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Arousal from hibernation and BAT thermogenesis against cold : central mechanism and molecular basis

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AROUSAL FROM HIBERNATION AND BAT THERMOGENESIS AGAINST COLD: CENTRAL MECHANISM AND MOLECULAR BASIS

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1. INTRODUCTION

As a general definition hibernation is the over all decrease in metabolic rate and body temperature exhibited by some species, from a variety of classes of animals, in response to cold, dark environments when food resources are limiting. Whether this physiological response to harsh environmental conditions has evolved on numerous occasions or whether it represents the utilization of an ancestral physiology remains speculative. While the technological problems that result from the large changes in body temperature severely limit chronic in-vivo investigations, recent advances in the understanding of hibernation physiology have been obtained from molecular biology. However, the results of *in vivo* and *in vitro* studies serve to highlight the fascinating and extraordinary physiology of hibernators, that will undoubtedly provide a rich resource for future biomedical applications. Researchers of hibernation physiology straddle the problems associated with the transitions between the warm and the physiologically well-characterized state and the cold and the physiologically alien state. This path provides a physiological and a philosophical challenge the nature of which currently tests our understanding and invites speculation, theory and experimentation.

Although hibernation is a seasonally dependent behavior under natural conditions, hibernators are able to enter into hibernation under laboratory-controlled environments provided certain key conditions such as photoperiod, temperature, food are appropriately manipulated (Fig. 1). The environmental conditions necessary to induce hibernation differ between species. Two different states, torpor and hibernation, both are associated with hypometabolism and hypothermia but differed in the degree and duration of these associated responses. The somewhat arbitrary distinction between the two states being related to degree and duration of the response. At present the hibernation cycle can be conveniently divided into four stages. A preparatory phase immediately before entrance, an entrance or cooling stage, a maintenance stage where body temperature remains low but stable and an arousal stage where body temperature increases to cenothermia. These gross divisions will be refined upon more complete understanding of the physiology associated with each stage. Hypometabolism causes hypothermia and vice versa since lowering temperature decreases the rate of every biochemical reaction. Hypothermia is, of course, not only caused by hypometabolism but also induced by an increase in heat loss to low ambient temperature when metabolic heat production remains unchanged. Like warm blooded animals, incapable of hibernation, hibernators when cenothermic regulate their body temperature within 2~3 °C around 37 °C. This temperature range is in sharp contrast to the amplitude of the annual

fluctuation of body temperature which, in the most extreme case as in arctic ground squirrels (*Spermophilus parryii*), approaches 40 °C (Barnes, 1989). When homeothermic animals are exposed to cold, most invoke mechanisms that facilitate energy metabolism and inhibit heat dissipation from the body surface to maintain their body core temperature. In the case of a comparatively short exposure to the cold, cutaneous vasoconstriction and piloerection are used in addition to increased heat production. In contrast, these thermoregulatory mechanisms must be inhibited during the entrance to hibernation whilst the reserve should occur to facilitate increase of body temperature during arousal (Hammel et al., 1973). Indeed, it would appear that during all the stages of hibernation temperature regulation is a carefully controlled active process.

Does the central nervous system (CNS) retain control of the body temperature irrespective of behavioral state of hibernator? Experimental evidence indicates that during each stage of hibernation the animal is capable of responding to changes in its external environment with increases in body temperature and/or oxygen consumption in response to cold stimulation (Hammel et al., 1968; Heller and Hammel, 1972; Heller and Colliver, 1974; Florant and Heller, 1977) or mechanical stimulation (Pakhotin et al., 1993). The decrease in temperature during the entrance stage is now accepted not to be simply a process of passive heat loss but rather it is a process of active suppression of metabolic- and temperature-regulatory mechanisms (Heller et al., 1977a; Heller and Glotzbach, 1977b; Nedergaard and Cannon, 1990). In arousal from hibernation these mechanisms that were inhibited are now engaged but it would be too simple to agree that the physiological mechanisms responsible for rewarming during arousal are solely the result of disinhibition of the mechanisms inhibited during cooling.

Two modes of heat production are classified, namely shivering thermogenesis that uses skeletal muscle movement and non-shivering thermogenesis (NST). Although shivering is the most potent method for heat production in body temperature rise, the inhibitory effect of low temperatures on innervating motor neuron and muscle function determines that shivering is inefficient at the very low body temperatures typically observed in the maintenance stage of hibernation. Skeletal muscles are thought to contribute significantly to thermogenesis once the body has warmed to a temperature appropriate for efficient motor neuron function. Despite a paucity of direct evidence, NST is considered as the mechanism whereby body temperature is raised from that maintained during hibernation to that which can be efficiently augmented by the enlistment of shivering thermogenesis. NST is localized in brown adipose tissue (BAT) - a highly vascularized tissue that is localized in close proximity to a number of crucial

organs, interscapular with its venous connections close to the heart, around carotid vessels and between skin and vertebra. This pattern of distribution contributed to it being considered crucial for arousal from hibernation (Wunnenberg and Merker, 1978).

The contribution of BAT to total NST is estimated to be about 60 % in rats (Foster and Frydman, 1978) and 50 % in cenothermic Jangarian hamsters (Heldmaier et al., 1983). The remaining 40-50% of NST may be derived from brain, liver and possibly skeletal muscles (Horwitz, 1978; Colquhoun et al., 1990; Eldershaw et al., 1996). Chronic exposure to cold environment leads to hypertrophy of BAT and increased efficiency of BAT thermogenisis in both non-hibernators and hibernators. Kuroshima and co-workers showed that removal of interscapular BAT led to a loss of improved cold tolerance and a significant reduction of NST in rats (Kuroshima et al., 1984). Heat production by BAT is controlled by direct neuronal input from sympathetic nerves and humoral factors, e.g. adrenaline and glucagone (Kuroshima, 1993).

This review will be focused towards studies on CNS control of BAT function, the molecular mechanism of NST by brown adipocytes, the relevance to hibernation will be summarized and recent studies will be introduced. Terminology of this paper follows 'Glossary of terms for thermal physiology' edited by IUPS Thermal Commission, which appeared in Jpn. J. Physiol. 51: 245-280 (2001).

2. CNS CONTROL OF NST/ *CNS sites relating to BAT thermogenesis*

Hypothalamic contribution

A wealth of experimental evidence has demonstrated that the hypothalamic region is the principal thermoregulatory center of the brain. Within the hypothalamus, the ventromedial hypothalamic nuclei (VMH) have been the most intensely studied region in the search for a command center that facilitates BAT thermogenesis. Electrical and chemical stimulations of the VMH induce a robust increase in BAT thermogenesis (Rothwell, 1989; Halvorson et al., 1990) and neuronal activity, as estimated by glucose uptake, which was preferentially enhanced in the VMH neurons upon cold exposure (Morimoto et al., 1986). Moreover, cold-induced BAT thermogenesis was inhibited by local anesthetic microinjection into the VMH (Imai-Matsumura et al., 1984).

Although numerous studies support the conclusion that a functional VMH is essential for BAT thermogenesis, some contra-indicative results from recent studies are note worthy. Fos protein expression, an intracellular indicator of activated neurons, in VMH neurons was not clear in rats exposed to cold

stimuli for less than 24 hr but was prominent in rats exposed for 14 days (Miyata et al., 1995). More recently, retrograde labelling of sympathetic innervation of BAT by injection of pseudorabies virus into intrascapular BAT by Bamshad and co-workers (1999) failed to demonstrate virus infection in VMH neurons despite evidence for viral particles in almost all other hypothalamic nuclei such as paraventricular nuclei, medial preoptic area and lateral hypothalamic area. This apparent contradiction between the results of the electrophysiological and histochemical studies requires closer examination and suggests a long multi synaptic pathway between BAT and the VMH.

The preoptic area of the anterior hypothalamus (PO/AH), contains thermosensitive neurons, and so has long been a focus of investigations aimed at determining putative CNS control over NST (Kanosue et al., 1998). However, despite numerous descriptive studies outlining the thermal characteristics of hypothalamic neurons a demonstration of the involvement of the POA in the regulation of NST remains to be forthcoming.

Midbrain/ prepontine

In the last decade, remarkable progress has been made in understanding the role of midbrain inhibitory mechanism on BAT thermogenesis. Prepontine transection increased core and BAT temperature, however, if the cut was just rostral of the mammilary body, then these changes did not occur. Shibata and co-workers suggested that a mechanism generating tonic inhibition of BAT thermogenesis existed in the lower midbrain in rats (Shibata et al., 1987). This conclusion was strengthened when microinjections of procaine (a local anesthetic) into the mid brain in and around the retrorubral field and rubrospinal tract evoked increased BAT temperature while microinjection of glutamate (an excitatory amino acid) into the same region decreased BAT temperatures (Shibata et al., 1996; Shibata et al., 1999b). Electrical stimulation of the same midbrain region also decreased BAT and rectal temperatures in anesthetized rats (Hashimoto et al., 1998a). On the basis of these studies it was concluded that neurons constituting a tonic inhibitory input on BAT thermogenesis were probably located in the medial area of the rubrospinal tract and in and around the retrorubral field of the rat. Hashimoto and co-workers (1998b, 1999a) reported that this midbrain tonic inhibitory mechanism existed in the homologus region of the Syrian hamster (*Mesocricetus auratus*) (Fig.2, Fig3). Although the lower mid brain region is recognized as a CNS loci involved in shivering mechanism, shivering was not observed during the transection that induced BAT thermogenesis, thus the CNS mechanism controlling shivering appears to be independent of the lower midbrain mechanism inhibiting BAT thermogenesis in rats and probably hamsters.

Lower Brainstem

The sympathetic nervous system provides the principal efferent pathway regulating BAT thermogenesis irrespective of whether the stimulus is cold exposure, diet or immobilization (Himms-Hagen, 1995; Rothwell and Stock, 1997). However, the efferent neuronal pathway from the midbrain to the sympathetic preganglionic neurons in the spinal cord innervating BAT is not completely determined. Recent observations by Shibata and co-workers demonstrate the involvement of neurons in the inferior olive (IO) in the CNS circuit controlling BAT function in anesthetized rats (Shibata, 1999a; Uno and Shibata, 2001). These workers showed that the increase in BAT temperature induced by removal of the tonic midbrain inhibition, induced by procaine microinjections, was accompanied by the appearance of c-Fos-positive neurons in the IO and the intermediolateral (IML) cell column of the thoracic spinal cord. Electrical stimulation and glutamate microinjections into the IO both facilitate BAT thermogenesis. Reciprocally, midbrain procaine-induced BAT thermogenesis was blocked by electrolytically produced IO lesions. These workers concluded that central thermal signals produced from the lower midbrain are transmitted to BAT through the IO and IML. Morrison and co-workers have demonstrated that the Raphe pallidus neurons are also involved in the central mechanism regulating BAT thermogenesis in rats (Morrison, 1999a; Morrison et al., 1999b; Morrison et al., 2000; Morrison, 2001).

Other brainstem regions including peritrigeminal, paratrigeminal, supramamillary, lateral parabrachial nuclei, the dorsal part of the pontine reticular formation, nucleus of the solitary tract have also been demonstrated to be responsive to acute 3 hr cold exposure in rats (Baffi and Palkovits, 2000), of these regions the parabrachial nucleui (PBN) in pontine region could be involved in the regulation of NST. Although the majority of the PBN neurons respond to noxious stimuli, a sub set appears to respond to innocuous thermal stimuli from spinal sensory neurons (Menendez et al., 1996; Bester et al., 2000). Recent studies by Kobayashi and co-workers (2001) showed that electrical stimulation of the PBN increased BAT heat production while heat production in response to cold stimulation was significantly inhibited by PBN ablation in rats.

3. CNS CONTROL OF NST DURING HIBERNATION

The understanding of the CNS mechanism controlling NST in rats has progressed, much less is known about the role of, or the mechanisms that control, NST in hibernation. It seems clear that thermoreceptivity of CNS persists during hibernation. Hibernating animals increased oxygen

consumption and maintained body temperature without arousal in response to cold stimulation (Lyman and O'Brien, 1972; Mills and South, 1972; Hammel et al., 1973; Heller and Colliver, 1974; Weidler et al., 1974). Whether NST contributes to temperature control during the maintenance stage of hibernation is unknown. What is clear is that the contribution of NST to the increase in body temperature during arousal varies from species to species. When shivering was blocked by curarization, hibernating bats were able to arouse at a normal rate, but hibernating golden hamsters and dormice took more than twice the normal time to reach cenothermic temperature (Hayward and Lyman, 1967).

Hypothalamic contribution

Ground squirrels with ablation of rostral region of the anterior hypothalamus entered hibernation and maintained body temperature slightly above room temperature for several days but failed to arouse (Satinoff, 1967). Such "oneway" experiments should be interpreted with caution, however, this suggests a regional distinction between loci involved in entrance into and maintenance/arousal stages of hibernation (Kanosue et al., 1998).

The question whether CNS neurons are capable of generation and propagation of action potentials (AP) at body temperatures near freezing is paramount in determining how receptivity to external stimuli is translated into temperature-compensatory response and how recruitment of neurons occurs to induce arousal. In both in vivo and in vitro experiments, persistence of firing activity or spontaneously firing activity in CNS neurons during hibernation were confirmed even in several hibernator species when body temperatures were low (Wunnenberg et al., 1986; Hashimoto et al., 1998c). CNS neurons of non-hibernators like guinea pigs, cats and dogs did not show any spontaneous activity at temperature below 27 °C (Wunnenberg et al., 1986). Indeed the distinction between hibernators and non-hibernators could be characterized by their ability to sustain the activity of CNS neurons under extreme hypothermic conditions. However, electrophysiological recordings from brain slices showed no clear difference in thermal characteristics of CNS neurons between Wistar rats and European hamsters (Hashimoto et al., 1998c). In both animals PO/AH neurons exhibited spontaneous AP but the propagation rate and amplitude was dramatically reduced at low temperature. It remains to be determined if these small, slow APs were capable of inducing post-synaptic depolarization. Biochemical analysis by Goldman and Willis (1973) found no differences between sodium-potassium dependent ATPase activity in rat and hamster brain at temperature from 5 to 38 °C consistent with the similarity in thermal characteristics of PO/AH neuron in rats and hamsters.

The SCN is though to be the major brain locus generating circadian rhythmicity (Zucker et al., 1983; Kilduff et al., 1989; Dark et al., 1990). Since the entry of torpor is synchronized with the entry into sleep, the clock mechanism in SCN has been thought to be intimately associated with the induction of hibernation. A variety of evidence from divers hibernating species confirmed a relationship between SCN and hibernation mechanism (Satinoff, 1967; Kilduff et al., 1982; Kilduff et al., 1989; Dark et al., 1990; Kilduff et al., 1990; Bitting et al., 1994; Grahn et al., 1994; Ruby et al., 1996; Ruby et al., 1998; O'Hara et al., 1999). The contribution of SCN to circadian fluctuation of the body temperature is well documented in cenothermic animals and the existence of the circadian rhythmicity in body temperature was also reported in golden-mantled ground squirrels during the maintenance stage of hibernation (Grahn et al., 1994). Neuronal activity of SCN, however, was not persistent. No cell was observed to fire at temperatures below 16 °C. In general, as with the PO/AH, most properties of SCN cells from hibernators (golden mantled ground squirrels) and non-hibernators (rats) were similar (Miller et al., 1994). If the persistence of circadian rhythmicity is a function of AP-dependent neurotransmission from the SCN, then one would expect that hibernation with a body temperature maintained below 16 °C should be characterized by an absence of circadian fluctuations in temperature (Miller et al., 1994). Furthermore, from observations of clock gene-mutant Syrian hamsters, Oklejewicz and coworkers (2001) suggested that the circadian system does not control periodicity of hibernation and arousal onsets in hibernation, at least in this species. The SCN may play a certain role in the mechanism of entry into hibernation, however, the possibility that this nucleus participates in triggering mechanism for arousal from hibernation seems very slight.

Hippocampus

Hippocampal participation in the initiation of the mechanism of arousal from hibernation was first report by Chatfield and Lyman (1954) in hamsters, and was followed by studies on other hibernator species (Chatfield and Lyman, 1954; Mihailovic, 1972; Hooper et al., 1985; Horowitz et al., 1987; Eckerman et al., 1990; Igelmund and Heinemann, 1995). Although the onset of electrical activity in hippocampus preceded that in other brain regions during arousal from hibernation, the neuronal activity was not detected until the brain temperature reached about 17 °C in hamsters (Chatfield and Lyman, 1954; Mihailovic, 1972) and was completely blocked in brain slices below 16 - 14 °C in hamsters (Horowitz et al., 1987; Igelmund and Heinemann, 1995). It is unlikely that hippocampus takes any part in the initiation of arousal from hibernation.

A possible role of Midbrain/Prepontine

BAT thermogenes is reported to be involved in thermogenesis during arousal from hibernation (Hayward and Lyman, 1967) however, a functional role for the above-mentioned midbrain tonic inhibitory mechanism of BAT thermogenesis has yet to be demonstrated. Hibernating Syrian hamsters were forced to arouse by handling. The head was mounted in a stereotaxic apparatus and standard procedures were employed to microinject glutamate or saline into the midbrain inhibitory region whilest the temperature of interscapular BAT and rectum were recorded (Hashimoto and Kuroshima, 2001b). The rates of temperature increase in BAT of hamsters treated with glutamate was significantly slower than that in saline-injected control animals (Fig 4). Decerebration, by transection rostral to the midbrain region inhibited the rates of temperature increase in BAT of the forced arousal, while transection caudal to the midbrain inhibitory region did not change the rate of temperature increase in BAT during the forced arousal (Hashimoto et al., 2001a). These results are consistent with the suggestion that the activation of NST during arousal requires the inhibition of the activity of the midbrain tonic inhibitory mechanism, possibly originates from the rostral hypothalamic area. At present it is unknown if the APs recorded in PO/AH neurons (Satinoff, 1967; Hashimoto et al., 1998c) connect to the midbrain inhibitory network or if they are capable of inducing functional post-synaptic events.

At present there are very few studies directed towards examining neurotransmitter levels in any brain region during hibernation but the few results from separate brain regions can be assembled to present a cohesive picture of synaptic events in non-essential brain regions, speculatively hippocampal and higher cortical areas. In ground squirrels, immunohistochemical mapping showed a clear increase in histaminergic fiber density when animals were sacrificed during the maintenance stage of hibernation although histamine decarboxylase mRNA expression was not increased relative to non hibernating animals (Sallmen et al., 1999). This result is consistent with reduced release of neurotransmitter into the synaptic space. Although not directly related to histamine, decreased levels of extracellular GABA have been demonstrated *in vivo* in hibernating ground squirrels (Osborne et al., 1999). Reversible ultra structural changes in synaptic innervation of hippocampal neurons, that would be expected to inhibit synaptic release of neurotransmitters, has been demonstrated in hibernating ground squirrel (Popov and Bocharova, 1992a; Popov et al., 1992b).

It is clear that hibernation is controlled by the central nervous system as mentioned above. However, it is still unclear how it is participating in control of hibernation, and the nerve mechanism

which may have been concerned with hibernation control is left behind as a future examination subject. Alternatively, research followed to a center from the effector molecules which should be controlled in the effector mechanism as a way which clarifies the control mechanism of hibernation has also been advanced. Also about the substances which changes synchronizing with each phase of hibernation, the function of the substances is clarified comparatively and it is also becoming clear that the substance is controlled by the nerve nature. It will be important to investigate behavior of those effector molecules in detail in a series of the time course of hibernation. Then, it surveys about the molecule mechanism which shows change correlated with hibernation.

4. MOLECULAR PROSPECTIVE OF HIBERNATION

Numerous substances have been documented to change concentration in blood and other tissues during a bout of hibernation. It is clearly important to draw a clear distinction between secondarily induced changes that are consequent of physiological compensations to reduced body temperature and changes essential to the mechanisms controlling hibernation, although in reality this demarcation is vague. Molecular-orientated investigations offer a methodology appropriate for the investigation of the globally coordinated responses that facilitate and perhaps control hibernation and also offers the possibility determining whether a ubiquitous intracellular control rather than a "command center" approach which directed earlier eras of hibernation research. The localization of tissue actively involved in the regulation of hibernation might be indicated by examining levels of mRNA transcription and protein translation provided the rate of degradation between genes is constant at hibernating and cenothermic temperatures. Unfortunately the majority of molecular biologically oriented studies do not allow for repeated sampling from the same animals at different stages of hibernation and thus between treatment analysis may be complicated by comparisons to cold acclimated control groups that did not/could not hibernate. Whether mRNA and protein are newly synthesized during hibernation is a question which should be also resolved. Although the overall level of mRNA appears slightly decreased or constant during hibernation (Srere et al., 1992; Frerichs et al., 1998) the rate of *in vivo* synthesis of cerebral mRNA is 8-fold reduced (Bocharova et al., 1992) - a factor which should be considered when determining if changes measured in the level of mRNA transcription result from changes during hibernation or are residual from changes implemented preceding entrance to the hibernating state.

A number of research groups have focused on the physiological changes that occur within the

preparatory period immediately prior to the entrance to hibernation. This is a difficult because no practical predictive biochemical marker of the onset of hibernation has been demonstrated. Initial transfusion experiments demonstrated the existence of a blood-born "hibernation inducing trigger (HIT)" with seasonal profile compatible for a role in hibernation (Dawe and Spurrier, 1969; Oeltgen et al., 1978). The temperature and metabolic depressing effect of these opioid-like proteins (Oeltgen et al., 1982; Bruce et al., 1987), was to some extent consistent with the state-dependent effect of intracerebral opioids on temperature (Tanaka et al., 1985) Although a role for HIT in hibernation remains controversial these results demonstrate that some aspects of the hibernation physiology may involve opioid receptor stimulation (Horton et al., 1998) which may have practical applications in cardiology. Kondo and co-workers have demonstrated that synthesis of liver derived "hibernation specific protein complex (HSPC)", isolated from the blood of chipmunks (*Citelus lateralis*) but not non hibernators, was inhibited during hibernation consistent with an annual rhythm where the lowest levels coincided with the hibernation period (Kondo and Kondo, 1992). A stimulatory and inhibitory regulatory mechanism involving testosterone and thyroxine, respectively, has been demonstrated (Kondo et al., 2000). Inconsistent with the role for HSPC as a principal factor inducing entrance or arousal from hibernation is the observation that plasma HSPC levels remain low during interbout cenothermia. However critical studies utilizing a subunit of HSPC capable of passage into the brain (Kondo et al., 2000) are yet to be published. In contrast to the decreased levels of plasma HSPC in chipmunks, blood alpha2 macroglobin levels and activity and liver mRNA are increased in hibernating ground squirrel. These increases probably occurred in the preparatory phase preceding hibernation (Srere et al., 1992).

Within the last decade, a number of genes were identified as being differentially expressed during hibernation (Squire and Andrews, 2000) while *in vivo* quantification of protein translation has produced some interesting results (Frerichs et al., 1998). A partial list of changes in gene expression associated with hibernation includes: increased alpha-globulin mRNA and protein (Srere et al., 1992) and decreased hibernation-related proteins (Takamatsu et al., 1993) in liver, increased UCP-1 mRNA in BAT (Boyer et al., 1998; Liu et al., 1998; Hashimoto et al., 1999b), increased lipase and UCP-2 mRNA in white adipose tissue (Wilson et al., 1992; Boyer et al., 1998), increased glyceraldehyde-3-phosphate dehydrogenase and UCP-3 mRNA in skeletal muscle (Soukri et al., 1996; Boyer et al., 1998), increased pancreatic lipase mRNA and protein and pyruvate dehydrogenase kinase mRNA and protein in heart (Andrews et al., 1998), increased in early immediate genes and decreased prostaglandin synthase mRNA

in brain (O'Hara et al., 1999) and increased c-Fos in SCN of the hypothalamus and neuropeptide Y mRNA in the arcuate nucleus (Bitting et al., 1994; El Ouezzani et al., 2001).

At present, it would appear that the majority of these changes in mRNA transcription occur in the preparatory and arousal stages of hibernation. Judicious monitoring of the animals brain temperature and respiratory rate at the time of sacrifice may assist in more accurately determining in what stage of hibernation did the changes in mRNA expression actually occur. At present, it is not routinely determined if the changes in mRNA transcription levels represent newly transcripted mRNA while differential rates of *in vivo* degradation between the mRNA may also influence apparent changes in levels of mRNA. Determination of *in vivo* protein synthesis, by quantifying the rate of labelled leucine incorporation into proteins, demonstrated that protein synthesis in brain, heart and liver was greatly inhibited by the entrance and maintenance stage of hibernation (Frerichs et al., 1998). Inhibition of protein synthesis results from reversible increase of phosphorylation of ribosomal elongation factor 2 (Chen et al., 2001). This result has far reaching implications for hibernation physiology.

UCP and hibernation

UCP-1, the "thermogenic protein of BAT" is particularly interesting from the view of regulation of NST during hibernation (Nedergaard et al., 2001) while several UCP-gene families are reported. Somewhat paradoxically, the mechanisms controlling NST have been more thoroughly investigated in mice and rats than in hibernators. The UCP-gene family which should be discussed in the context of this paper includes 3 subtypes, coding for UCP-1, -2 and -3 proteins (Bouillaud et al., 2001). At cenothermia, it is considered that a positive relationship exists between newly synthesized mRNA content and the level of protein production and biological function. However, for NST ability this relationship is probably complicated by the action of endogenous purine nucleotides like GDP (Klingenberg and Echtay, 2001). *In vitro* GDP binds to UCP1 on the inner mitochondrial membrane to inhibit the thermogenic function of UCP 1 protein althought the natural inhibitor is probably ATP because of its cellular abundance. The degree of *in vitro* binding is used as an index of biologically functional UCP-1 protein *in vivo* because GDP dose not interfere with the oxidative phospohorylation system (Klingenberg, 1999). The factors influencing the binding of GDP to UCP-1 remains controversial but evidence exists to support reduced binding in response to increased sympathetic stimulation and noradrenaline induced by cold exposure (Rothwell and Stock, 1984; Young et al., 1984; Desautels and Dulos, 1988; McDonald et al., 1988).

Acclimation to cold in mice, rats, squirrels and gerbils has been shown to cause increases in the amount of BAT and in the mitochondria content of BAT, in UCP-1 mRNA and UCP-1 protein and probably also in the amount of functional UCP-1 protein by unbinding of GDP (Milner et al., 1989; Nizielski et al., 1989; Nizielski et al., 1995). Hamsters utilize a modified strategy in that although NST ability is increased, this is not the result of hypertrophy of the existing large stores of BAT but rather results from enhanced UCP-1 synthesis and unmasking (Himms-Hagen, 1984). Hamsters, unlike other hibernators, do not undergo a pre-hibernation fattening and subsequent aphagic period (Boyer and Barnes, 1999) but require constant access to food. These differences in behavior may be reflected as differences in the profile of differentially regulated gene expression during the hibernating period. As an example, hibernation in jerbils would appear to activate genomic processing that is related to food restriction (El Ouezzani et al., 2001) - processes that should not be applicable to hamsters.

At this moment, only UCP-1 is known to be increased in amount and uncoupling efficiency in response to cold stimulation in cenothermic animals (Nedergaard et al., 2001) whilst mice lacking the ability to express UCP-1 could not maintain their body temperature in a cold environment (Enerback et al., 1997; Nedergaard et al., 1999; Nedergaard et al., 2001). Since the changes in amounts and/or activity of UCP-1 protein correlate well with the intensity of the NST in non-hibernators and hibernators at cenothermia, it is speculated that UCP-1 may play a significant role in regulation of body temperature changes during the maintenance and arousal stages of hibernation. The role of the other UCP-gene family proteins is yet to be determined although mRNA transcription appears to be responsive to cold stimulation. Changes of the level of expression of UCP-1 mRNA and homologues (UCP-2, UCP-3) in BAT during hibernation were measured in arctic ground squirrels (Boyer et al., 1998), in Daurian ground squirrels (Liu et al., 1998; Liu et al., 2001) and in golden hamsters (Fig.5) (Hashimoto et al., 1999b). In each species, UCP-1-mRNA levels in BAT removed from hibernating animals, with body temperatures near freezing, were increased relative to the summer active animals but were not significantly different from cold acclimated animals. Levels of mRNA for UCP -2 and UCP -3 in other tissues are clearly increased in hibernation (Boyer et al., 1998). In arousing ground squirrels UCP-1 mRNA was not different from hibernating squirrels but levels of functional UCP-1 were increased relative to levels in animals during maintenance stage of hibernation but unchanged from cenothermic levels (Liu et al., 1998). Although it is thought that NST is mediated by the amount and activity of UCP-1 in infant and adult non hibernators, the evidence for such a relationship in hibernation is not evident on the basis of

mRNA levels alone. The relationship could be clarified if future studies also examined functional UCP-1 levels by tests of GDP binding. Interestingly, increased level of UCP-1 gene expression, increased GDP binding and enhanced NST can be induced by a variety of stressors in rats (Gao et al., 2001). The existence of such a cross induction of NST in wild (squirrels) or tame (hamster) hibernators could be envisaged to have adaptive significance but will serve to complicate the relationship between UCP-1 levels and hibernation.

5. CONCLUSION AND PERSPECTIVES

After the first description of BAT by Konrad von Gesner in 1551 (Trayhurn and Nicholls, 1986), it has long been suspected that BAT contributes to NST but there were only a small number of studies which could be said to provide direct evidence of a relationship (Foster and Frydman, 1978; Heldmaier et al., 1983; Kuroshima et al., 1984). Recently, molecular biological techniques have strengthened the conviction that BAT has a role in NST at cenothermia by demonstrating that mice genetically deficient of BAT lose the ability to maintain body temperature in a cold environment (Lowell et al., 1993; Klaus et al., 1998; Stefl et al., 1998) and BAT lacking UCP-1 does not respond to noradrenaline (Nedergaard et al., 1999; Nedergaard et al., 2001). In hibernation, the evidence for a role of BAT and NST in maintenance of body temperature during hibernation or in initiating the increase in body temperature during arousal to a level at which shivering thermogenesis can be efficiently utilized is suggestive but incomplete. Like most of the questions in hibernation physiology the development of analytical techniques that enable serial samples from the same animal across the hibernating cycle will clarify many controversies.

Hibernating animals maintain decreases in metabolic rate and temperature accompanied by ultrastructural changes in CNS tissue (Popov and Bocharova, 1992a; Popov et al., 1992b) and restructured molecular biosynthesis (Frerichs et al., 1998), despite this they retain the ability of graded and relatively rapid responses to external stimuli such as temperature or tactile stimulation (Raths and Hensel, 1967; Heller and Hammel, 1972; Heller and Colliver, 1974; Florant and Heller, 1977). Furthermore, in the absence of such stimuli, they can exhibit periodic, brief arousals to cenothermia before returning to hibernation.

The mechanism of arousal is another aspect that is obscure. It could be considered, for example, that arousal resulting from external stimulation is subject to a different mechanism of control

than is periodic arousal. The ability to respond to external stimuli requires a functional neuronal circuit that can recruit the whole animal to arousal. Electrophysiological studies (Hashimoto et al., 2001a) demonstrate that some parts of the midbrain and brain stem are capable of generating modified APs, which may translate to functional intercellular communications. Electrophysiological (Horowitz et al., 1987; Igelmund and Heinemann, 1995) and histological (Popov and Bocharova, 1992a; Popov et al., 1992b) studies clearly demonstrate that other parts of the brain do not possess a communicative ability at very low body temperatures. How some circuits maintain functional ability remains obscure. However, one can speculate that one such circuit may link the sensory input to the BAT via the hypothalamus and midbrain inhibitory pathways.

The problem to be addressed in periodic arousal is of a different nature. The early phase of hibernation is associated with an almost total inhibition of protein synthesis (Frerichs et al., 1998; Chen et al., 2001). This, however, is a component of a widespread reduction in biochemical activity. Just how this reduction in metabolic activity is orchestrated and also is related to the preparatory phase of hibernation remains unknown. It is, therefore, pertinent to ask the question ‘once the animal is in hibernation and its cellular molecular activity severely depressed, how does it reactivate metabolism to normal aroused levels?’ One possibility is that molecular mechanisms involved in energy production are subject to inhibition by a variety of inhibits that bind to regulator units on the effector molecules. The binding and degradation characteristics of these inhibitors will differ at body temperatures found in the hibernator. So as time in hibernation passes some inhibitors will degrade or unbind, the sequence of unbinding may have specificity which could free the genomic machinery so that it now starts to function at low temperature, eventually sufficient metabolic activity would ensure that the threshold for arousal occurs.

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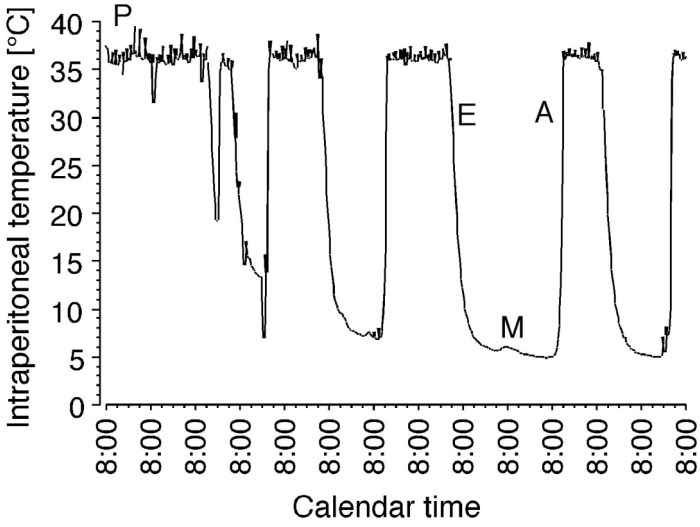

Fig. 1. Body temperature changes during hibernation in a golden hamster housed in the laboratory. Environmental conditions were room temperature of 5 °C, constant darkness and free access to food and water. P, preparatory stage of hibernation; E, entrance stage; M, maintenance stage, A, arousal stage

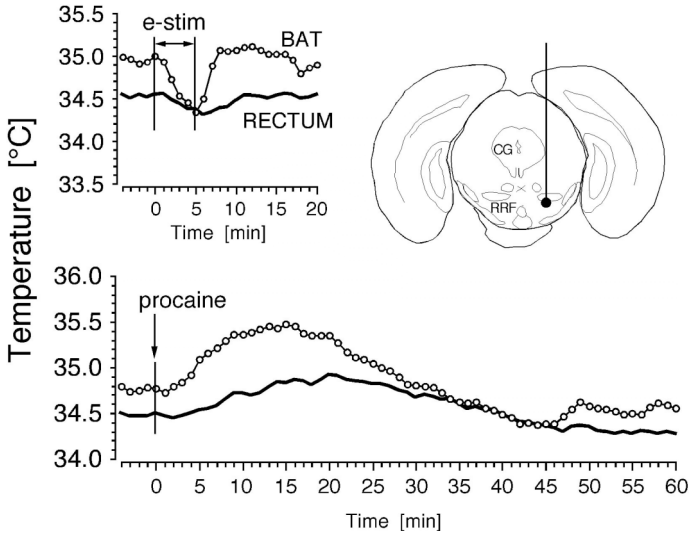

Fig. 2. Effects of electrical stimulation and local anesthetic microinjection on temperatures of interscapular brown adipose tissue (BAT) and rectum in anesthetized golden hamster. Electrical stimulation (e-stim: rectangular current, 1ms, 1mA, 10 Hz) and local anesthetics (procaine: 10%-procaine hydrochloride, 800 nl) microinjection at 1.0 mm left to the midline, 2.5 mm rostral to vertical inter-aural zero plane (IA0), 2.5 mm dorsal to horizontal IA0 were given to hamster while temperatures of rectum (RECTUM) and BAT were measured. CG: central gray matter, RRF: retrorubral field

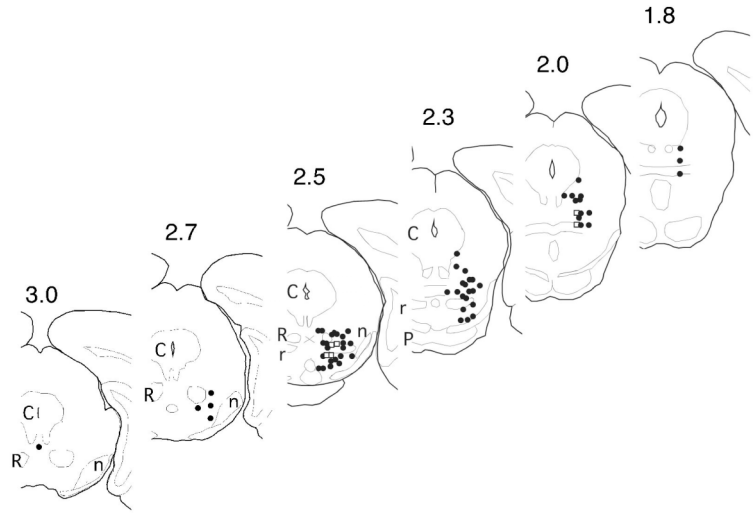

Fig. 3. Distribution of neuronal elements in the golden hamster brain probably constituting a tonic inhibitory mechanism for BAT thermogenesis (modified from (Hashimoto et al., 1999a). C: central gray matter, n: substantia nigra, P: pontine nuclei, r: retrorubral field, R: Red nucleus

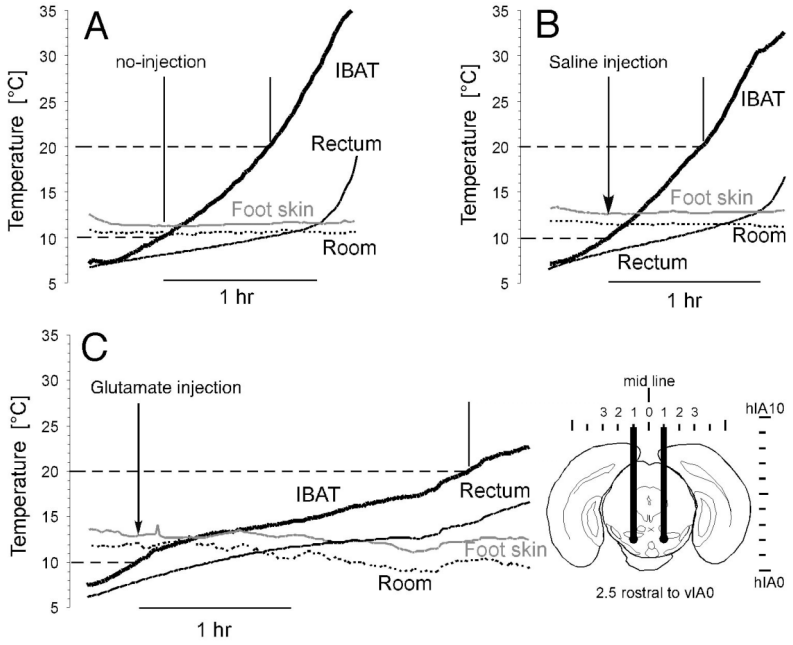

Fig. 4. Effects of excitatory amino acid microinjection on temperature changes of interscapular brown adipose tissue (IBAT) and rectum in golden hamster arousing from hibernation. Sodium glutamate (0.1 M, 800 nl) was bilaterally microinjected when IBAT temperature reached about 10 °C. Note the rate of increase in BAT thermogenesis is suppressed by excitation of tonic inhibitory mechanism with glutamate injection.

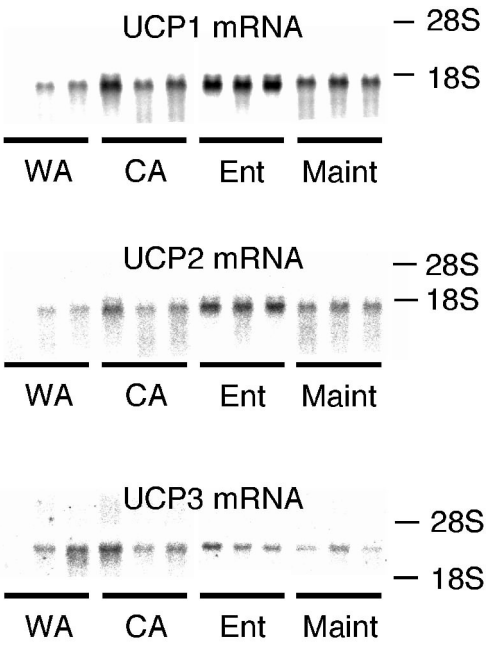

Fig. 5. Expression of uncoupling protein (UCP) subtypes mRNA in BAT of golden hamster. BAT was removed from warm acclimated cenothermic animals housed at ambient temperature of 25 °C (WA), cold acclimated cenothermic animals housed at 5 °C for 2 ~ 4 weeks (CA), from hamster in the entrance stage of hibernation (Ent, body temperature of 28 ~32 °C) and from hamsters in the maintenance stage of hibernation (Maint, body temperature of 5 ~ 7 °C). Absolute amount of UCP-1 mRNA was 40-fold more than UCP-2 mRNA and 100-fold more than UCP-3 mRNA in WA-animals. Radioactive labeling for UCP-2- and UCP-3-mRAN detection was done before detecting UCP-1-mRNA. 28S, 18S: internal standard ribosomal RNA.