. Total RNA was isolated from right cerebral cortex, liver and skeletal muscle of each animal by using Trizol (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's instructions. Final RNA concentrations were determined spectrophotometrically. RPAs and qPCR were used to measure mRNA levels of *UCP2* (cerebral cortex, muscle, liver), *UCP3* (muscle), *UCP5* (cerebral cortex) and *PGC1* (cerebral cortex, muscle). For RPAs, antisense RNA probes complementary to the coding region of *UCP2*, *UCP3* and *UCP5* were synthesized by run-off transcription from a linearized DNA template using the MAXiScript *in vitro* transcription kit (kit (Ambion, Austin, TX) and [α -³²P]UTP (New England Nuclear-Du Pont, Natick, MA). RPAs were performed using the RPAII™ kit (Ambion). Real time qPCR was performed as already described (10). Sequence Detection System 5700, Perkin Elmer, Foster City, CA). Reverse transcription reactions were carried out in parallel on DNase I digested pooled total RNA from 8hS, 8hSD, 8hW (n = 6 rats/group), and from TSD and TSC rats (n = 7/group). PCR was done in quintuplicate for each sample condition assayed and relative quantities determined based on the equation of the line of best fit derived from the standard curve ($R^2 \geq 0.985$).

RESULTS

Table I shows mean values (\pm SEM) of wakefulness, NREM sleep and REM sleep for the last 8 hours before sacrifice for all experimental groups. As expected since rats are nocturnal, 8hS animals were spontaneously asleep for most of the light period, while 8hW animals were spontaneously awake for most of the dark period. Since 8hS and 8hSD rats were sacrificed at the same time of day but in opposite behavioral state, and 8hSD and 8hW rats were sacrificed 12 hours apart but in the same behavioral state, the effects of time of day (day/night) and of behavioral state (sleep/wakefulness) on UCP expression could be dissociated. Table I also shows that the DOW method was effective in enforcing prolonged wakefulness in the long-term sleep deprived rats and in allowing their yoked controls to maintain most of their sleep. Indeed, during the entire SD period, TSD rats lost on average (mean \pm S.E.M.) $67\% \pm 5$ and $92\% \pm 5$ of their daily baseline values of NREM and REM sleep,

respectively. Their yoked controls, by contrast, lost $35\% \pm 9$ and $42\% \pm 25$ of their daily baseline values of NREM and REM sleep, respectively.

Tab. I. Percentages of wakefulness, NREM sleep and REM sleep for the last 8 recording hours before sacrifice in the 4 experimental groups.

Eight hours represent the entire duration of the experiment for rats in groups 1-3, and are representative of any time period during the 7 days of SD in TSD and TSC rats.

Experimental groups			Behavioral state		
			wakefulness	NREM	REM
1. spontaneous sleep	8hS (n = 6)	Asleep	21.7 (2.1)	63.6 (1.2)	14.7 (0.5)
		Sleep	94.2 (0.4)	5.8 (0.4)	0.0
2. short-term total sleep deprivation (novel objects)	8hSD (n = 6)	Deprived	71.7 (1.9)	23.3 (2.1)	5.0 (1.7)
		Awake	81.7 (3.2)	17.8 (2.3)	0.5 (0.1)
3. spontaneous wakefulness	8hW (n = 6)	Asleep	62.5 (1.5)	33.7 (0.9)	3.8 (1.9)
		Awake	81.7 (1.9)	23.3 (2.1)	5.0 (1.7)
4. long-term (7 days) total sleep deprivation (disk-over-water method)	TSD (n = 7)	Sleep	81.7 (3.2)	17.8 (2.3)	0.5 (0.1)
		Deprived	62.5 (1.5)	33.7 (0.9)	3.8 (1.9)
	TSC (n = 7)	Yoked Controls	62.5 (1.5)	33.7 (0.9)	3.8 (1.9)

Figure 1 summarizes expression levels of UCPs in brain and peripheral tissues of all experimental groups. Levels of mRNA were measured with RPAs and/or qPCR, which gave similar results, and are expressed as percentage change relative to the sleeping animals (8hS). In the cerebral cortex, we found no change in UCP2 and UCP5 expression between rats spontaneously asleep and rats spontaneously awake for 8 hours. By contrast, relative to the 8hS group, UCP2 and UCP5 mRNA levels were increased (40-50%) after short-term SD and slightly decreased (10-30%) after long-term SD. Cortical expression of PGC1, a coactivator of nuclear receptors that stimulates mitochondrial biogenesis and increases UCP2 expression (26), did not change in any experimental group. Despite the use of several combinations of primers, we were not able to measure UCP4 expression in the cerebral cortex using qPCR.

In the liver, UCP2 mRNA levels increased 2-fold in 8hSD rats, 8-fold in TSD rats, and 6-fold in their yoked controls (TSC). In the skeletal muscle, UCP3 mRNA levels increased to the same extent (~ 3-fold) in 8hSD, TSC and TSD animals relative to 8hS rats. By contrast, UCP2 mRNA levels showed little change in 8hSD and TSC rats relative to 8hS rats, but were more than 4-fold higher in TSD rats relative to all other experimental groups. Figure 2 shows UCP2 and UCP3 expression levels in the skeletal muscle as measured by RPAs.

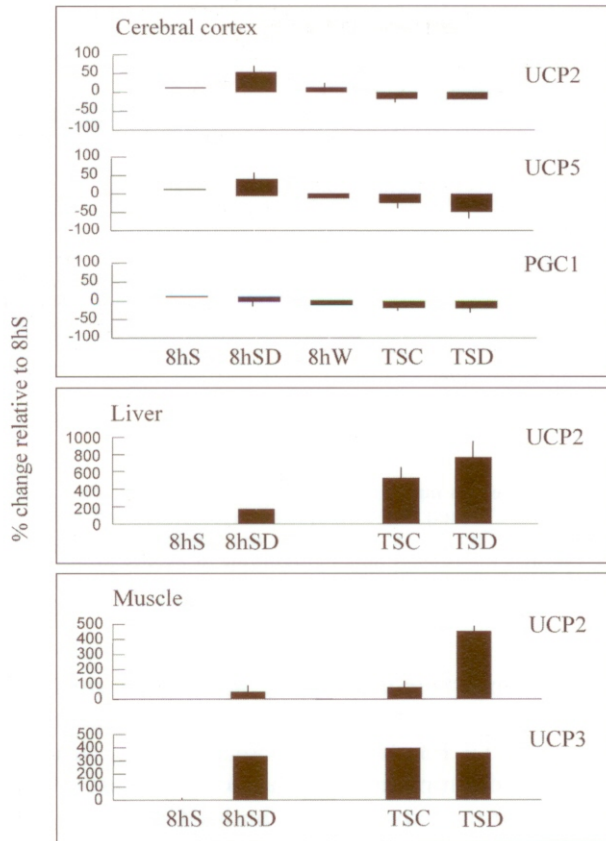


Fig. 1. mRNA levels of UCPs and PGC1 in cerebral cortex, liver, and skeletal muscle during sleep (8hS), spontaneous wakefulness (8hW), and after short (8hSD) and long (7 days, TSD) sleep deprivation

TSC rats are the yoked controls for TSD rats. Expression data for liver and muscle in 8hW animals were not available. Expression mRNA levels refer to qPCR and/or RPAs experiments, which gave similar results, and are expressed as % change relative to 8hS. Note that the y axes have different scales.

DISCUSSION

Until a few years ago, the only characterized uncoupling protein was UCP1, whose expression is restricted to the brown adipose tissue and whose induction is largely dependent on the noradrenergic system (18). UCP1 plays a crucial role in cold-induced thermogenesis in rodents and in human neonates. However, UCP1 is not expressed outside the brown adipose tissue and therefore cannot be a major determinant of resting EE. Recently, four new UCPs have been identified and, because of their widespread distribution and established uncoupling properties, UCP2 and UCP3 are more likely to mediate overall changes in EE (9). The uncoupling properties of UCP2 and UCP3 were first inferred from their sequence homol-

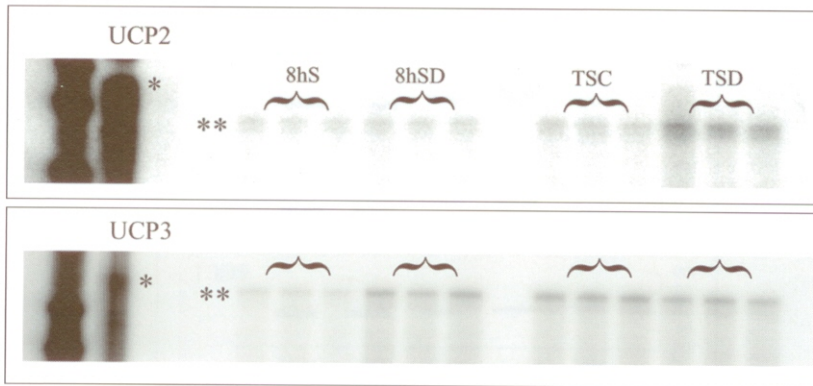


Fig. 2. Representative examples of RPA experiments showing mRNA levels of UCP2 and UCP3 in the skeletal muscle.

A β -actin antisense riboprobe was used to normalize the amount of sample RNA. Lane 1 (from left): molecular weight markers. Lane 2: UCP2 and UCP3 riboprobes hybridized with 10 μ g yeast RNA, incubated without RNase mixture. Most of the signal is given by the full-length UCP2 or UCP3 (*) probe. Lanes 3-14: UCP2 and UCP3 probes hybridized under conditions of excess probe with 2 μ g of pooled skeletal muscle RNA (3 replicas/pool). The protected fragments are indicated by (**).

ogy to UCP1 and by measuring their uncoupling activity *in vitro*. More recently, experiments *in vivo* had provided more direct evidence (e.g. 11, 12, 16). For instance, mice overexpressing UCP3 show an increase in EE, associated with elevated uncoupling and increased temperature in the skeletal muscle (11).

The present study shows that the expression of both UCP2 and UCP3 is increased in animals that are not allowed to sleep, and that UCP2 may be particularly important for the changes in EE related to sleep loss. UCP3 mRNA levels in skeletal muscle were similarly high in all experimental groups relative to sleeping rats, i.e. in short-term sleep deprived rats that were kept awake for only 8 hours, in long-term sleep deprived rats that lost >80% of their total sleep during 7 days of SD, and in yoked controls that lost 30 to 40% of their sleep. UCP2 mRNA levels, on the other hand, were high in liver and skeletal muscle of long-term sleep deprived animals but not (muscle), or much less so (liver), in yoked controls that experienced sleep restriction or in rats that were sleep deprived only for a short period of time. Thus, UCP2 expression in peripheral tissues seems to be a function of prior time awake. Since the skeletal muscle is the largest tissue in the body, an increased muscular expression of UCP2 could significantly influence the overall resting EE and therefore represent an important mechanism by which SD can increase resting metabolic rate.

It is becoming increasingly clear that thermogenesis may not be the only or the most important function of UCPs (15, 25). In fact, by promoting partial uncoupling of the respiratory chain, UCPs not only dissipate energy in the form of heat, but also diminish the production of superoxides, and decrease the likelihood of calcium entry in the mitochondrial matrix. Consistent with this view, UCP2 overexpressing ani-

mals show a decrease in brain lipid peroxidation (16), while UCP2 knockout animals show an increased free radical production by monocytes, which may explain their increased resistance to infections (2). Moreover, superoxides can activate mitochondrial UCPs and may be the main controllers of their transcription (13). Thus, because of their ability to reduce free radical production, UCPs are emerging as potential antioxidants.

Several hypotheses about the functions of sleep rest on the assumption that wakefulness represents an oxidative challenge for the brain. It has been claimed, for instance, that sleep may allow the removal of free radicals accumulated in the brain during wakefulness (23). In a recent study (14), however, we have shown that long-term SD does not increase oxidant production, protein oxidation or lipid peroxidation, neither in the cerebral cortex nor in the liver or skeletal muscle. In that study we speculated that the noradrenergic system of the locus coeruleus, which is active during waking and much less so or not at all during sleep (3), could be at least partially responsible for the absence of oxidative stress during SD. Norepinephrine can inhibit lipid peroxidation *in vitro* (19) and can also promote the survival of dopaminergic neurons by directly reducing oxidant production (24). Interestingly, the expression of UCP2 and UCP3 in the skeletal muscle is sensitive to stimulation by beta-adrenergic agonists (8). Moreover, in humans, UCP2 and UCP3 muscle mRNA levels are highly correlated with nocturnal urinary norepinephrine excretion (5). Thus, it cannot be excluded that sleep deprived subjects are protected against oxidative stress through the activation of UCPs, possibly mediated by the noradrenergic system.

SUMMARY

In both humans and animals sleep deprivation (SD) produces an increase in food intake and in energy expenditure (EE). The increase in EE is a core element of the SD syndrome and, in rats, is negatively correlated with survival rate. However, the mechanisms involved are not understood. A large component of resting EE is accounted for by the mitochondrial proton leak, which is mediated by uncoupling proteins (UCPs). We measured UCP2, UCP3, and UCP5 mRNA levels in rats during the spontaneous sleep/waking cycle and after short (8 hours) and long (7 days) SD. During spontaneous sleep and waking there was no change in the level of mitochondrial uncoupling as measured by UCPs expression, either in the brain or in peripheral tissues. During SD, by contrast, UCP3 expression in skeletal muscle was elevated, but the increase was similar, compared to sleep, after both short-term and long-term SD. UCP2 expression, on the other hand, was strongly increased in the liver and skeletal muscle of long-term sleep deprived animals and much less so, or not at all, in yoked controls or in rats that lost only 8 hours of sleep. Since the skeletal muscle is the largest tissue in the body, an elevated muscular expression of UCP2 is likely to affect the overall resting EE and may thus contribute to its increase after SD.

Acknowledgments. - This study was supported by a grant from NIMH.

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