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Published online: 11 Oct 2017.

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Changes in the cerebellar cytoarchitecture of hibernating hedgehog *Erinaceus europaeus* L. (Mammalia): an immunocytochemical approach

E. RODA[1,2], M. G. BOTTONE[1], V. INSOLIA[1], S. BARNI[1], & G. BERNOCCHI [1]

[1]*Department of Biology and Biotechnology "L. Spallanzani", Laboratory of Cell Biology and Neurobiology, University of Pavia, Pavia, Italy, and* [2]*Laboratory of Clinical & Experimental Toxicology and Poison Control Centre and National Toxicology Information Centre, Toxicology Unit, ICS Maugeri Spa Benefit Corporation, IRCCS of Pavia, Pavia, Italy*

Abstract
Hibernation is an amazing animal strategy to survive when the environmental temperature is very low and food resources are scarce. Successful hibernation requires a variety of complex biological adaptations, in which the brain plays a central regulatory role. Currently, little information is available regarding the morphology and functional activity of specific neurons within the cerebellar cytoarchitecture of hibernating animals. In the present study, we investigated the immunohistochemical expression of essential proteins in the cerebellum of a mammalian hibernator (i.e. hedgehog *Erinaceus europaeus* L.), focusing on (i) Purkinje neurons, the sole output cells of the cerebellar cortex; (ii) selected neurotransmitters involved in hibernation processes; (iii) intracellular calcium homeostasis, considering that calcium is also an important regulator of neurotransmission mechanisms; and (iv) cytoskeletal proteins, involved in maintenance of neuronal shape and axon calibre. Specifically, we studied *in situ* immunocytochemical changes during the torpor state of hibernation (November–March) versus the activity phase (April–September). We employed different selected markers, i.e. glutamic acid decarboxylase (GAD67) and postsynaptic glutamate ionotropic receptor GluR2-3, different calcium-binding proteins (i.e. calbindin, parvalbumin and calretinin) and cytoskeletal components (i.e. pNF-H and MAP2). In summary, our data in hibernating animals demonstrated: (i) downregulation of GAD67, indicating loss/changes of synaptic contacts on Purkinje somata and dendrites; (ii) GluR2-3 upregulation in Purkinje neurons, with a drastic decrease of calbindin expression; and (iii) decrease of normal mechanisms regulating intracellular calcium homeostasis. We also found a decrease/modification in the distribution of cytoskeletal proteins, particularly evident for pNF-H. Changes in the functional activity of Purkinje cells were accompanied by some morphological dendrite alterations, signs of degeneration in cell somata and flattened basket pinceaux at the Purkinje axon hillock. These findings confirm that hibernation makes heterothermic animals a valuable model to study physiological adaptations to adverse conditions, and also for understanding cellular and molecular mechanisms aimed at preserving mammalian organs from full degeneration and death.

Keywords: *Hibernating mammals, cerebellum, neurotransmission, calcium homeostasis and cytoskeleton, immunocytochemistry*

Introduction

Hibernation is an exceptionally intricate biological event occurring in both non-mammalian and mammalian vertebrates, and also in some invertebrate species, essentially involving the central nervous system (CNS). Hibernating mammals undergo rapid and extreme physiological changes compared to active mammals. During torpor, the physiological characteristics would result in death for most mammals (Andrews 2007). However, hibernators can survive both frequent and dramatic fluctuations of body temperature and blood flow, caused by periodic arousals, without any signs of neurological damage (Frerichs et al. 1994; Frerichs 1999), as well as in experimental conditions against neurological insults (Frerichs et al. 1994; Frerichs & Hallenbeck 1998; Frerichs 1999; Zhou et al. 2001, 2002). Notably, the hibernating mammal brain, which has a central regulatory role in the hibernation (Drew et al. 2007), is protected against a variety of insults detrimental to humans

and other non-hibernating species (Drew et al. 2001; Dave et al. 2012). Specific brain areas remain active during deep torpor (Heller 1979), even when the hibernator's body temperature drops close to the freezing point of water and the brain becomes electrically quiescent to surface electroencephalography (Heller & Ruby 2004). More specifically, different brain regions show different activity during torpor and arousal phases: the cerebral cortex is the first brain area to lose activity as the animal enters torpor, while the hypothalamus remains active throughout the entire hibernation season (Heller 1979) to maintain homeostasis and several physiological processes. As a consequence, differently from the hypothalamus, the cerebral cortex undergoes extensive structural plasticity during torpor (von der Ohe et al. 2007; Schwartz et al. 2013), showing that diverse brain regions implement different strategies during hibernation (Schwartz et al. 2013).

Interestingly, hibernating animals retain coordinated postures and the ability to rouse in response to external stimulation (Heller 1979). These characteristics suggest the involvement of the cerebellum in the torpor phase compared to the activity period, even though only a few studies have demonstrated overt metabolic changes in the cerebellar cortex of a deep-hibernator mammal, during hibernation (Bernocchi et al. 1986; Giacometti et al. 1989).

Cerebellar cortex is usually considered a valuable model for *in situ* studies due to its regular cytoarchitecture. In particular, the Purkinje neurons, the sole neurons projecting outside the cerebellar cortex on deep nuclei in the white matter (Ito 2006), reflect the functional activity of the cerebellum in normal and altered neuroarchitecture, although the involvement of other neuronal types should not be overlooked.

In this context, the present experimental study was conducted to investigate *in situ* the cellular and molecular bases of changes occurring in the cerebellar cortex of hibernating hedgehog (*Erinaceus europaeus* Linnaeus, 1758) to provide insight into whether and how neurons survive or die in response to different physiological body conditions, focusing on potential neuroprotective strategies adopted by the cerebellum that are not yet fully explored. Specifically, attention was devoted to two different seasonal conditions, i.e. the hibernation torpor state (November and March) and the activity phase (April to September), with the main goal to examine whether the inactivity affects either cerebellar neurotransmission systems or cytoarchitecture integrity, with particular attention to Purkinje neurons.

Purposely, we employed immunohistochemistry to demonstrate the expression/localisation changes of representative molecules of neuronal functionality: (i) two specific neurotransmission markers, i.e. glutamic acid decarboxylase (GAD)67 and postsynaptic ionotropic glutamate receptor GluR 2–3; (ii) three calcium-binding proteins, i.e. calbindin, parvalbumin and calretinin; and (iii) two cytoskeletal components, i.e. 200 kDa phosphorylated neurofilament protein (pNF) and microtubule associated protein 2 (MAP2).

Methods

Animals

Male hedgehogs (*Erinaceus europaeus* L.) were collected in their natural environment, in the countryside near Pavia in the North-west of Italy, (i) in the hibernation torpor phase (five animals were collected asleep) at the end of November (4–9°C; two animals) and at the beginning of March (5–9°C; three animals); and (ii) in the activity phase, i.e. in April, June and September (18–30°C; six animals, two per month, were collected awake). Animals in the torpor phase were kept in a cold room (5°C) before sacrifice.

The animals were slightly anesthetised with ether before decapitation; cerebella were immediately excised, washed in NaCl 0.9%, and fixed by immersion for 48 h at 22°C in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), dehydrated in alcoholic scale, and finally embedded in paraffin. Twelve micron-thick sections of cerebellar hemispheres were cut in the sagittal plane; six sections were collected on each silane-coated slide (20 slides per hemisphere per animal).

Animal maintenance and experimental procedures were performed in compliance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes. All animals used in this research were treated according to the institutional guidelines; all efforts were made to minimise animal suffering, and to reduce the number of specimens.

Immunohistochemistry

To avoid possible staining differences due to small changes in the procedure, the immunoreactions were carried out simultaneously on slides of hibernating and active animals. Paraffin-embedded sections were deparaffinised in xylene, rehydrated through a series of graded alcohol treatments and rinsed in phosphate-buffered saline (PBS, Sigma).

Light microscopy

Endogenous peroxidases were suppressed by incubation of the sections with 3% H_2O_2 in 10% methanol in PBS for 7 min. Subsequently, the sections were washed in PBS and incubated for 20 min in normal

serum at room temperature in order to block non-specific antigen binding sites.

Localisation of the different markers was achieved by employing the following commercial primary polyclonal and monoclonal antibodies: rabbit monoclonal anti-GAD67 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal antibody anti-calbindin 28kD, anti-CB (1:5000, Bellinzona, Switzerland); mouse monoclonal anti-parvalbumin, anti-PV (1:5000; Swant, Bellinzona, Switzerland); rabbit monoclonal anti-calretinin, anti-CR (1:1000; Swant, Bellinzona, Switzerland); rabbit monoclonal anti-heavy phosphorylated neurofilament, anti-pNFH (1:100, Sigma, St. Louis, MO); rabbit polyclonal anti microtubule associate protein 2, anti-MAP-2 (1:250, Santa Cruz Biotechnology). Biotinylated anti-rabbit secondary antibody and an avidin biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA) were used to reveal the sites of antigen/antibody interaction. Furthermore, 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) with 0.01% H_2O_2 in Tris-HCl buffer (0.05M, pH 7.6) was used as a chromogen. Sections were dehydrated in ethanol, cleared in xylene and mounted in Eukitt (Kindler, Freiburg, Germany). For control staining, some sections were incubated with PBS instead of with the primary antibody. No immunoreactivity was present in this condition.

The slides were observed with an Olympus BX51 microscope, and the images were recorded with an Olympus Camedia C-5050 digital camera.

Fluorescence microscopy

The immunocytochemical localisation of GluR2-3 was achieved using a rabbit polyclonal antibody anti-GluR2-3 (1:200; Chemicon, Teecula, CA). After washing, sections were incubated for 1 h with secondary antibodies containing an Alexa Fluor 488-conjugated anti-rabbit at a dilution of 1:100 (Molecular Probes, Space, Milano, Italy) in PBS overnight in a dark, moist chamber. After PBS washing, coverslips were mounted in a drop of Mowiol (Calbiochem, San Diego, CA).

Sections were observed by fluorescence microscopy with an Olympus BX51 equipped with a 100-W mercury lamp used under the following conditions: 450–480 nm excf, 500-nm dm, and 515 nm bf for Alexa 488. Images were recorded with an Olympus Camedia C-5050 digital camera. Images were optimised for colour, brightness and contrast using Paint Shop Pro 7 software (Jasc Software Inc). For control staining, some sections were incubated with PBS instead of the primary antibodies; no immunoreactivity was detected in these.

Number of observations and figures

For each marker, five slides (30 sections) per animal were analysed.

Purkinje neurons with signs of degeneration were also counted in the hibernating animals (about 10% out of the total number of Purkinje neurons observed).

The figures show the most representative changes of cerebellar cortex/Purkinje cell layer for each immunohistochemical reaction.

Fluorescence intensity evaluation and statistical analysis

GluR2-3 labelling extent was evaluated on the acquired digitised section images under exposure time avoiding any pixel saturation effect. The labelling intensity was measured by means of densitometry analysis (Image-J 1.46p; NIH, Bethesda, MA, USA). The mask shape was adjusted depending on the spatial distribution of the Purkinje cell body under measurement; the labelling was measured as the mean intensity value over the area.

The immunocytochemical fluorescence intensity for GluR2-3, indicated as optical density (OD), was evaluated in 10 neurons per animal, for each month. Results were recorded on Microsoft Office Excel Software spreadsheets and are expressed as the means ± standard deviation (SD). Statistical differences between hibernating and active animals were evaluated by Unpaired Student's *t*-test.

Results

Here we describe the immunocytochemical expression pattern of selected markers related to (i) GABAergic and glutamatergic neurotransmission systems, i.e. the enzyme responsible for GABA synthesis, glutamic acid decarboxylase (GAD67) and postsynaptic ionotropic glutamate receptor GluR2-3; (ii) different calcium-binding proteins, i.e. calbindin (CB), parvalbumin (PV) and calretinin (CR); and (iii) cytoskeletal components, i.e. Heavy phosphorylated Neurofilament 200 kDa (pNF-H) and Microtubule Associate Protein 2 (MAP2).

Similar to what was observed in the cerebellum of other vertebrate species, e.g. rat and mouse (Bernocchi & Scherini 1980, 1983; Bernocchi & Barni 1985), in the hedgehog the immunocytochemical response was localised in distinct cell types or cell structures (i.e. soma, dendrites, axons and synapses). Briefly, the hibernation torpor induced evident changes in the Purkinje cells or Purkinje cell layer within the cerebellar cytoarchitecture.

Neurotransmission markers: GAD67 and glur2-3

GAD67 immunoreaction. GAD67 is usually expressed in the axons of inhibitory neurons (molecular layer interneurons and Golgi neurons), as well as in the Purkinje cell bodies, axons and dendrites (Kang et al. 2001; Mason et al. 2001; Obata et al. 2008; Piccolini et al. 2015).

During hibernation, specifically in November (Figure 1(a)), numerous GAD67 immunopositive nerve puncta and basket cell-descending axons surrounded the weakly labelled Purkinje cell somata. Flattened, intensely stained pinceau, formed by basket interneurons, at the axon hillock region of Purkinje cells were clearly identified. Several immunopositive cells, nerve puncta and axons were detected in the molecular layer (ML). Rosettes originating from immunopositive Golgi cell axon terminals are distinguished in the glomeruli of the internal granular layer (IGL).

Differently, in March (Figure 1(b)) a loss of labelled axons and nerve terminals around Purkinje neurons was found. Purkinje cells appeared pale, sometimes characterised by signs of "light degeneration". Shrinkage of some Purkinje cells was also recognisable, and the pinceaux at the axon hillock region were hardly identifiable.

Either intensely stained enlarged nerve puncta or axons were present in the ML and around Purkinje
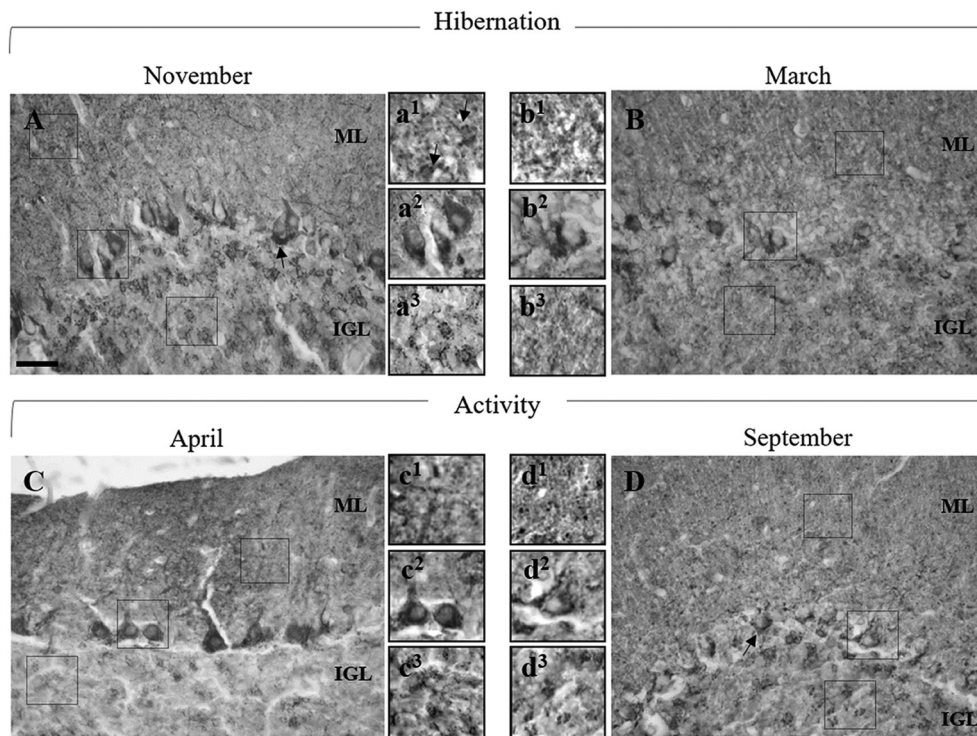

Figure 1. Micrographs showing representative cerebellar cortex sections after GAD67 immunostaining.
(a) Several fine immunopositive puncta, together with immunolabelled interneurons (insert a[1] arrows), are detected in the molecular layer (ML), where reactive dendrite branches of Purkinje cells are also observed. Purkinje cells, some of which display also weakly labelled cytoplasm, appeared encircled by immunopositive nerve puncta and axons (insert a[2]). Flattened, intensely stained pinceaux, formed by basket interneurons, at the axon hillock region are identifiable (thin arrow). In the internal granular layer (IGL), rosettes originating from immunopositive Golgi cell axon terminals are distinguished in the cerebellar glomeruli (insert a[3]). (b) In the ML, enlarged immunostained nerve puncta are observed (insert b[1], arrows). A few labelled nerve terminals around a pale Purkinje neurons (light degeneration?) are shown (left, insert b[2]) or enlarged and strongly immunostained axons surround Purkinje cell soma (right, insert b[2]). In the IGL, a general disorganisation, mainly due to wholly unlabelled glomeruli, is apparent in some areas (insert b[3]). (c) In the ML, fine nerve puncta and immunopositive Purkinje cell dendrites are shown (insert c[1]). Purkinje cell soma is surrounded by strongly labelled puncta; an intense immunopositive annular band is observed around the Purkinje cell nuclei (insert c[2]). Typical glomeruli rosettes are identifiable in the IGL (insert c[3]). (d) In the ML, intensely stained punctate nerve terminals and immunopositive interneurons (arrow) are shown (insert d[1]). Weakly labelled Purkinje cell soma is bordered by numerous immunoreactive nerve puncta and axons (insert d[2]); pinceaux at the Purkinje cell axon hillock are identifiable in some areas (thin arrow). In the IGL, immunopositive fibre rosettes are visible in the glomeruli (insert d[3]). Scale bars: a–d = 50 μm; inserts a[1]–d[3] = 25 μm.

cells. Either disorganisation or lack of immunoposi-tive Golgi cell rosettes in the cerebellar glomeruli was found in the IGL.

During the activity period, from April to September (Figure 1(c and d)), strongly labelled axons, originating from basket interneurons, sur-rounded Purkinje cell somata; the pinceaux at the Purkinje cell axon hillock were identifiable. In parti-cular, in April, an intense immunopositivity was localised around the Purkinje cell nuclei.

In the ML and IGL, punctate nerve terminals and immunopositive glomeruli rosettes were identified, respectively.

Glur2-3 immunostaining. GluR2-3 labelling is loca-lised at postsynaptic level in both Purkinje neuron somata and dendrite branches, in granule cells and glomeruli in the IGL (Hafidi & Hillman 1997; Pisu et al. 2003, 2004).

During the annual cycle, no relevant differences were observed in the ML and IGL. Specifically, in the ML immunopositive Purkinje cell dendritic branches are seldom recognisable, and in the IGL clusters of immunopositive cells or glomeruli coex-isted with weakly labelled areas.

In contrast, there were clear differences between hibernation and activity in the GluR2-3 immunopo-sitivity of Purkinje cells, as better shown by the OD evaluations (Figure 2(a)).

During hibernation, cerebellar convolutions dis-played Purkinje cells characterised by immunolabelled cytoplasm and primary dendrite (Figure 2(b)); in March (Figure 2(c)) Purkinje cell somata were more strongly immunoreactive. During the activity period (Figure 2(e–f)), Purkinje cell somata had the same immunopositivity as GluR2-3 as in November.

Considering the OD evaluations of Purkinje cell somata, the results highlighted statistically significant differences when comparing November (mean 43.8; SD ± 8.8) with March (mean 85.2; SD ± 21.2) and March with April (mean 29.6; SD ± 4.3) ($p < 0.01$ and $p < 0.001$, respectively). No significant differ-ences were detectable comparing April with June, June with September or September with November.

Calcium-binding proteins

CB immunoreaction. The fast buffering protein CB 28 kD is exclusively present in the Purkinje cells, showing a specific localisation in somata, dendritic branches and spines, and axons (see for review Schwaller et al. 2002; Bastianelli 2003). CB D-28 k may also be associated with cytoskeletal structures.

Changes in the CB immunolabelling are indicative of changes in the level of the protein, since it has
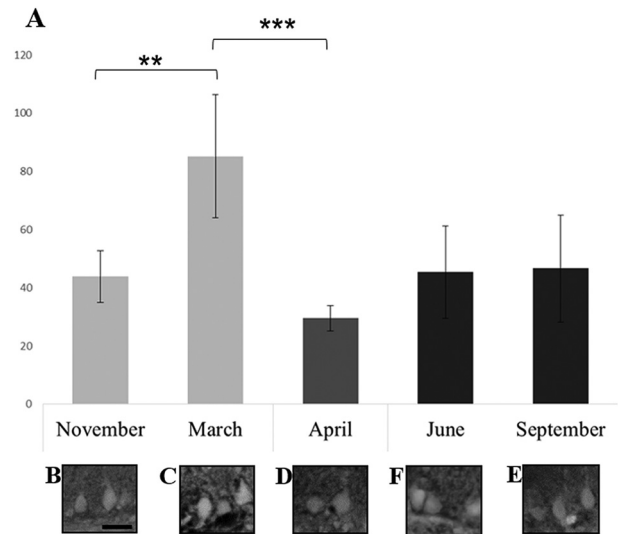

Figure 2. GLUR 2–3 immunofluorescence staining pattern in Purkinje cells.
(a) Histogram showing the optical density (OD) trend of Purkinje cell soma. The OD was significantly higher after a long hiberna-tion period (in March), compared to that measured during the other evaluated periods. Statistically significant differences were highlighted comparing November (mean 43.8; SD ± 8.8) with March (mean 85.2; SD ± 21.2) and March with April (mean 29.6; SD ± 4.3) with respective p-values of p < 0.01 and p < 0.001. No significant differences were detectable comparing the other months. (b–e) Purkinje cell somata are immunopositive, more intensely in March. Scale bars: b–e = 25 μm.

been shown that CB loss after commissural kindling stimulation is due to a decrease in the protein, as shown by quantitative evaluations in granule cells of dentate gyrus (Braun 1990).

During hibernation (Figure 3(a)), CB immunoreac-tivity was very intense in the Purkinje cell population, mostly localised in both cytoplasm and the main den-dritic branches. These observations referred to animals collected in November. Notably, destroyed dendrite branches were also observed. Differently to what was observed in rat Purkinje cells, the main proximal den-drite was remarkably long. A drastic decrease in immu-noreactivity was observed in the hibernating hedgehogs collected in March (Figure 3(b)), when several cere-bellar convolution areas displayed immunopositivity only in the nuclei of Purkinje cells.

During the activity period, from April to September (Figure 3(c and d)), there was intense CB labelling in the Purkinje cell population, in both somata and whole dendrites (proximal stem dendrite, main and thin branches); nuclei in some Purkinje cells were immunoreactive too.

PV immunoreaction. Similarly to CB, PV, a slow-onset buffering protein, is expressed mainly in
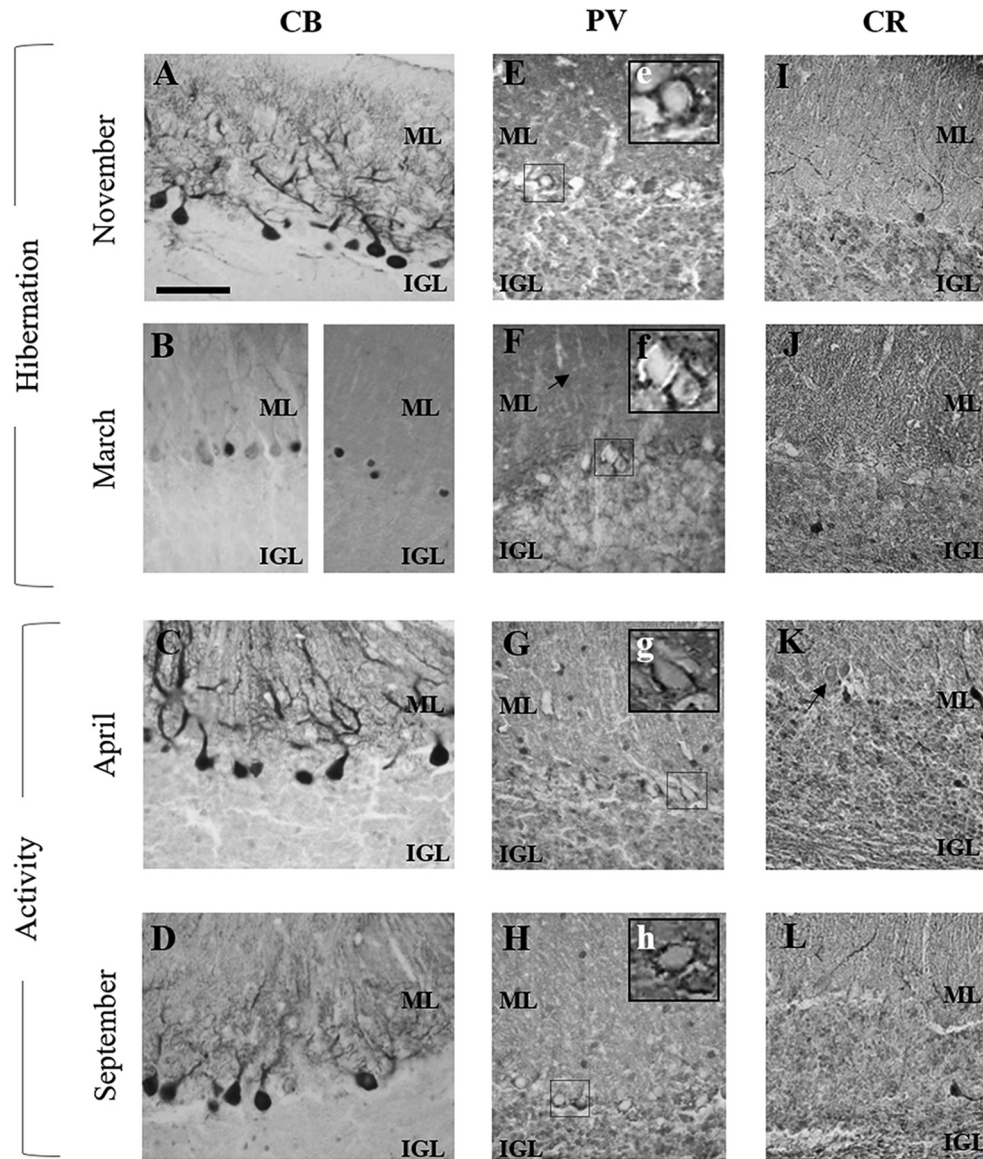
Figure 3. *Calcium-binding proteins (CBPs)* immunohistochemical labelling in cerebellar cortex cytoarchitecture.
Calbindin. (a) Purkinje cells displayed strong reactivity, mainly localised in cytoplasm and main dendrites; fine branches are also immunopositive. Notably, the main proximal stem dendrite appears long, and destroyed in places. (b) A general drastic decrease in the immunoreactivity response is observed; in several convolution areas, only immunopositive Purkinje cell nuclei are distinguished. (c) Purkinje cell cytoplasm and dendrites (even the main branches) show strong immunolabelling and are sometimes still destroyed. (d) Primary dendrites and thin branches of immunolabelled Purkinje cell are identifiable; notably, the cytoplasm has strong positivity. Parvalbumin. (e) Immunonegative or weakly immunopositive Purkinje cell soma appears to be encircled by thickened nerve puncta and labelled axons (insert e). The molecular layer (ML) as well as the internal granular layer (IGL) are immunopositive. (f) Around Purkinje neurons a decreased immunolabelling is observed (insert f). Similarly, ML interneurons wholly lack immunoreactivity (thin arrow). (g, h) Regarding the Purkinje cells, the observed immunostaining pattern is similar to that observed in panel F, but with a progressive increase of thin labelled axons or nerve puncta encircling immunonegative soma (inserts g and h); in the ML interneurons are labelled. Calretinin. (i) Most of the Purkinje cells were wholly immunonegative. Dendrite branches, mainly ascribable to Golgi neurons, are detected in the lower ML. In the IGL, weakly labelled granule cells are observed; Golgi cells show immunoreactive cytoplasm (arrow). (j) All Purkinje cells lack immunoreactivity; in the IGL, between weakly labelled granule cells there are even labelled Golgi cells (arrow). (k) Immunopositive Purkinje cells (thin arrow) are shown in some cerebellar convolution regions. In the ML the presence of a few fine immunoreactive fibres is observed. In the IGL, labelled granule cells and Golgi neurons are identifiable. (l) Purkinje cells appear immunonegative. In the ML, thin fibres and dendrite branches (likely ascribable to Golgi cells) are identifiable. In the IGL, between immunopositive granules cells there are strongly immunostained Golgi cell somata. Scale bars: a–l = 100 μm; all inserts = 25 μm.

axons, dendrites and spines of Purkinje cells. Additionally, it is localised in a subpopulation of inhibitory ML interneurons, i.e. stellate and basket cells, while no interaction with other cell components has been documented yet (Schwaller et al. 2002; Bastianelli 2003).

During hibernation (Figure 3(e–f)), in November, enlarged labelled axons surrounded the immunonegative or weakly immunopositive somata of Purkinje cells. The ML interneurons were PV-immunostained, as well as the granule cells in the IGL. In March (Figure 3(f)), loss of labelled axons and nerve puncta was found, particularly around the Purkinje cells.

Notably, the immunostaining pattern of the activity period (Figure 3(g and h)) was characterised by thin labelled axons surrounding the immunonegative somata of Purkinje cells and immunoreactive ML interneurons. Occasionally (not shown), some cerebellum convolution areas showed strongly immunopositive Purkinje cell somata and proximal dendrites.

CR immunoreaction. The fast calcium-buffering protein CR is essentially expressed either in granule cells and parallel fibres, or in sparse neuronal subtypes of IGL, i.e. unipolar brush cells, Lugaro cells and Golgi neurons (Schwaller et al. 2002; Bastianelli 2003). CR may also localise in Purkinje cell somata (Wierzba-Bobrowicz et al. 2011), and can be associated with intermediate filaments and microtubules (Schwaller et al. 2002).

During hibernation, in November (Figure 3(i–j)), most of the Purkinje cell population showed immunonegative cytoplasm, even though rare immunopositive neurons were observed. In the lower ML, nerve punctum terminals were observed, together with immunopositive dendrite branches, mainly ascribable to Golgi neurons. In the IGL, weakly labelled granule cells were evident, accompanied by Golgi cells with immunopositive cytoplasm. In March (Figure 3(j)), the immunostaining pattern showed a lower intensity than in November and signs of cell degeneration such as cell lightening were visible in some Purkinje cells.

In the activity period (Figure 3(k and l)), most Purkinje cells were immunonegative; intense labelling was observed occasionally in the Purkinje cell somata. In the ML, labelled nerve puncta, likely ascribed to parallel fibres, were found; there were also immunopositive dendrite branches, likely of Golgi cells. In the IGL, immunopositive granule cells and Golgi neurons were still present.

Cytoskeletal proteins

Heavy phosphorylated neurofilaments (pNF-H). Neurofilaments are structural components of nerve cell cytoskeleton (Riederer 1990; Burgoyne 1991). They are involved in the maintenance of neuronal shape and axon calibre. The phosphorylation of NF regulates neurofilament interaction and assembly, as well as interaction with other cytoskeletal elements and calcium binding. pNF-H are mainly located in axons (Sternberger & Sternberger 1983; Lee et al. 1987).

In rat cerebellar cortex, pNF-H labelling is detected in the ML interneurons, and in both the Purkinje cell somata and axons; notably, this staining is also detected in the pinceaux at the Purkinje cell axon hillock.

Furthermore, the presence of pNF-H in the neuronal somata would be indicative of cellular damage such as in neurodegeneration events (Scherini & Bernocchi 1994).

During hibernation, in November (Figure 4(a)), intensely immunoreactive axons, often characterised by enlarged calibre, surrounded the weakly immunostained Purkinje cell somata. Several labelled axons were identifiable in the lower ML. The immunostaining pattern was drastically reduced or even absent in March (Figure 4(b)), when signs of shrinkage/light degeneration were detectable in the Purkinje cell layer (~10%).

During the whole activity period (Figure 4(c)), intensely labelled thin axons formed a continuous ring-shaped band around the weakly stained Purkinje cell somata; however, pinceaux at the axon hillock region were rarely recognisable. Immunopositive fibres and puncta were also observed in the lower ML.

Microtubule associate proteins (MAPs). Within the MAP family, MAP2 is one of the most studied proteins; in the mammalian brain it is expressed mainly in dendrites and axons (Matus 1988; Riederer 1992; Ludin & Matus 1993; Riederer et al. 1995), where it is involved in the formation of cross bindings between microtubules and neurofilaments and in microtubule assembly and stabilisation (Riederer 1990; Weisshaar & Matus 1993; Sánchez et al. 2000). In rat cerebellar cortex, MAP2 labels cell somata, even though the strongest staining is evident in the Purkinje cell's main dendrite (Bernhardt & Matus 1984; Scherini & Bernocchi 1994); additionally, labelling is also identifiable in the ML (basket and stellate interneurons) as well as in the IGL (granule cells and Golgi cells) (Bernhardt & Matus 1984).
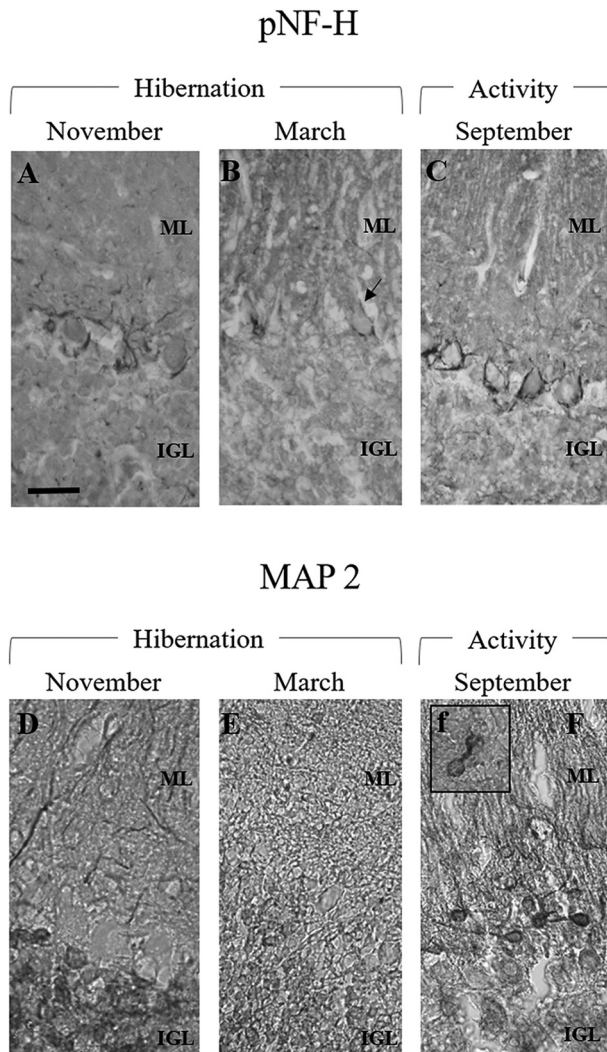
pNF-H

MAP 2

Figure 4. Representative micrographs showing cerebellar cortex architecture after cytoskeletal protein (i.e. *pNF-H and MAP2)* immunohistochemistry.
pNF-H. (a) Intensely immunoreactive axons, belonging to basket cells, surround the weakly stained Purkinje cell soma. In the lower molecular layer (ML), several immunopositive axon tracts, characterised by an enlarged calibre, are identifiable. (b) The immunolabelling appears drastically reduced around the Purkinje cells, some of which show signs of shrinkage or "light degeneration" (arrow). (c) Several intensely stained, fine axons, from basket cells, surround the very weakly stained somata of Purkinje cells. MAP2. (d) Purkinje cell somata are immunonegative, while in the ML, intensely immunostained structures, likely Purkinje cell dendrites or interneuron axons, are identifiable. In the internal granular layer (IGL), labelled granule cells are present. (e) A severe general reduction of immunolabelling is detected throughout the whole cerebellar cortex. (f) Purkinje cells are still unlabelled or weakly stained, whereas in the ML, clusters of immunopositive interneurons are visible (arrows). In the IGL, labelled granule cells and strongly immunoreactive brush cells (insert) are observed. Scale bars = 50 μm.

Increased MAP2 staining, mainly in form of aggregates or tangles, is usually indicative of

neurodegeneration (Caner et al. 2004; Wang & Liu 2008; Riederer et al. 2013).

During hibernation, in November (Figure 4(d)), Purkinje cells displayed pale somata, together with intensely immunostained main dendrite branches. In the IGL, labelled granule cells were observed, while in the ML no immunopositive interneurons were found. In March (Figure 4(e)), the loss of labelling in the ML was evident; however, some immunolabelled cells, likely brush cells, were present in the IGL (not shown).

During the whole activity period (Figure 4(f)), Purkinje cells were still characterised by immunonegative or weakly stained somata and dendrite branches, while clusters of strongly immunolabelled interneurons (likely basket cells) were found in the ML. Strongly immunoreactive cells (likely brush cells) were still observed in the IGL.

Discussion

In the current investigation, conducted using a mammalian hibernator model, i.e. the hedgehog *E. europaeus*, we demonstrated that morphological and functional changes occurred in the cerebellar cortex cytoarchitecture during the hibernation torpor phase, and were particularly evident in March. We demonstrated the central role played by Purkinje neurons, further correlating Purkinje cell morphology and immunopositivity with the other neurons forming the cerebellar circuits. Additionally, the investigated markers were related to the immunohistochemical expression of pivotal neurotransmitters as well as to intracellular calcium proteins, taking into consideration that calcium is also an important regulator of neurotransmission mechanisms (Mattson 1989, 1992, 2008; Mattson et al. 1993). Moreover, typical markers indicative of cytoskeletal stabilisation were also examined (Hindle & Martin 2013), since the involvement of cytoskeleton in synaptic reorganisation and neural plasticity has been amply demonstrated (Popov & Bocharova 1992; Popov et al. 1992; von der Ohe et al. 2007; Hindle & Martin 2013).

Two fundamental phases of the annual cycle of hedgehog were considered: the torpor phase of the hibernation period, lasting from the autumn through the winter season (based on environmental temperature from the end of November to the beginning of March) and the activity phase, taking place during the spring–summer season (from April to September).

During winter months, when food supplies become more scarce and environmental temperature becomes too low, hedgehogs enter into a hibernation period, a kind of long-term torpor frequently interrupted by brief, spontaneously rewarming (Lyman & Chatfield 1955; Frerichs et al. 1994; Morris 2006).

The mechanisms that initiate torpor are believed to involve interactions between neural systems regulating endogenous timing, metabolism and homeostasis. Numerous, potentially neuroprotective adaptations of hibernation physiology (e.g. immunosuppression, leukocytopenia, inhibition of protein synthesis, enhanced antioxidant defence and metabolic suppression (Frerichs et al. 1994; Frerichs & Hallenbeck 1998; Drew et al. 2007), permit hibernators to survive in harsh conditions without neurological damage (Frerichs & Hallenbeck 1998; Zhou et al. 2001). However, brains during the hibernating state show many structural changes (Jacobs 1996; Azzam et al. 2000), which may be related to energy conservation and/or hypofunction.

Neurotransmission

Neurotransmitters and neuromodulators play a role in hibernation; GABA and glutamate are considered key regulatory molecules during torpor (Egebjerg et al. 2001; Drew et al. 2007), being the major inhibitory and excitatory neurotransmitters, respectively, largely represented in the cerebellum (Nguyen et al. 2001).

In the present investigation, the two selected neurotransmission markers displayed different trends during the torpor phase of hibernation, especially in March, when a decreased expression of GAD67 was paralleled by an enhanced GluR2-3 immunoreactivity. Moreover, we demonstrated that the neurotransmitter trend in the cerebellum differed compared to that observed in other brain areas. For instance, in the suprachiasmatic nucleus, involved in hibernation, glutamate immunoreactivity decreases during torpor, at the same time that GABA immunoreactivity increases (Nurnberger et al. 2000), while in the striatum, glutamate remains constant (Zhou et al. 2002) and GABA decreases (Osborne at al. 1999). Previous experimental data demonstrated peculiar neurotransmitter characteristics typical of different brain regions. Indeed, it has to be considered that in hibernating species, some neurons generate and conduct impulses and initiate synaptic transmission at the low temperature associated with deep torpor, albeit with reduced capacities (Harris & Milsom 1995; Milsom & Jackson 2011). On the other hand, recent studies in hamster demonstrated that during the torpor phase of hibernation several metabolites, i.e. GABA, glutamate, glutamine and alanine, are present in the striatum, cortex, hypothalamus, and brainstem; many central receptors retain a stimulatory function capable of inducing arousal from hibernation. In fact, changes in tissue metabolite levels

occur prior to or during entrance into torpor but not during torpor (Osborne & Hashimoto 2008).

GABA is the principal inhibitory neurotransmitter in the CNS. The GABA-synthesising enzyme (Houser & Esclapez 1994) represents a good marker for GABAergic function (Martin & Rimvall 1993; Sheikh et al. 1999) and was used to mark the distribution of GABAergic neurons, such as Purkinje cells (Erlander & Tobin 1991; Martin & Barke 1998; Esclapez & Houser 1999; Sheikh et al. 1999). In particular, GAD67 marks axons and neuronal cell bodies (Scherini et al. 1992; Pisu et al. 2004; Popp et al. 2009). Our findings on GAD67 expression indicated a loss of labelling in the axons surrounding Purkinje cell somata, particularly in March after a long hibernation period, indicating functional alterations within this neuronal population. We demonstrated a decrease in labelled GAD67 basket axons and thickened nerve terminals around Purkinje cells, the latter often demonstrating signs of degeneration (shrinkage or lightening). According to the previous literature (Rohkamm 1977; Scherini et al. 1987; Monteiro et al. 1994), this finding suggests a possible onset of neuronal damage. In contrast, in the activity phase, thin labelled axons surrounded Purkinje cell somata. Moreover, a peculiar feature, i.e. the presence of an annular immunopositive band around nuclei, was observed at arousal, suggesting that GAD67 immunopositivity was localised in cytoplasmic organelles encircling the nucleus, such as Golgi bodies (Oertel et al. 1982), where the synthesis of GAD occurs.

Glutamate has been reported to regulate neurogenesis, neurite outgrowth, synaptogenesis and neuron survival (Hollmann & Heinemann 1994; Ozawa et al. 1998; Kew & Kemp 2005; Mattson 2008). The alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) class of glutamate receptors has been studied extensively and its roles in synaptic transmission and plasticity are well established (Malenka 2003). In this study, the AMPA receptor GluR2-3 was used to label Purkinje cells and, less intensely, granule cells (Hafidi & Hilman 1997; Grabs et al. 2008). An upregulatory pattern of GluR2 has been recently demonstrated in hypothalamic nuclei of hibernators (Alò et al. 2011); the increase in GluR2 was associated with an increase in the anti-apoptotic molecule Bcl-2, suggesting a neuroprotective role of this AMPA receptor (Mele et al. 2015). In addition, the altered high expression of the postsynaptic AMPA receptor GluR2-3 in the Purkinje cells is modulated by a decreased expression in the synaptic contacts by granule cells, and in parallel modulates the presynaptic function mediated by GABA (Grabs et al. 2008). Regarding the ionotropic glutamate receptor GluR2-3, which regulates

neuronal membrane depolarisation and calcium influx (Yang et al. 2011), data from the previous literature on hippocampal neurons showed that a decrease in presynaptic inhibition of the perforant path would increase glutamatergic neurotransmission, resulting in an increase in the glutamate receptors' reactivity in surviving dendrites and axons of (hippocampal) dentate gyrus (DG) granule cells, and/or upregulation of glutamate receptor production in the Purkinje cells of hibernating hedgehogs (Lynd-Balta et al. 1996). From this view, our data suggest the presence of GluR2-3 during the activity phase, but most of the Purkinje neurons displayed more intensely GluR2-3-immunopositive cytoplasm (and dendrites) in torpid animals in March, than in November. The functional significance of this altered pattern in the torpid hedgehogs may be simply due to changes in the neurotransmission.

The increased GluR2-3 expression observed in the Purkinje cell population of torpid animals in March may be owing to (i) a decreased control of presynaptic parallel fibre contacts, as well as (ii) a diminished inhibition of GABAergic synaptic contacts. However, it has to be taken into careful consideration that the AMPA receptors become rapidly desentisised to glutamate and the increased expression of AMPA receptors may be a compensatory mechanism to protect against further excitotoxic damage linked to excessive accumulation of calcium in the cell (Choi & Hartley 1993). Therefore, as suggested by Lynd-Balta et al. (1996), the robust immunoreactivity for GluR2-3 may represent a functional reorganisation, but also an adaptive mechanism to reduce calcium influx or a loss of mechanisms regulating calcium homeostasis (see below).

The maintenance and regeneration of synapses is another interesting feature of hibernating species possibly correlated with neurotransmission. Popov and coworkers reported that during torpor, dendritic branching and synaptic profiles are less complex, and reduced in size and number, but rapidly recover upon arousal (Popov & Bocharova 1992; Popov et al. 1992).

As previously mentioned, loss of functionally active GAD67 labelled synapses surrounding Purkinje cell somata was found after the long hibernation period, in March, while during the activity period, the normal morphology of fibres surrounding Purkinje cell somata did not exclude recovery phenomena. In particular, the annular distribution of GAD67 immunoreactivity around nuclei supports the recovered synthesis of GABA once the activity period begins, in April.

Intracellular calcium homeostasis

Changes in neurotransmitter marker expression have been correlated with alterations of the intracellular calcium concentration and calcium buffering. Neurotransmitters can affect neurite outgrowth and cell survival by calcium modulation. Loss of calcium homeostasis, resulting in elevated calcium concentration, can cause structural damage to neurons and can initiate the cell death process (Mattson 1992; Mattson et al. 1993). Calcium-binding proteins (CBPs) primarily regulate calcium balance. Interaction of calcium with these proteins represents one of the mechanisms by which the second messenger controls many biological processes. Although these proteins share an ability to bind calcium, they belong to different families. PV, CB and CR belong to a family of low-molecular-weight CBPs. They have been localised in many different tissues beside the nervous system and their highly conserved structure throughout evolution suggests that they should play some fundamental roles, such as calcium buffering (Goodman et al. 1979).

The CBPs were systematically localised in the CNS, as markers of specific neuronal populations in the mammalian cerebellum (Celio 1990; Baimbridge et al. 1992; Celio et al. 1996; Schwaller et al. 2002; Bastianelli 2003), and their presence has been demonstrated also in the hedgehog brain (Ferrer et al. 1992). In the absence of CBPs, there is high calcium accumulation inside the cytosol, causing hyperexcitability, which often leads to neurodegeneration (Schwaller et al. 2002; Bastianelli 2003). In particular, downregulation of PV and CB content might cause alterations in calcium homeostasis, finally leading to Purkinje cell death (see for review Schwaller et al. 2002; Lee et al. 2010).

In the current study, the very strong immunoreactivity for CB, detected in the Purkinje cell population of hibernating hedgehogs in November, could be indicative of its involvement as a buffering protein of excessive calcium influx (Braun 1990), when neurons need to be modulated, i.e slowed down in their activity. Curiously, this possible modulation occurred at the beginning of the hibernation period, when Golgi inhibitory neurons in the IGL also showed strong CR labelling. However, the protective role of CB against calcium influx seemed to precede the later increase in the GluR2-3 immunoreactivity. In March, after several months of hibernation, there was a drastic decrease in the CB labelling and, then, a loss in the calcium buffering protein. Otherwise, this loss could be associated with decreased calcium accumulation in the cells, as reported in isolated nerve endings in hibernating ground squirrels (Gentile et al. 1996; Storey 1997; Carey et al. 2003). A link between calcium concentration and GluR2-3 has not been yet demonstrated (Choi & Hartley 1993), but our data showed a peculiar hibernating process, i.e. neuronal

rescue against adverse conditions, probably requiring a difficult balance in the neuronal activity, as shown in the first month of activity (arousal), when weak GluR2-3 expression correlated with an increased CB immunoreactivity. However, in spite of this balance, cytoarchitectural signs of changed functional activity occurred in March, as shown also after GAD67, PV and pNF-H immunoreactions. On the other hand, previous immunohistological studies in ground squirrels demonstrate a 50–65% loss of synapses over the entire brain during entry into torpor (Von Der Ohe et al. 2007; Osborne & Hashimoto 2008; Dave et al. 2012). Beyond this, the length and branching of the dendritic arbor as well as the number of dendritic spines are decreased in the hippocampus (Popov et al. 1992, 2007; von der Ohe et al. 2007), thalamus and cortex (Popov et al. 2007; Ruediger et al. 2007). Actually, in our investigation, finer branching and more homogeneous CB labelling were found in the Purkinje cell dendrites during the activity phase, while the dendritic stems and branches appeared strongly immunoreactive and destroyed at the beginning of hibernation phase.

Moreover, a new and intriguing finding emerged after the long hibernation period, in March: CB immunoreactivity drastically decreases in the Purkinje cell somata and dendrites and is mainly located in the nuclei only. This pattern confirms the heterogeneity we previously described concerning the different condensed chromatin and different metabolic activity of Purkinje cells in the hedgehog (Bernocchi 1985; Bernocchi et al. 1986). From a functional point of view, the nuclear CB has been supposed to regulate nuclear calcium signals (German et al. 1997) and therefore to influence calcium-regulated gene expression (Sheng et al. 1990). Calcium can regulate the subcellular localisation of CB, and inositol triphosphate-sensitive nuclear membrane calcium stores may regulate the entry of small molecules into the cell nucleus.

Concerning the other CBPs, PV and CR integrated some important information in IGL granule cells and ML interneurons, respectively. With regard to PV distribution and expression, our data indicate that ML interneuron labelling was lower in the hibernation period than during the activity period. During hibernation, these neurons were generally found to be immunonegative, even though they were surrounded by PV immunopositive thickened axons. These latter processes belong to ML basket interneurons (Celio 1990), which resulted in a flattened basket at the Purkinje cell axon hillock, or in weak labelling for GAD67. This finding could indicate a reduced inhibitory action on Purkinje cell somata, accompanied by a

compensative protection mechanism exerted by calcium homeostasis changes.

Considering CR protein, the relevant modification was the loss of labelling in the ML and IGL. As previously demonstrated in other animal species (Bastianelli 2003), during activity CR labels IGL granule cells and Golgi neurons; punctate nerve terminals (likely parallel fibres) or dendrite branches of Golgi neurons were present in the ML and some Purkinje cells were also marked. Differently, during hibernation torpor, particularly in March after a long hibernation period, granule cells and fibres were weakly stained, while Golgi neurons maintained unaltered immunopositivity in their somata. The general immunostaining trend was almost similar for CB and CR (a marked decrease in the labelling) and suggested a coordinated role for CBPs in several districts of the cerebellar cortex.

The cytoskeleton

In the adult CNS, an impressive degree of plasticity is retained through morphological and molecular rearrangements in the pre- and post-synaptic compartments, which underlie the strengthening or weakening of synaptic pathways. The molecular underpinnings of synaptic plasticity are actively studied and it is well known that the cytoskeleton is a key substrate for many cues that affect plasticity (Gordon-Weeks & Fournier 2014), regeneration of axons and its regulation (Li et al. 2014). The cytoskeleton comprises highly dynamic fibrous protein networks that undergo constant and rapid reorganisation during cellular processes (Oláh et al. 2013). In CNS damage, the cytoskeleton components are misregulated in their physiology and this might be responsible for the toxicity leading to pathological conditions and to neuronal death (Perrot et al. 2008; Heimfarth et al. 2013).

Among the neuroprotective processes in mammalian hibernators (Hindle & Martin 2013), a mechanism for cytoskeletal stabilisation has been enumerated, including in the synaptic contacts. Furthermore, cytoskeletal reorganisation is known to occur on return to euthermy (Dave et al. 2012). Neural retraction across multiple brain regions has been described: dendritic spines and synaptic profiles shrink, accompanied by general cytoskeletal breakdown as microtubules disassemble. Yet the hibernator's brain is capable of rapid dendritic regrowth and synaptic rebuilding during each interbout arousal (Popov & Bocharova 1992; Popov et al. 1992). It was demonstrated that the entry into torpor is associated with 50–60% loss of synapses in golden-mantled ground squirrel (*Callospermophilus lateralis*),

as a result of decreased co-localisation of pre- and post-synaptic protein markers, although their abundance does not change. The majority of proteins varying with hibernation physiology were associated with the cytoskeleton, highlighting its importance in defining the torpid and aroused states (Von Der Ohe et al. 2007).

Our data provide interesting insight into fundamental proteins involved in the stabilisation of cytoskeleton in neuronal cell bodies and fibres, able to maintain and protect cerebellar cytoarchitecture during hibernation and activity periods. Downregulation of pNF 200 kDa expression occurred in the torpid hedgehogs, especially after a long period of hibernation, in March: a decrease of immunopositive axons and synaptic contacts was demonstrated in the Purkinje cell layer where signs of degeneration are also identifiable. In the activity phase, the typical morphology of this cerebellar layer has been achieved: Purkinje cell somata appeared surrounded by fibres and synaptic puncta, originating in the low ML, where their inhibitory interneurons are located. The changes in pNF 200 kDa mentioned above overlapped with those of the GAD 67 immunolabelling pattern. However, additional information on the cytoskeleton protein called microtubule associated protein 2 (MAP2) came from our results. In fact, immunonegative Purkinje cell somata were detected both in the torpid animals and in the active ones. Conversely, during the hibernation phase, markedly immunostained Purkinje cell dendritic branches were scattered in the ML, while pale immunolabelling was detected in the IGL. Both the increased and decreased staining in MAP2 immunoreactive structures, consistent with that observed for pNF, may be indicative of microtubule disassembly or changes in neurofilament and microtubule interaction, in which MAP2 has a role (Riederer 1990).

Concerning the fibres, our data from the hedgehog are in line with those we previously obtained in hibernating frogs (Pisu et al. 1998). Moreover, the absence of pNF and MAP2 storage in the neuronal cell bodies avoided diffuse cell degeneration and death, although degenerative signs are restricted to a small part of the Purkinje cell population.

The involvement of cytoskeletal proteins highlights Ca^{2+} homeostasis as an element of cell stabilisation during inactivity–activity phases. Ca^{2+} signaling has wide-reaching effects in regulating general cell functions. As previously reported, upregulation of CBPs could protect neurons from degeneration upon entry into hibernation, but loss of CBPs, particularly CB, after several months of hibernation, could explain not only the quiescent or decreased neuronal activity but also the signs of axon and neuron damage.

Conclusions

In the hibernating hedgehog CNS, the cerebellum requires highly orchestrated activity among cells and their synaptic contacts, with modulation of specific molecules. This is the only way to assure the architectural preservation of the tissues, avoiding marked degeneration, as well as to guarantee the animal's survival.

Purkinje neurons, the sole output cells of the cerebellum, undergo changes which affect the regulation of synaptic transmission, during the hedgehog annual cycle. For the first time, we demonstrated *in situ* interesting immunocytochemical differences between (i) the torpor phase of hibernation compared to the active time, and (ii) the beginning versus the end of the hibernation period. In fact, after a long period of torpor, downregulation of GAD 67, paralleled by a decrease in CB expression, was demonstrated, suggesting a loss of functionally active inhibitory synaptic contacts on Purkinje cell bodies and dendrites, and an absence/decrease of the normal mechanisms regulating calcium homeostasis. Further, the CB decrease can be related to the enhancement of the ionotropic receptor of glutamate, GluR2-3; this finding may represent a compensatory mechanism against calcium accumulation in the cells (Choi & Hartley 1993). Moreover, based on pNF-H and MAP2 data, we proved that cytoskeletal regulation and reorganisation occur, probably as a strategy to adapt neuronal functional activity to a lower CNS metabolic activity in torpid-hibernating animals (Bernocchi et al. 1986; Giacometti et al. 1989).

From a morphological point of view, neurotransmission changes within the Purkinje cell population are clearly indicated by altered pinceaux at the axon hillock, as well as by the presence of distorted dendrite branches or disintegrated cytoplasm in some Purkinje cells (Caner et al. 2004); these latter are signs of the so-called "cellular light degeneration" (Rohkamm 1977, pp. 13–77). Previous papers reported that during torpor, dendrite branches and synaptic profiles are less complex, and reduced in size and number, but rapidly recover upon arousal. However, comparing hibernation with the activity phase, we considered that the above-mentioned changes were recovered (in both morphology and function) at arousal (Popov & Bocharova 1992; Popov et al. 1992). Nevertheless, we cannot confirm the reversibility of the evidenced changes without neurological damage, although aroused hedgehogs, entering into the activity period, displayed GAD67-

immunoprecipitates encircling the nucleus, ascribable to Golgi bodies where synthesis of GAD occurs (Oertel et al. 1982); this finding suggests a restoration of active neuronal metabolism.

Further, although the lack of accumulation of both pNF-H or MAP2 in the Purkinje cell cytoplasm indicates that changes are not so massive as to lead to neuronal death (for a review see Scherini & Bernocchi 1994), degeneration signs deserve to be deeply investigated, while also studying potential recovery due to cell renewal.

Our recent findings on cell death during frog hibernation (Cerri et al. 2009) showed that, compared to active frogs, in the hibernating animals an increased proliferating cell nuclear antigen (PCNA) -immunoreactive cell number was observed in both the brain ventricles and parenchyma. This increase was primarily evident in the lateral ventricles, a region known to be a proliferating "hot spot". The increase in the number of apoptotic cells in these brain areas occurs when cell proliferation is higher in the corresponding ventricular zones. We suggested that the high number of dying cells found in the parenchymal regions of hibernating frogs might provide the stimulus for the ventricular zones to proliferate. Instead, Popov et al. (2011) demonstrated in the dentate gyrus of the hibernating ground squirrel a decreased mitotic activity, in spite of the presence of a large population of immature cells.

In conclusion, our study showed the strategy adopted by the cerebellum to adapt itself to and survive adverse conditions without profound damage. In this context, hibernation as a natural condition offers the possibility to investigate neuroprotective mechanisms, also representing a good model for the study of adult neurotransmission, synaptic plasticity and natural neuroprotection, offering significant contributions in the study of some pathological conditions.

Acknowledgements

Declaration of interest

The authors declared no conflict of interest. The authors alone are responsible for the content and writing of the paper. The authors have no relevant affiliation or financial involvement with any organisation or entity with a financial interest or conflict concerning the information presented in this manuscript.

ORCID

G. Bernocchi ⓘ http://orcid.org/0000-0001-5816-7418

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