Ultrastructural analysis of coiled body assembly and disassembly in cell nuclei of hibernating dormice

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SUMMARY

Hibernating mammals are characterised by intense periodic changes of their metabolic activity through quiescence-reactivation cycles. During these transitions, the structural constituents of the cell nucleus undergo various modifications. In particular, large number of coiled bodies (CBs) accumulate during the hibernation bouts and disappear upon arousal in cell nuclei of different tissues. CBs are enigmatic nuclear organelles occasionally occurring in cells of non-hibernating species, they shuttle between nucleoplasm and nucleolus, and are thought to play a role in the storage and intranuclear transport of nucleoplasmic and nucleolar splicing factors as well as in the regulation of chromatin organization and transcriptome activity. However, CB origin, formation and disassembly is still unclear, and hibernating mammals may represent a suitable model to study these phenomena under physiological conditions. In this work, we investigated by transmission electron microscopy the structural evolution of CBs and their content in their marker protein p80-coilin in liver and brown adipose tissue of the hazel dormouse *Muscardinus avellanarius* during the euthermic-hibernating-arousal cycle. We found that CBs form in the nucleoplasm at early hibernation as aggregates of threads already containing p80-coilin and other splicing factors, and only in deep hibernation they move to the nucleolus; at arousal, CBs undergo rapid disaggregation in both the nucleoplasm and nucleolus, thus releasing their molecular components in the intranuclear milieu.

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Introduction

Mammals are able to produce endogenous heat for maintenance of a high and constant body temperature. However, under adverse environmental conditions (*e.g.*, cold or food or water scarcity), this function can exceed the available energy. To overcome this problem, some mammals enter a hibernating state, characterised by a fall in the body temperature and a drastic reduction of all metabolic and physiological functions (Lyman *et al*., 1982; Carey *et al.*, 2003). During entry into hibernation, a progressive decrease in heart and respiratory rate and body temperature occurs, together with a lowering of the overall metabolic activity. In deep hibernation, all physiological functions are kept at minimum, and the body temperature is maintained close to the ambient one (sometimes even near 0°C). Upon arousal, the production of endogenous heat by non-shivering thermogenesis and the mobilization of the stored substrates rapidly restore all euthermic activities.

Consequently, tissues and cells of hibernating mammals are characterised by intense periodic changes of their activity through quiescence-reactivation cycles. During these transitions, the structural constituents of the cell nucleus are severely modified: in particular, nucleoplasmic and nucleolar ribonucleoprotein (RNP)-containing components undergo quantitative changes and intranuclear relocation (Zancanaro *et al*., 1993; Malatesta *et al*., 1995, 1999, 2000, 2008, 2009, 2011; Tamburini *et al*., 1996). Among them, a large number of coiled bodies (CBs) have been found to accumulate during the hibernation bouts and disappear upon arousal in cell nuclei of different tissues of dormice (Malatesta *et al*., 1994a; 2001).

CBs are enigmatic nuclear organelles: they occur in many cell types of non-hibernating species (although much less frequently); they are generally found in the nucleoplasm and can make contact with the nucleolus (Malatesta *et al*., 1994b; Ochs *et al*., 1994); they contain several processing factors for pre-mRNA and pre-rRNA, thus suggesting a role in the storage and intranuclear transport of these molecules as well as in the biogenesis of spliceosomal RNPs and the modification of non-coding RNAs (recent reviews in Meier, 2017; Staněk, 2017). It has also been suggested that CBs participate in the regulation of chromatin organization and transcriptome activity (Sawyer *et al*., 2016).

In fact, we are still far from completely understanding the role(s) of CBs in the nuclear functional activities, and there is still uncertainty as to their origin, formation and disassembly (review in Dundr, 2013). In this view, the hazel dormouse *Muscardinus avellanarius* is a suitable model to investigate these phenomena under physiological conditions.

In this study, we investigated by transmission electron microscopy the structural evolution of CBs and their content in the typical marker protein p80-coilin (review in Machyna *et al*., 2015) in two tissues - liver and brown adipose tissue (BAT) - at different phases of the euthermic-hibernating-arousal cycle.

Materials and Methods

This is a retrospective study on embedded tissue samples from

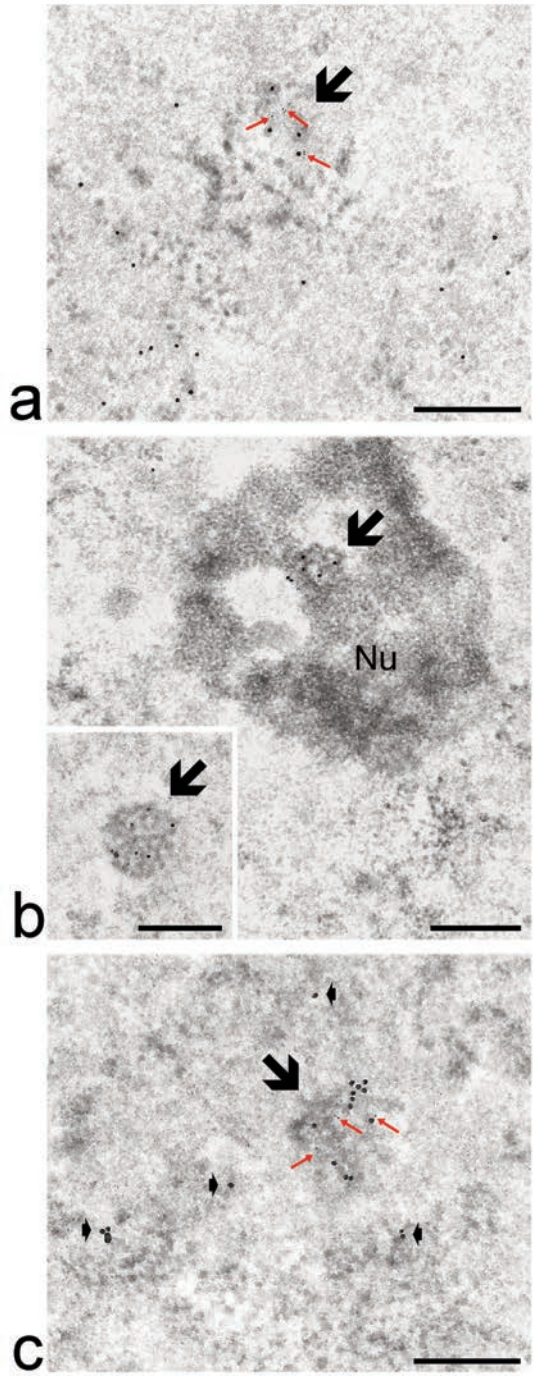

Figure 1. Electron micrographs of liver (a,b) and BAT (c) nuclei of early hibernating (a), deep hibernating (b) and arousing (c) hazel dormice. (a) Early hibernation: the assembling CB (arrow) contains both coilin (6 nm colloidal gold gran, red arrows) and snRNPs (15 nm gold grains). (b) Deep hibernation: CBs (arrows) labelled for p80-coilin occur in the nucleoplasm (inset) as well as inside the nucleolus (Nu). (c) Arousal: the disassembling CB (arrow) contains both coilin (15 nm colloidal gold granules) and fibrillarin (6 nm gold granules, red arrows). Some coilin molecules are distributed in the nucleoplasm (arrowheads). Scale bars: 200 nm.

eleven individuals of the hazel dormouse *Muscardinus avellanarius* (Gliridae) trapped in 1989-1992 in Switzerland, with permission from local authorities, for the purpose of multiple investigations (as reported in Zancanaro *et al*., 1993). The animals were maintained in external animal houses and provided with food and bedding material; under such conditions, they spontaneously began to hibernate in November and awoke in March. Five animals were killed during hibernation (two in late November, in early hibernation *i.e*., 24 hours after entering torpor; and three in January, in deep hibernation *i.e*., after at least three days of continuous torpor), three during arousal (March) and three during the euthermic period (June-July). Dormant animals were taken from the cage and immediately killed by cervical dislocation. Arousing animals were allowed to awake undisturbed apart from placing a thermistor probe on the abdomen (they were typically coiled up with the tail on their head) and killed as above when their body temperature reached 26°C. Euthermic animals were deeply anaesthetised before cervical dislocation. Samples of liver and BAT were quickly removed, and small fragments were fixed by immersion in 4% paraformaldehyde in 0.1 M Sörensen phosphate buffer at 4°C for 2 h. After washing in Sörensen buffer and in phosphate buffered saline (PBS), free aldehydes were blocked in 0.5 M NH_4Cl in PBS at 4°C for 45 min. Following washing in PBS, the specimens were dehydrated through graded concentrations of ethanol and embedded in Lowycril K4M resin. Ultrathin sections were placed on grids coated with a Formvar-carbon layer and then processed for immunocytochemistry. To this aim, a rabbit polyclonal anti-p80-coilin antibody (Andrade *et al*., 1993); a mouse monoclonal anti-(Sm)-snRNP antibody (Lerner *et al*., 1981) and a rabbit polyclonal anti-fibrillarin antibody (Lapeyere *et al*., 1990) were used. Sections on nickel grids were floated for 3 min on normal goat serum (NGS) diluted 1:100 in PBS and then incubated for 17 h at 4°C with the primary antibodies diluted with PBS containing 0.1% bovine serum albumin (Fluka) and 0.05% Tween 20. After rinsing, sections were floated on NGS, and then reacted for 30 min at room temperature with the secondary gold-conjugated antibody (Aurion) diluted 1:3 in PBS. Following the last incubation, all sections were rinsed and air-dried. As controls, some grids were treated with the incubation mixture without the primary antibody, and then processed as described above.

The samples were observed in a Philips CM12 electron microscope operating at 80 kV.

In order to evaluate quantitatively the intranuclear distribution of the CB marker protein p80-coilin, 80 micrographs (final magnification x25,000) of nuclei of hepatocytes and brown adipocytes from euthermic, early hibernating, deeply hibernating and arousing dormice (20 micrographs per animal group) were analysed. The areas of nucleoplasm, nucleoli and CBs were measured using a computerised image analysis system (Image Pro-Plus), and the gold grains occurring over each nuclear compartment considered were counted per square micrometre. Background values were assessed in section areas devoid of tissue.

The labelling densities were then expressed as the mean ± standard error of the mean (SE) values. Statistical analysis of the results was performed by the non-parametric Mann-Whitney U test and the statistical significance was set at $p \leq 0.05$.

Results and Discussion

The ultrastructural observations carried out on hepatocyte and brown adipocyte nuclei of early hibernating hazel dormice revealed the first phases of formation of CBs in a physiological *in vivo* model. At the beginning of the hibernating state, the CBs were not morphologically recognizable; in fact, only one or few small electron dense threads already contained the marker protein p80-coilin, as well as splicing factors such as snRNPs (Figure 1a) and fibrillarin (*not shown*). These coilin-containing structures (we may call pre-CBs) were found exclusively in the nucleoplasm without any contact with nucleoli, were quite rare (being present as single threads in about 10% of the sectioned nuclei of both tissues), and appeared differently organized suggesting different phases of formation. No evident difference in their frequency or morphological features were observed between the hepatocyte and brown adipocyte nuclei. Our findings differ from what reported by Ochs *et al*. (1995) in hepatocytes of estrogen-treated roosters, where CBs were found to arise from the nucleolar surface and increase in number by replicative division. However, this discrepancy may be related to the different experimental models used in the two studies.

Table 1. Mean values ± SE of anti-p80-coilin labelling densities (number of gold grains/µm²) over CBs, nucleoplasm and nucleoli of hepatocyte nuclei from euthermic, early hibernating, deeply hibernating and arousing hazel dormice. In each column, values identified with asterisks are not significantly different each other. The values underlined are not significantly different from background value (0.56±0.06 gold grains/µm²).

	CBs	Nucleoplasm	Nucleolus
Liver			
Euthermia	-	0.43±0.11	0.00±0.00
Early hibernation	81.18±10.61	5.59±0.76*	0.72±0.23
Deep hibernation	44.05±6.28*	1.11±0.10	1.50±0.34*
Arousal	36.88±4.55*	7.74±0.50*	1.12±0.39*
BAT			
Euthermia	-	0.65±0.08	0.10±0.10
Early hibernation	73.81±5.95	2.42±0.40	0.43±0.42
Deep hibernation	36.74±5.06*	0.67±0.10	1.23±0.47
Arousal	42.52±5.60*	0.40±0.09	0.65±0.30

Whatever the site of CBs formation, it is known that these nuclear structural components are able to self-assemble (Kaieser *et al*., 2008) in association to specific gene loci, thus contributing to genome activity, regulation and maintenance (Dundr, 2013).

As previously reported (Malatesta *et al*., 1994a, 1994b), in deeply hibernating dormice the CBs become quite frequent (about 25% of sectioned nuclei of both tissues exhibited at least one CB) and occur not only in the nucleoplasm (Figure 1b, inset) but also in association with the nucleolus (Figure 1b), according to their role in both nucleoplasmic and nucleolar functions (Trinkle-Mulcahy and Sleeman, 2017).

Upon arousal, in hepatocyte and brown adipocyte nuclei only few CB remnants were observed (in BAT they were especially rare), which still contained their typical molecular marker p80-coilin as well as splicing factors such as fibrillarin (Figure 1c). Disassembling CBs were again found both in the nucleoplasm and inside the nucleolar body (Malatesta *et al*., 1994b).

In euthermia, CBs were never observed in hepatocytes or brown adipocytes.

The quantitative evaluation of the marker protein p80-coilin (Table 1) revealed that in euthermic animals no significant level of this protein is detectable in any nuclear component of both tissues examined. During early hibernation, CBs showed the highest anti-p80-coilin labelling densities both in liver and BAT, suggesting that, during their formation, CBs accumulate large amounts of p80-coilin. The protein labelling density decreased in the mature CBs found in deep hibernation, probably due to the protein distribution inside their structure. This labelling density was maintained in CBs during the disassembly upon arousal. No difference in p80-coilin labelling density was found in CBs of the two tissues analysed.

In the nucleoplasm, the coilin density was the highest in early hibernation (when probably many free molecules are needed to promote CB formation), whereas in deep hibernation it decreased close to the background levels probably because most of the protein is bound to CBs (only in hepatocyte nuclei coilin density remained significantly high). Upon arousal, a striking difference in the nucleoplasmic coilin labelling was found between the two tissues: in hepatocyte nuclei, the coilin level increased to the early hibernation values, whereas in the brown adipocyte nuclei it remained at the background level. These differences could be due to the different physiological role played by the two tissues in the early phases of the arousal. In fact, BAT is immediately activated, due to its role in the thermogenic processes (recent review in Nedergaard and Cannon, 2018), while the liver starts later its functions. Consequently, in hepatocyte nuclei the disassembly of CBs could be at an earlier stage, with larger amounts of free coilin still dispersed in the nucleoplasm than in brown adipocytes. Accordingly, the residual CBs were found more frequently in hepatocyte than in brown adipocyte nuclei.

As for nucleoli, both hepatocytes and brown adipocytes showed a negligible labelling in early hibernation and a significant - but always low - coilin content in deep hibernation. Upon arousal, the nucleoli showed different trends of coilin content in liver and BAT: the nucleolar signal decreased to background in brown adipocytes whereas it remained at significant levels in hepatocytes, according to the nucleoplasm labelling.

In conclusion, the CBs occurring in cell nuclei of hazel dormice form in the nucleoplasm at early hibernation as aggregates of threads already containing their typical molecular factors and only in deep hibernation they move to the nucleolus; at arousal, CBs undergo rapid disaggregation in both the nucleoplasm and nucleolus, thus releasing their molecular components in the intranuclear milieu. It is likely that CB formation and disaggregation during the hibernation-arousal cycle is related to their regulatory role on genome and transcriptome (Dundr, 2013; Sawyer *et al*., 2016), which both undergo drastic seasonal modifications in hibernating mammals (Carey *et al*., 2003; Storey, 2015).

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