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BRAIN INFLAMMATORY CYTOKINES AND MICROGLIA MORPHOLOGY CHANGES THROUGHOUT HIBERNATION PHASES IN SYRIAN HAMSTER

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ABSTRACT

Hibernators tolerate low metabolism, reduced cerebral blood flow and hypothermia during torpor without noticeable neuronal or synaptic dysfunction upon arousal. Previous studies found extensive changes in brain during torpor, including synaptic rearrangements, documented both morphologically and molecularly. As such adaptations may represent organ damage, we anticipated an inflammatory response in brain during specific hibernation phases. In this study, signs of inflammation in the brain were investigated in the Syrian hamster hippocampus (*Mesocricetus Auratus*) both during hibernation (torpor and arousal phases) and in summer and winter euthermic animals.

mRNA expression of the pro-inflammatory cytokines TNF- , IL-6 and IL-1 was quantified by RT-qPCR. Morphological changes of microglia were studied by immunohistochemistry staining for IBA-1. Activation of microglia based on retraction and thickening of the dendritic branches and an increase in cell body size was quantified by calculation of cell body size to total cell size ratio.

Expression of pro-inflammatory cytokines was upregulated early in arousal (90 min), and normalized after 8h of arousal. Substantial loss of microglia ramification was found throughout torpor and early arousal together with a 2 fold increase in the cell body size to total cell size ratio. Notably, microglia changes were fully reversed in late arousal (8 h) to euthermic levels.

These results demonstrate an upregulation of inflammatory cytokines and signs of microglia activation during hibernation, which completely resolves by late arousal. Activation of this response may serve to prevent or offset brain damage resulting from the substantial physiological changes accompanying torpor and their rapid change during early arousal.

Keywords: brain; microglia; inflammatory cytokines; neuroinflammation; hibernation; Syrian hamster

1. INTRODUCTION

Hibernation is characterized by hypometabolism and hypothermia promoting survival of heterothermic mammals in times of limited resource availability and extreme temperature/energetic conditions (Dave et al., 2012). Hibernation consists of torpor phases featuring metabolic suppression and lasting 6-40 days, interrupted by short arousal periods lasting less than 24h. Throughout torpor, hibernators undergo large physiological changes, including a decrease in body temperature, a reduction in heart and respiratory rate, reduction of cerebral blood flow to below 10% of normal (Frerichs et al., 1994) and a suppression of the immune response. All these physiological changes are rapidly and completely reversed during the transition from torpor to arousal (Carey et al., 2003; Drew et al., 2007; Drew et al., 2009). Torpor and arousal phases in the Syrian hamster, under laboratory condition, typically last 4-6 days and less than 24 h, respectively (Trefna et al., 2017).

Remarkably, hibernators endure the repetitive cycles of low body temperature and rapid reperfusion without any apparent damage to organs, including brain. Consequently, post-hibernation, animals are free from noticeable neuronal histopathology or disturbances in synaptic activity, despite the profound reduction of cerebral blood flow in torpor and the subsequent rapid reperfusion during arousal (Bogren and Drew, 2014; Dave et al., 2006; Dave et al., 2009). The exact mechanism underlying this natural tolerance to repetitive cycles of lowered body temperature and rapid reperfusion is still poorly understood. Likely, multiple mechanisms are involved to protect and preserve the vital brain function, including suppression of protein synthesis, and alleviation of excitotoxicity, inflammatory responses, oxidative stress and cell death pathways (Dave et al., 2012; Drew et al., 2004; Yenari and Hyung, 2012).

In non-hibernating mammals, activation of the inflammatory response after cerebral ischemia is a key factor in the ischemic cascade that results in cell damage and death. Abundant release of pro-inflammatory mediators disrupts the blood-brain barrier and subsequently causes infiltration and adherence of circulating leucocytes to the site of injury. Failure to attenuate the pro-inflammatory cascade will consequently result in damage to the brain parenchyma (Ceulemans et al., 2010). In fact, chronic activation of the brain's immune system is the most common and prominent feature shared by all neurodegenerative diseases (Amor et al., 2010, McGeer et al., 2006).

Suppression of the neuroinflammatory response is one of the mechanisms thought to play a neuroprotective role in hibernators. Previous studies indicate that during hibernation the innate and adaptive immune responses are suppressed. Torpor induces about a 90% reduction in the number of circulating leukocytes in several hibernators (Soumalainen and Rosokivi, 1973; Reznik et al., 1975; Bouma et al., 2010), partly resulting from storage of lymphocytes in secondary lymphoid organs (Bouma et al., 2011), reduced leukopoietic activity in the bone marrow (Drew et al., 2001; Szilagyi and Senturia, 1972) and fat-soluble factors released from brown fat tissue that act as immunosuppressant (Atanassov et al., 1995; Drew et al., 2001; Sidky et al., 1969). Depletion of neutrophils and inhibition of leukocyte migration to the ischemic tissue may possibly explain the absence of brain pathology in hibernation. In the CNS, microglia represent the resident immune cells, accounting for 5-20% of the glial population. Under resting conditions, ramified microglia continuously scan the environment for danger signals associated with pathogens or injury. In response to alterations in the brain microenvironment, microglia cells undergo morphological changes to become activated (Ulvestad et al., 1994). Stages 1 and 2 of microglia activation are characterized by thickening

of the branching processes and change in cell body shape. Stage 3 is characterized by retraction and thickening of the dendritic processes and an increase in the cell body size to total cell size ratio. Stage 4 is characterized by an overall increase in the cell body size to total cell size ratio and only a few noticeable dendritic branches. Eventually microglia assume an amoeboid form capable of phagocytosis (Kreutzberg, 1996).

To date, little is known about the neuroinflammatory state in the hibernating brain. Zhou et al. (2001) showed inhibition of microglial activation and migration following implantation of a microdialysis probe in torpid Arctic ground squirrel as opposed to euthermic animals. Further, microglial activation and migration are inhibited by mild therapeutic hypothermia as found in brain ischemia in rats, in which a 54% reduction in microglial activation was found (Seo et al., 2012). Therefore, in the present study, we examined markers of inflammation throughout different phases of hibernation in the brain of hibernating Syrian hamster using (semi) quantitative immunohistochemical staining for microglia activation (IBA-1) and mRNA expression levels of pro-inflammatory cytokines with emphasis on the hippocampus.

2. MATERIALS AND METHODS

2.1. Animals and hibernation induction

Syrian hamsters (*Mesocricetus auratus,* n=51) were obtained from Harlan (Horst, The Netherlands) at the age of 11 months old and initially raised under summer conditions (14h:10h light:dark at 21±1°C, 55% humidity). The hamsters were housed in Macrolon type 3 cages on sawdust bedding, with ad libitum water, food and hay as nesting material. These conditions were maintained for another 9 weeks during which animals received 80 g of food

per 14 days. Entry into torpor was induced by transferring the animals to a climate controlled room, wherein conditions were changed from summer to a short-day of photoperiod (8:16h, light: dark, 21±1˚C, 55% humidity) for 8 weeks. Subsequently, temperature was maintained at 5±1°C and the light was changed to continuous dim red light (<0.5 lux). Torpor periods were identified by using simultaneous body temperature measurements and PIR activity logging. The hamsters were divided into multiple groups, based on the phase of hibernation wherein they were sacrificed, comprising: torpor early (TE) sacrificed after 24h entering torpor (n=3), torpor late (TL) sacrificed after 72h of torpor (n=10), arousal early (AE) sacrificed 1.5h after the onset of arousal (n=9) and arousal late (AL) sacrificed 8h after reaching euthermia (n=11). Winter euthermic hamsters (WE) served as control (n=8), being sacrificed after minimum two weeks of cold acclimatization without induction of torpor. Failure to enter torpor was confirmed by 24 h activity patterns showing no torpor and measurement of body temperature at euthanization. Furthermore, 10 animals were housed under normal summer conditions as summer euthermic (SE) controls. Animal experiments were approved by the Institutional Animal Ethics Committee of the University of Groningen.

2.2 Tissue preparation

For immunohistochemical (IHC) staining animals (n=27) were transcardially perfused with 0.1 M PBS, followed by 4% PFA in 0.1 M PBS. After dissection, brains were post-fixed in PFA for 24h at 4˚C and stored in 0.1M PBS, containing 1% sodium-azide, at 4˚C. For cutting slices (20 µm), brains were cryoprotected with a 30% sucrose solution and stored in PBS with 1% sodium azide, at 4˚C. For qRT-PCR, animals (n=24) were transcardially perfused with PBS and the brains were dissected in different areas, snap-frozen in liquid nitrogen and stored at -80˚C. qRT-PCR material from TE group was not available to be included in the analysis

due to shortage of TE animals and different methods of brain processing for IHC staining and molecular work.

2.3 Immunohistochemistry

Activation of microglia was investigated by IHC staining against the ionized calcium-binding adaptor 1 protein (IBA-1). Seven to nine dorsal hippocampus slices were included per animal. In total 216 brain slices were selected for the staining. Approximately 30 to 40 brain slices were selected per group. The IHC was performed on free floating-brain slices pre-treated with 0.3% H_2O_2 for 30 minutes at room temperature, followed by rinsing with 0.01M PBS. Slices were incubated with the primary anti-IBA-1 antibody (Wako Chemicals USA Cat# 019-19741) 1:2500 diluted in 0.1%Triton X, 1% BSA, 0.01M PBS for 72 hours at 4^{o}C. Slices were then rinsed with 0.01M PBS and incubated with the biotin conjugated secondary goat-anti-rabbit antibody (Jackson ImmunoResearch, Suffolk, UK, lot#11513), diluted 1:500 in 0.01M PBS for 2h at room temperature. Thereupon slices were rinsed and incubated with avidin-biotin comples (Vector ABC kit; Vector laboratories, Burlingame, CA, USA) diluted 1:500 in 0.01M PBS for 1h at room temperature. The slices were treated with 1 mg/ml diaminobenzidine (DAB) tablets (Sigma Aldrich MDL: MFCD00007725), activated by adding 100µl 0.1% H2O2, followed by rinsing with 0.01M PBS. Slices were then fixated on glass slides, dehydrated and coverslipped for microscopy analysis. Images of the stained sections were taken using imaging software (Leica QWIN, Leica Microsystems) at a 200x magnification focusing on specific subregions of the dentate gyrus hippocampus: molecular layer (MoDG), polymorph layer (PoDG) and dorsal hippocampal areas cornu ammonis 1(CA1), corpus ammonis 3 (CA3). Microglia activation was quantified by measuring the cell body size to total cell size ratio according to a protocol previously described by Hovens et al.

(2014), using Image-Pro Plus 6.0.0.26. The quantification and analysis of the morphological characteristics of microglia, namely, cell size, cell body size, size dendritic processes and cell body size to cell size ratio was performed using Image-Pro Plus 6.0.0.26. This software was developed as a semi-automatic image analysis method to analyze microglial activation in IBA-1 stained brain sections of rats based on the morphological characteristics of microglia (Kreutzberg, 1996). In order to measure the total cell size and total cell body size, an intensity threshold and size filter was applied based on the intensity of the staining, ensuring exclusive inclusion of pixels that are darker than the background. The total cell size measurement was acquired using the "automatic dark objects" function. Thereafter, the total cell body size was determined by applying an intensity threshold (140-170 pixels) and size filter (150-500 pixels). The size filter excludes dendritic processes with a high-staining intensity, smaller than a certain size, from the analysis. Consequently, only the total cell body size was measured. The measurements of microglial cell size were performed by an observer unaware of the state of the animal.

2.5 Real-time qPCR

RNA was extracted using the Nucleospin tissue kit (Macherey-Nagel, Duren, Germany). RNA (1µg) was reverse transcribed in a reaction mixture (20 µl) containing 1 µl of random hexamers, 0.5 µl of RNAse inhibitor, deoxynucleotide triphosphates (0.2 µl), 1 µl of reverse transcriptase, 4 µl RT buffer and 1,65 µl RNAse free H2O. The sample mixture was transferred to the thermal cycler and applied to the following protocol: 10 minutes at 20^{O}C, 30 minutes at 42^{o}C, 5 minutes at 99^{o}C and 5 minutes at 20^{O}C. Specific primers were: TNF-α (F: CCGCATTGCTGTGTCCTACG, R: TTGACCTCGGCACTGAGTCG IL-6 (F: TCTGGAACTTCCGGTGATAC, R: TGGTGCTCTGAATGACTCTG), IL-1β (F:

CGGCAGGTGGTGTCAGTCAT, R: GGAGCATCAGCCACGATCAG. β-actin (F: AAGATGACCC AGATCATGTTTGAG and R: ACGTACATGGCTGGGGTGTTG) was used as a housekeeping gene. SYBR Green real-time PCR was performed using Bio-Rad CFX384 C1000 (Bio-Rad Laboratories, Hercules, CA, USA) by 40 cycles of denaturation at 95°C (15 sec) and annealing at 60°C. Data were quantified with the Bio-Rad CFX manager 2.0 using reagents from the GoTaq qPCR Master Mix (Promega, USA). All data were normalized to β-actin.

2.6 Statistical analysis

Differences between the groups were compared using one-way ANOVA with a post hoc Bonferroni test for normally distributed data. For data that was non-normally distributed, statistical analyses were performed using Kruskal-Wallis test and Mann–Whitney U test. The Pearson correlation coefficient was used to assess the statistical evidence for a linear relationship among the variables. All p-values were considered statistically significant at a threshold of $p < 0.05$.

3.RESULTS AND DISCUSSION

To investigate the presence of a neuroinflammatory response in the hibernating brain, mRNA levels of inflammatory cytokines were quantified in the hippocampal area. mRNA expression of IL-1β showed the most substantial variation throughout the hibernation phases (Fig. 1A). IL-1β was significantly increased during AE and normalized during AL. Moreover, IL-1β mRNA expression level during TL was significantly lower than in all other groups. IL-6 was increased during AE and normalized in AL. Moreover, expression of IL-6 was significantly lower in TL compared to AE (Fig. 1C). While expression of TNF-α appeared to be

upregulated in AE compared to SE and WE, it did not reach a statistical significant difference (Fig. 1B). Further, expression levels of cytokines were positively correlated (Fig. 1D-E), with the strongest relation observed between TNF-α and IL-6 (Fig. 1D).

Taken together, the mRNA expression levels of the inflammatory cytokines follow a general pattern, i.e. transient upregulation during AE with normalization in AL. This upregulation was not seen in β-actin where mRNA expression levels remained at a constant level throughout the different phases of hibernation.

To further explore the presence of inflammatory markers in the brain, visualisation and quantification of microglia was performed throughout the different phases of hibernation. Microglia from SE, WE and AL (Fig. 2B,C,G) were characterized by numerous ramified dendritic branches, whereas in TE, TL and AE (Fig. 2D-F), the density of the microglial dendritic branches was decreased. To quantify morphological changes, cell body size to total cell size ratio was determined in dorsal hippocampus (all hippocampal areas of interest combined). Here, cell body size to total cell size ratio was increased by about 2-fold in TE, TL and AE compared to SE and WE control groups (Fig. 2H). Importantly, the cell body size to total cell size ratio normalized in AL to euthermic values. Similar changes in cell body size to total cell size ratio were found in the various hippocampal areas (Supplemental data). Moreover, cell body size was increased in TE, TL and AE compared to WE control groups (Fig. 2I). The opposite effect, namely a decrease in size of dendritic processes was observed in TE, TL and AE compared to SE and WE control groups (Fig. 2J). Importantly, both cell body size and size of dendritic processes normalized in AL to euthermic values.

Together, these results indicate signs of inflammation occurring in the brain throughout early and late torpor, and early arousal, which then normalizes upon late arousal to levels found in summer and winter euthermic conditions. As to our knowledge no previous study addressed

neuroinflammation during different hibernating phases, no comparisons can be made. However, Zhou et al. (2001) examined microglia activation and migration induced by implanting a microdialysis probe in brain of Arctic Ground squirrels and found reduced microglia activity in torpid animals compared to summer euthermic animals. Consequently, both our hamster and the ground squirrel study indicate that microglia behaviour markedly differs between summer euthermia and the topor phase.

Morphology of microglia is highly variable and changes during activation depending on the signal detected and the local environment (Nimmerjahn et al., 2005; Olah et al., 2011). The observed morphological changes of microglia in hamster, that seems uniform from early in torpor until early arousal, are consistent with the first stages of microglia activation previously described by Kreutzberg (1996), mainly showing graded retraction of dendritic branches and an increase in cell body size. Progression to more advanced stages of microglia activation, characterized by advanced loss of dendrites and a more pronounced amoeboid phenotype, were not observed, which seems consistent with the modest increase in mRNA synthesis of the neuroinflammatory cytokines. The absence of advanced morphological changes of microglia may actually indicate that neuronal injury in hibernation is limited or even absent. Such notion is consistent with the observation that hibernators do no exhibit signs of brain damage and are able to tolerate multiple cycle of cooling-rewarming (Dave et al., 2006; Dave et al., 2009). Given such lack of neuronal pathology and synaptic disturbances, the morphological changes microglia undergo might signify merely a response to changes in the microenvironment or deep cooling rather than activation of a true neuroinflammatory cascade.

Furthermore, our results show that pro-inflammatory cytokines, including IL-6 and IL-1β are only increased during early arousal, which may be related to reperfusion and rewarming from hypothermic conditions due to a burst production of reactive oxygen species (ROS), low ATP production and cellular Ca^{2+} overload (Rauen and de Groot, 2002; Talaei et al., 2011). Alternatively, the inflammatory response precipitated by microglial activation, which already commences in early torpor, may only be executed into mRNA synthesis of pro-inflammatory cytokines upon restoration of normothermia, i.e. during early arousal.

In our study, we report on the inflammatory state of microglia by measuring mRNA expression levels of pro-inflammatory cytokines and changes in microglia morphology. However, we acknowledge the importance of using multiple different approaches (behaviour, specific microglia activation markers, morphology, expression of pro-inflammatory cytokines) when reporting on the level of microglia activation (Norden et al., 2015). Unfortunately, due to lack of proper molecular tools in hamster, as also experienced by us, obtaining a more accurate picture of the inflammatory events occurring in the hamster hibernating brain will mostly rely on analysis of gene expression.

Therapeutic brain cooling has been shown to act on both cell death pathways including excitotoxicity, apoptosis, inflammation, free radical production as well as on neuroregeneration pathways (Dietrich et al., 2009; Yenari and Han, 2013). Several experimental studies found mild hypothermia (30^{o}- 34^{o}C) to decrease the release of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6, suppress the generation of NO and superoxide and inhibit microglia activation in animal models of brain ischemia and traumatic brain injury (Ceulemans et al., 2010; Kirchgessner and Hofer, 2009; Yenari and Han, 2006). While abundant data is available regarding the protective effects of hypothermia in animal

models of brain ischemia/hypoxia, little is known about the effect of hypothermia in the uninjured brain. One study has shown that therapeutic hypothermia has a negative effect on neurogenesis in the uninjured aged rat brain upon cooling the brain to 30°C for 21 h. However, under hypoxic/ischemic conditions in the rat brain, therapeutic hypothermia to 33°C showed beneficial effects on neurogenesis (Yenari and Han, 2013). Similar to mild hypothermia in the uninjured rat brain, low body temperature during torpor (below 10°C) restricts the proliferative capacity of new-born neurons in the hibernating hamster brain. However, upon arousal this proliferative capacity is recovered (León-Espinosa et al., 2016).

The activation of microglia and upregulation of the pro-inflammatory cytokines observed in hibernation differs from previous observations showing inhibition of the brain immune system under hypothermic conditions. However, hibernating and artificially cooled animals differ markedly in both metabolism, i.e. suppressed by > 90% *versus* mildly increased metabolism, and body temperature, i.e. 6-8°C *versus* 30°-34°C, which may underlie the difference in pro-inflammatory cytokines.

Further studies in this line of research are needed to gain a better understanding of the neuroinflammatory events and neuroprotective pathways occurring in the hibernating brain as this may guide the discovery of treatments that can mimic this natural tolerance to hypothermic/ischemic conditions.

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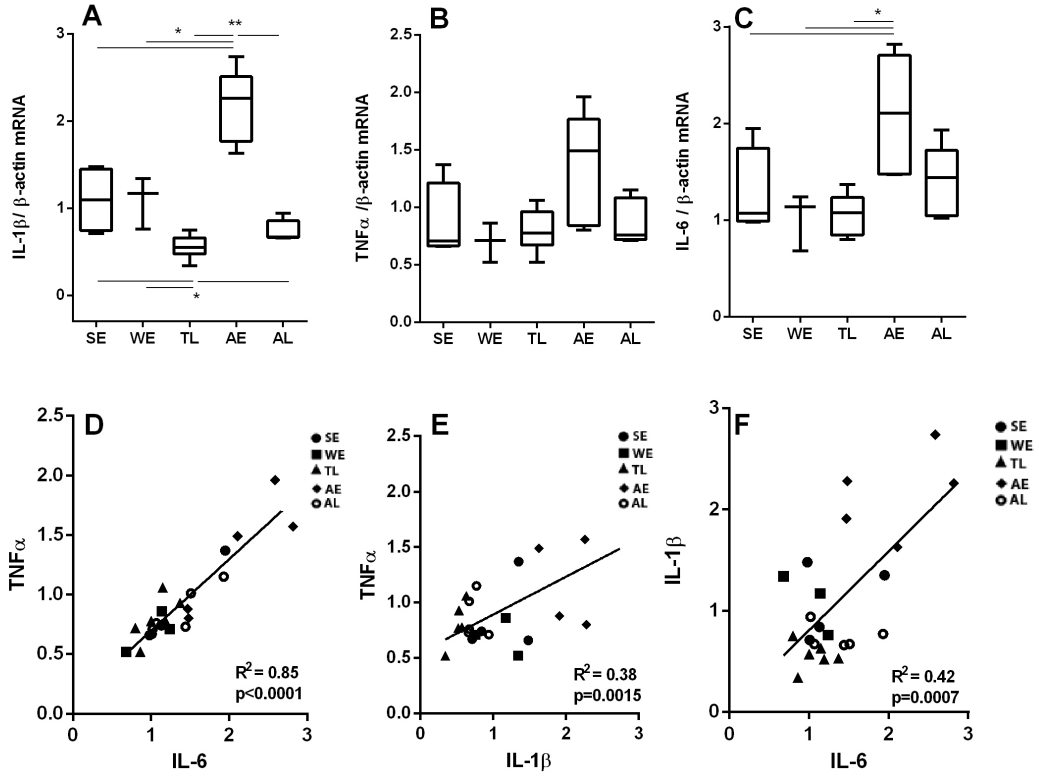

Figure 1. mRNA expression levels and correlation of pro-inflammatory cytokines in hippocampus during the different phases of hibernation and euthermia. Expression levels of IL-1β (A), TNF-α (B), IL-6 (C) are upregulated in AE and normalize in AL to SE and WE levels. Panel D-E: TNF-α is positively correlated with IL-6 and IL-1β . F: Il-1β is positively correlated with IL-6. SE: summer euthermia; WE: winter euthermia; TL: torpor late; AE: arousal early; AL: arousal late; *p < 0.05, **p<0.01.

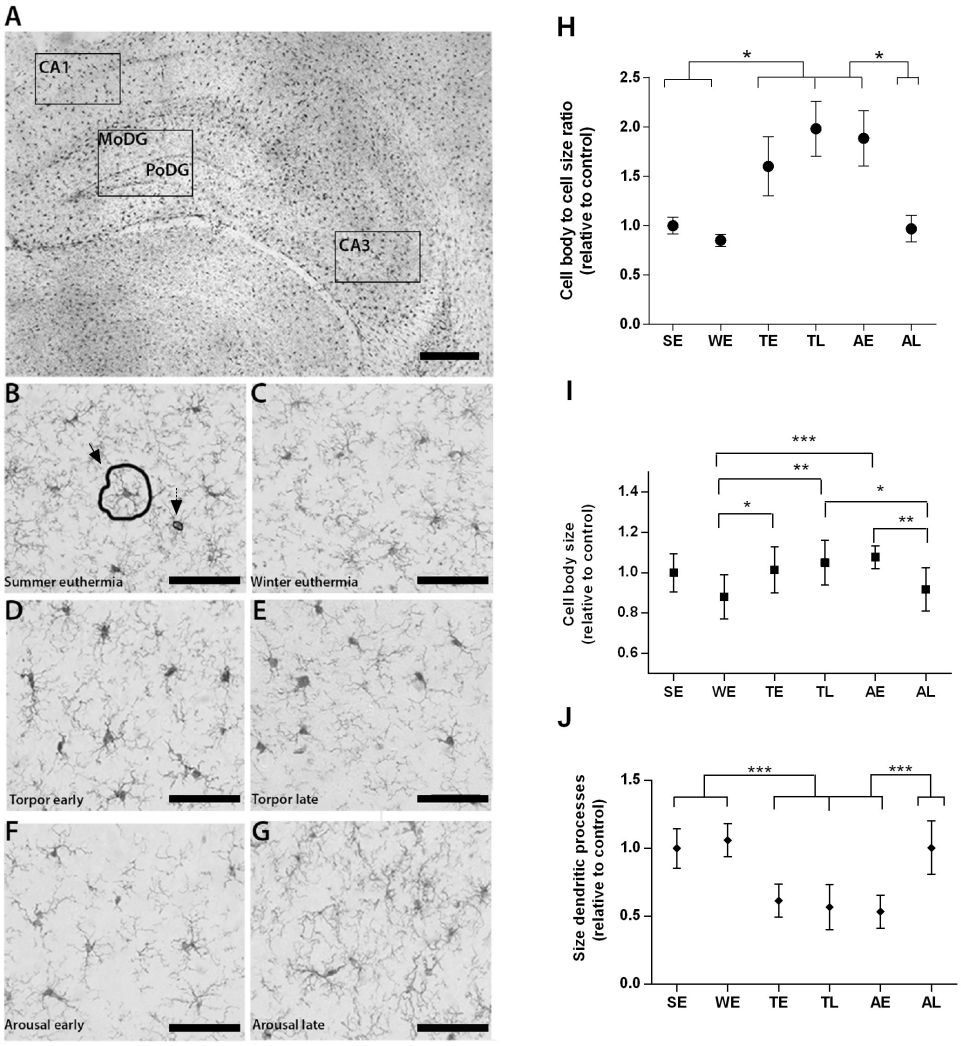

Figure 2. Microglia morphological changes and during the different phases of hibernation and euthermia. A: Overview of dorsal hippocampus and the areas of interest. B-G: In TE, TL and AE, the density of the dendritic branches decreases and the cell body size increases. In AL these changes normalize and microglia morphology is comparable to SE and WE conditions. H: Cell body size to total cell size ratio (semi) quantification of IBA-1 immunoreactivity in hippocampus total*. In TE, TL and AE, the ratio increases by about two fold and normalizes in AL to SE and WE levels. I-J: Cell body size and size dendritic processes (semi) quantification in hippocampus total*. Cell body size significanly increases in TE, TL and AE compared to WE and decreases in AL. In TE, TL and AE the size of dendritic processes decreases compared to SE and WE and returns to euthermic levels in AL. Straight arrow points to total cell body size and dashed arrow points to cell body size. SE: summer euthermia; WE: winter euthermia; TE: torpor early; TL: torpor late; AE: arousal early; AL: arousal late; CA1: cornu ammonis 1; CA3: cornu ammonis 3; PoDG: polymorphic layer of the dentate gyrus; MoDG: molecular layer of the dentate gyrus; *Hippocampus total includes: CA1, CA3, MoDG and PoDG. B-G pictures are taken at 200x magnification and are representative of CA1 area; *p <0.05, **p<0.01, ***p<0.001.

 The 2 fold increase in microglia cell body size to total cell size ratio (%) indicates activation of microglia throughout torpor and early arousal.

 Significant upregulation of proinflammatory cytokines IL-6 and IL-1β is present during early arousal.

 Upregulation of proinflammatory cytokines and changes in microglia morphology normalize to euthermic levels in late arousal.