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THEMED ISSUE: CANNABINOIDS

REVIEW

Endocannabinoid signalling: has it got rhythm?

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Endogenous cannabinoid signalling is widespread throughout the body, and considerable evidence supports its modulatory role in many fundamental physiological processes. The daily and seasonal cycles of the relationship of the earth and sun profoundly affect the terrestrial environment. Terrestrial species have adapted to these cycles in many ways, most well studied are circadian rhythms and hibernation. The purpose of this review was to examine literature support for three hypotheses: (i) endocannabinoid signalling exhibits brain region-specific circadian rhythms; (ii) endocannabinoid signalling modulates the rhythm of circadian processes in mammals; and (iii) changes in endocannabinoid signalling contribute to the state of hibernation. The results of two novel studies are presented. First, we report the results of a study of healthy humans demonstrating that plasma concentrations of the endocannabinoid, *N*-arachidonylethanolamine (anandamide), exhibit a circadian rhythm. Concentrations of anandamide are threefold higher at wakening than immediately before sleep, a relationship that is dysregulated by sleep deprivation. Second, we investigated differences in endocannabinoids and congeners in plasma from *Marmota monax* obtained in the summer and during the torpor state of hibernation. We report that 2-arachidonoylglycerol is below detection in *M. monax* plasma and that concentrations of anandamide are not different. However, plasma concentrations of the anorexigenic lipid oleoylethanolamide were significantly lower in hibernation, while the concentrations of palmitoylethanolamide and 2-oleoylglycerol were significantly greater in hibernation. We conclude that available data support a bidirectional relationship between endocannabinoid signalling and circadian processes, and investigation of the contribution of endocannabinoid signalling to the dramatic physiological changes that occur during hibernation is warranted.
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Abbreviations: 2-AG, 2-arachidonoylglycerol; ABH, alpha, beta hydrolase; AEA, *N*-arachidonylethanolamine; CB, cannabinoid; DGL, diacylglycerol lipase; DRN, dorsal raphe nuclei; ECS, endogenous cannabinoid signalling; FA, fatty acid; FAAH, fatty acid amide hydrolase; GDE, glycerophosphodiesterase; IGL, intergeniculate nucleus of the thalamus; LC–MS, liquid chromatography–mass spectrometry; MAG, monoacylglycerol; MGAT, monoacylglycerol acetyltransferase; MGL, monoacylglycerol lipase; NAAA, fatty acyl amide hydrolase with an acid optimum; NAE, *N*-acylethanolamine; NAPE, *N*-acyl-phosphatidylethanolamine; *N*-arach-PE, *N*-arachidonyl-phosphatidylethanolamine; PLC, phospholipase C; PLD, phospholipase D; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; REMS, rapid eye movement sleep; RHT, retinal hypothalamic tract; SCN, superchiasmatic nucleus; SWS, slow wave sleep; TAG, triacylglyceride; T_b, core body temperature; THC, Δ^9-tetrahydrocannabinol; TRPV1, transient receptor potential vanilloid type 1; WAT, white adipose tissue

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Introduction

We live in a world in which periodic environmental change, driven by geophysical cycles, dominates the activity of life (Foster and Roenneberg, 2008). The most obvious cyclical

change is the circadian/diurnal light–dark cycle, driven by the 24 h rotational period of the earth around its axis, which influences patterns of sleep and arousal. Because the metabolic and behavioral requirements of an organism are different between sleep and arousal states, many other physiological processes are also circadian (Lemmer, 2009). For example, the drive to eat is most efficiently expressed during the active period of the light–dark cycle. Similarly, metabolic rate and core body temperature exhibit clear circadian rhythms.

Temperate and polar regions experience yearly cycles in the intensity and duration of sunlight reaching the earth. To survive, plant and animal species that live in these regions must cope with circannual changes in the availability of food and water, and in external temperature (Foster and Roenneberg, 2008). One remarkable example of such adaptation is hibernation, a set of highly coordinated, physiological, biochemical and behavioral processes that occur during the season of winter (Carey *et al*., 2003a).

Endogenous cannabinoid signalling (ECS) is broadly utilized throughout the body as a mechanism to regulate intercellular communication. There is evidence that pharmacological manipulation of cannabinoid (CB) receptor signalling affects sleep/wake cycles (Murillo-Rodriguez, 2008b), temperature regulation (Maccarrone and Wenger, 2005), food consumption and fat storage (de Kloet and Woods, 2009), CNS regulation of autonomic (Pacher *et al*., 2005) and endocrine functions (Maccarrone and Wenger, 2005), reward-driven behaviour (Solinas *et al*., 2008), gastrointestinal function (Aviello *et al*., 2008), mood (Hill and Gorzalka, 2009) and sensory perception (Biro *et al*., 2009). All of these processes are altered in a cyclical manner.

The goal of this review was to present the hypotheses that ECS is influenced by circadian and circannual cycles, and that circadian and circannual cycles influence biology via changes in ECS. The interactions between ECS and circadian cycles have been the subject of several studies, and these are reviewed herein. However, there are no data regarding either the roles of the ECS in hibernation or the effects of hibernation on ECS; therefore, our goal in that section was to provide food for thought and ideas for future experiments.

Essentials of ECS

ECS consists of two arachidonate ligands, *N*-arachidonylethanolamine (AEA; anandamide) (Devane *et al*., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam *et al*., 1995; Sugiura *et al*., 1995), and at least two G-protein-coupled receptors, CB receptor types 1 (CB_1) (Matsuda *et al*., 1990) and 2 (CB_2) (Munro *et al*., 1993). In addition, intracellular AEA also targets the vanilloid receptor (TRPV1), which has led to the hypothesis that it could be a second messenger that regulates calcium signalling through this receptor (De Petrocellis and Di Marzo, 2009). Both AEA and 2-AG are the arachidonate members of larger lipid families: the *N*-acylethanolamines (NAEs) and the 2-monoacylglycerols (2-MAGs). While these other family members have overlapping synthetic and catabolic processes, with few exceptions (Hillard and Campbell, 1997), they do not act via the CB receptors.

Available data indicate that 2-AG is produced by post-synaptic neurons in response to metabotropic receptor activation and/or depolarization, and targets CB_1 receptors present on pre-synaptic terminals (Pan *et al*., 2009). Thus, 2-AG mediates activity-dependent retrograde inhibition of synaptic activity in many brain regions (Patel and Hillard, 2009). In all but one reported case (Azad *et al*., 2004), AEA is not involved in this process. Neither the mechanisms involved in the regulation nor in the function of AEA–CB_1 receptor signalling are well understood. Interestingly, AEA is a partial agonist of the CB_1 receptor (Kearn *et al*., 1999), and its extracellular concentration is lower than 2-AG (Caille *et al*., 2007). It is possible that AEA provides low intensity tonic activation of CB_1 receptor signalling, while 2-AG functions as a phasic high-intensity signal.

It is generally accepted that endogenous activation of the CB_1 and CB_2 receptors is regulated by processes that govern the biosynthesis and catabolism of the endocannabinoids AEA and 2-AG. AEA concentrations are regulated by the conversion of a minor phosphoglyceride, *N*-arachidonyl-phosphatidylethanolamine (*N*-arachPE), via either a phospholipase D (NAPE-PLD) (Okamoto *et al*., 2009) or a two-enzyme pathway that involves an alpha, beta hydrolase (ABH4) and a glycerophosphodiesterase (GDE1) (Simon and Cravatt, 2008). The mechanisms that regulate the activities of these enzymes and the specifics of their involvement in the synthesis of AEA are currently not well understood. At least three enzymes are involved in the catabolism of AEA: fatty acid amide hydrolase (FAAH) (Cravatt *et al*., 1996), a lysosome-localized fatty acyl amide hydrolase with an acid optimum (NAAA) (Tsuboi *et al*., 2005) and a recently identified FAAH-2 localized in lipid droplets (Kaczocha *et al*., 2009).

2-AG is synthesized via a two-enzyme cascade of phospholipase C (PLC) and diacylglycerol lipase (DGL). Recent evidence suggests that in neurons, the rate-limiting step for the synthesis of CB_1 receptor-targeted 2-AG is DGL (Bisogno *et al*., 2003; Yoshida *et al*., 2006; Gao *et al*., 2010). 2-AG is hydrolysed by several enzymes; in brain, 85% of the hydrolysis occurs via monoacylglycerol lipase (MGL), the remaining is hydrolysed by ABHD6 and ABHD12 (Blankman *et al*., 2007), enzymes about which far less are known. AEA and 2-AG are also substrates for some arachidonate oxygenases, including lipoxygenases (Edgemond *et al*., 1998) and COX 2 (Kozak *et al*., 2002).

Several generalities about ECS are supported by considerable experimental evidence. First, ECS is widespread. In addition to very prominent expression within the brain, ECS has been identified in the spinal cord (Hohmann, 2002), sympathetic nervous system (Ralevic and Kendall, 2009), enteric nervous system (Massa and Monory, 2006), liver (Mallat and Lotersztajn, 2008), adipose tissue (Nogueiras *et al*., 2009), immune system (Munro *et al*., 1993) and pancreas (Juan-Pico *et al*., 2006).

Second, ECS is modulatory. Within the CNS, the CB_1 receptor is located on pre-synaptic terminals, and functions to inhibit neurotransmitter release (Patel and Hillard, 2009). CB_1 receptors also regulate transmitter release from sympathetic terminals (Pakdeechote *et al*., 2007), enteric nerves (Tyler *et al*., 2000; Massa and Monory, 2006) and peripheral sensory nerves (Agarwal *et al*., 2007).

Third, ECS is plastic; in fact, multiple mechanisms have been identified for the regulation of ECS. As was outlined above, enzymatic mechanisms regulate the biosynthesis and catabolism of the endocannabinoids. In addition, conditions have been identified that regulate CB receptor expression. For example, hippocampal CB_1 receptors are down-regulated by chronic stress (Hill *et al*., 2005) and macrophage CB_2 receptor expression changes with alterations in cellular activation (Carlisle *et al*., 2002). At the systems level, the plasticity of ECS allows for context-dependent signalling.

Fourth, ECS serves a homeostatic role. At the synaptic level, this is seen in its function as an activity-dependent retrograde mediator of synaptic transmission (Freund *et al*., 2003). At glutamatergic synapses, endocannabinoids are mobilized in response to glutamate activation of metabotropic receptors and/or depolarization, and serve to reduce excitatory drive. At a systems level, activation of the ECS contributes to recovery from activation of the hypothalamic–pituitary–adrenal (HPA) axis, for example (Di *et al*., 2003).

ECS and the circadian cycle

ECS is intertwined with the circadian rhythm in several respects. Amounts of the endocannabinoids, their degradative and synthetic enzymes and their receptors all show tissue-specific diurnal changes, indicating that ECS is 'downstream' of circadian regulators. On the other hand, exogenous and endogenous CBs affect many important physiological processes that exhibit a circadian rhythm: sleep–wakefulness, body temperature, HPA endocrine secretions, food intake, learning and memory and locomotor activity. These findings indicate that ECS is 'upstream' of circadian processes. Therefore, a central thesis of this review is that ECS serves as a link between circadian regulators, such as the intrinsic clock of the suprachiasmatic nucleus (SCN) and the physiological processes that they affect.

The circadian rhythm of ECS components

There is evidence that ECS exhibits a circadian rhythm with variations reported in endocannabinoid tissue contents (Valenti *et al*., 2004; Murillo-Rodriguez *et al*., 2006), CB_1 receptor number (Martinez-Vargas *et al*., 2003; Rueda-Orozco *et al*., 2008) and in the enzymes controlling the synthesis and degradation of endocannabinoids (Valenti *et al*., 2004).

Because endocannabinoids are mobilized 'on-demand', their concentrations in lipid extracts of isolated brain regions are hypothesized to be proportional to their concentrations in the synapse. In Sprague-Dawley rats, significant diurnal variations in AEA and 2-AG contents have been demonstrated in CSF, hypothalamus, hippocampus, pons, nucleus accumbens, prefrontal cortex and striatum (Valenti *et al*., 2004; Murillo-Rodriguez *et al*., 2006). With respect to AEA concentrations, two patterns have been reported. In the pons (Valenti *et al*., 2004; Murillo-Rodriguez *et al*., 2006), nucleus accumbens, prefrontal cortex, hippocampus and striatum (Valenti *et al*., 2004), AEA content is higher in tissues harvested during the active phase of the rats (i.e. when the lights are off) than during the inactive phase. An opposite pattern is seen in CSF and hypothalamus, where AEA concentrations are higher in the inactive than in the active phase (Murillo-Rodriguez *et al*., 2006). These studies suggest that AEA-mediated signalling varies with time of day and that multiple mechanisms link the circadian rhythms with changes in AEA biosynthesis and/or clearance. In a few cases, the mechanism could involve changes in the activity of FAAH. One study found that FAAH activity was negatively correlated with AEA content in the hippocampus and striatum, suggesting that circadian changes in FAAH activity could underlie the changes in AEA content in those regions (Valenti *et al*., 2004). However, a recent study comparing FAAH activity at the midpoint of the light and dark phases did not find differences in either striatum or hippocampus, but did report small but significant differences in FAAH activity in the cerebellum and periaqueductal gray at these time-points (Glaser and Kaczocha, 2009).

Interestingly, the tissue contents of 2-AG were opposite to those of AEA in tissues where both were measured. 2-AG contents were higher during the inactive phase (day) in nucleus accumbens, prefrontal cortex, striatum and hippocampus (Valenti *et al*., 2004). In the striatum, the activities of both MGL and DGL are higher during the inactive than active phase (Valenti *et al*., 2004); increased DGL activity in particular is consistent with higher turn-over of 2-AG during the inactive phase in the striatum. On the other hand, no changes in MGL or DGL were seen in the hippocampus (Valenti *et al*., 2004), suggesting divergent mechanisms for the regulation of 2-AG between striatum and hippocampus.

There is evidence that CB_1 receptor density in rat brain is regulated in a circadian manner. In both pons (Martinez-Vargas *et al*., 2003) and hippocampus (Rueda-Orozco *et al*., 2008), the density of CB_1 receptor protein is approximately 5% higher during the inactive than the active phase. The pattern of protein expression is similar in both regions, with peaks at the midpoint in the light period and troughs 12 h later. CB_1 receptor mRNA expression exhibits a more substantial diurnal variation, with increases of 11% in the pons (Martinez-Vargas *et al*., 2003) and 50% in the hippocampus (Rueda-Orozco *et al*., 2008). In both regions, mRNA concentrations are out of phase with the changes of CB_1 receptor protein over a 24 h period, but the patterns are slightly different. Neither protein nor mRNA for CB_1 receptors varies significantly in the striatum (Rueda-Orozco *et al*., 2008).

The changes in endocannabinoid content and CB_1 receptor density with time of day in the pons and hippocampus display interesting relationships. In both brain regions, AEA content and CB_1 receptor protein concentration are nearly perfectly out of phase with each other (Figure 1A,B). For the majority of the inactive phase, CB_1 receptor density is high and AEA content is low in the hippocampus. We hypothesize that CB_1 receptor signalling is in a state of low basal tone (due to low AEA concentrations) and high sensitivity to 2-AG activation (Figure 1C). The finding that hippocampal 2-AG content is higher in the inactive phase suggests that its synthesis is greater or clearance is reduced during this phase, which could also contribute to a situation in which CB_1 receptor activation by 2-AG is potentiated. When the animals are awake and active, AEA tone is high, while CB_1 receptor density is slightly lower. We hypothesize that this results in

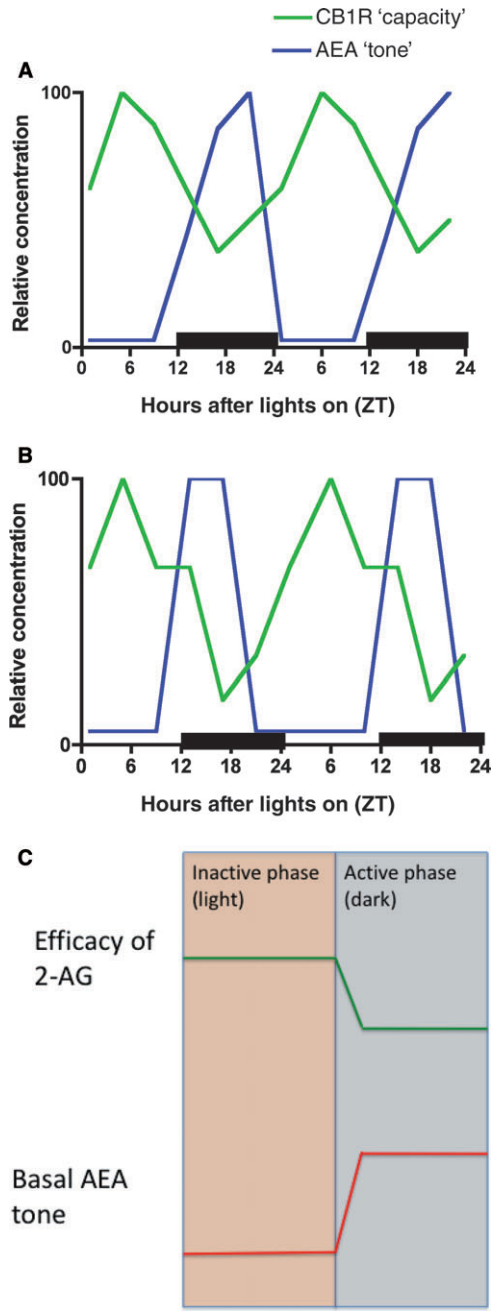

Figure 1 Schematic of the relationship between time and changes in AEA content and CB_1 receptor protein expression in hippocampus (A) and pons (B). The data plotted were taken from Martinez-Vargas *et al*. (2003), Valenti *et al*. (2004), Murillo-Rodriguez *et al*. (2006) and Rueda-Orozco *et al*. (2008); changes are displayed relative to the high and low values reported. (C) Hypothesis of the dual regulation of the effectiveness of 2-AG to activate CB_1 receptor-mediated signalling by AEA concentrations and the CB_1 receptor density. In both of these brain regions, AEA content is low in the inactive phase of the day, while CB_1 receptor protein is at its highest concentration. We hypothesize that this allows for high efficacy of 2-AG activation of CB_1 receptor signalling. In the active phase of the day, CB_1 receptor protein is lower, while AEA content is high; we hypothesize that this results in higher basal receptor tone and lower maximal efficacy for 2-AG than in the inactive phase.

higher basal tone, but reduced efficacy of 2-AG activation (Figure 1C). We propose a similar mechanism is operative in the pons; however, the period of time during which AEA content is high, and thus CB_1 receptor signalling is less sensitive to 2-AG, is confined to the first half of the active phase. Previous studies demonstrating that CB_1 receptor agonists increase neuronal activity in the locus coeruleus (Muntoni *et al*., 2006) and that neuronal activity in the locus coeruleus neurons is greater in the active period than the inactive period (Aston-Jones *et al*., 2001) suggest the intriguing hypothesis that high AEA concentrations in the active phase contribute to the awake state supported by high locus coeruleus output. Interestingly, AEA content in the hypothalamus exhibits the opposite relationship to time which, if the hypothesis presented above is correct, would result in high basal CB_1 receptor signalling during the inactive phase.

Time of day and sleep deprivation affect plasma endocannabinoids in humans

The endocannabinoids are also present in the circulation, although their source and targets are not well understood. In a small pilot study, we explored the circadian rhythms of circulating endocannabinoids in humans. Plasma was obtained in the late evening, early morning and early evening from five healthy humans who had consistent sleep/wake cycles for at least 5 days prior to sampling. Lipid extracts from plasma were obtained and concentrations of AEA and 2-AG were determined using liquid chromatography–mass spectrometry (LC–MS) as outlined in Hill *et al*. (2008). Blood samples were obtained during a 24 h stay at the clinical research centre at the University of Chicago; the subjects remained in bed with lights out from 2230 at day 1 until 0700 at day 2. AEA and 2-AG contents were determined in plasma obtained at 2200 at day 1, and at 0730 and 1730 at day 2 (Figure 2A). No significant relationship between 2-AG concentrations and time of day was found; however, there was a highly significant effect of time on AEA concentration ($F_{2,14}$ = 114; $P < 0.0001$). Tukey's multiple comparison test revealed a significant difference between the concentration in blood obtained at 2200 at day 1 and at 0730 at day 2 ($q = 20$; $P < 0.001$), and between blood obtained at 0730 and 1730 at day 2 ($q = 15$; $P < 0.001$). The difference in AEA concentrations between blood obtained at 2200 at day 1 and at 1730 at day 2 was not significant ($q = 3.5$; $P > 0.05$). These data suggest that circulating AEA concentrations rise during sleep, although the exact pattern of change remains to be determined.

In a second phase of the pilot study, we explored the effects of sleep deprivation on plasma endocannabinoids. The same five individuals spent a second night in the clinical research centre during which they were not allowed to sleep. The session (first or second) at which sleep deprivation was imposed was randomized. Plasma endocannabinoids were determined in blood drawn at 2200 at day 1 and at both 0730 and 1730 at day 2 (Figure 2B). As evidence of the stability of circulating AEA concentrations, there was no difference in AEA concentrations in blood drawn at 2200 between the two

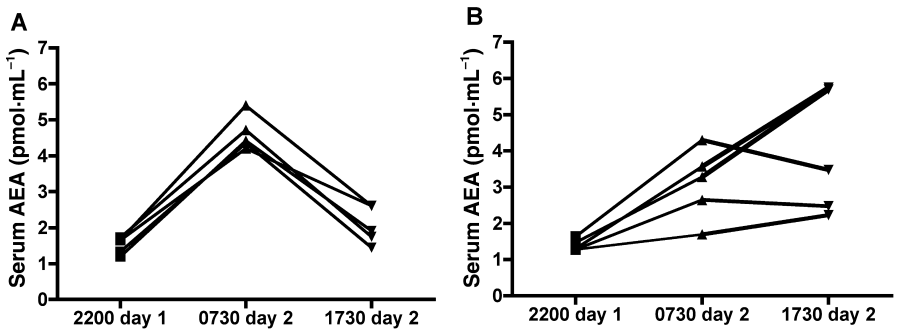

Figure 2 Blood was obtained from five (three male) subjects during two 24 h sessions in the clinical research centre of the University of Chicago. The two sessions were 1 week apart. During one of the sessions, the subjects were in bed with the lights out from 2230 (day 1) until 0700 (day 2) (A; normal sleep session); during the other session, the subjects did not sleep at all (B; sleep deprivation session). The two sessions were randomized with respect to deprivation. A study staff monitored the subjects during both visits to ensure compliance with the sleep or deprivation conditions. Plasma was obtained in Chicago, frozen within 2 h of collection and shipped to Milwaukee for analysis on dry ice. Endocannabinoids were extracted and measured as reported previously (Hill *et al.*, 2008). Lines connect data points obtained from the same subject. This study was approved by the Institutional Review Boards of the University of Chicago and the Medical College of Wisconsin. Please see text for the statistical comparisons.

clinic visits, which were 1 week apart (paired $t = 1.46$; $P = 0.22$). One-way ANOVA of the AEA concentrations by time of blood draw in the sleep deprivation arm also indicated a significant effect of time ($F_{2,14} = 8.9$, $P < 0.01$); however, the only comparison that reached significance in the Tukey's *post hoc* tests was between 2200 at day 1 and 1730 at day 2 ($q = 5.8$; $P < 0.01$), a comparison that was not significant in the same subjects allowed to sleep normally. The q value for the comparison between 2200 at day 1 and 0730 at day 2 was 3.9, and between 0730 and 1730 at day 2 was 1.9; both $P > 0.05$. Therefore, these data indicate that lack of normal sleep produces a significant dysregulation of circulating AEA. Intriguing as these data are, it is difficult to interpret their functional significance because we know little about the regulation of AEA concentrations in the circulation and the target of this mediator. These data are at odds with a previous report that sleep deprivation did not significantly alter plasma AEA concentrations in blood drawn between 1000 and 1200 the day following sleep deprivation (Koethe *et al.*, 2009). However, close inspection of data reported in that paper indicates that sleep deprivation increased the variance in plasma AEA concentrations in accord with the present results.

ECS and circadian rhythms

The primary circadian clock in mammals is located in the SCN, a distinct group of cells located in the hypothalamus. Destruction of the SCN results in the complete absence of a regular sleep/wake rhythm (Weaver, 1998). The clock can be entrained or tuned by environmental factors, called zeitgebers (Roenneberg and Merrow, 2007). The most well-studied zeitgeber is light; other zeitgebers are environmental temperature, availability of food and social interactions (Roenneberg and Merrow, 2007). The SCN receives information about environmental illumination through photoresponsive retinal ganglion cells. These cells project to the SCN via the retinohypothalamic tract (RHT) (Okamura, 2003). Other important inputs to the SCN are afferents from the intergeniculate leaflet (IGL) of the thalamus, which integrates photic and non-photic information (Hastings *et al.*, 1997), and the serotonergic projections of the raphe nuclei, which entrain the clock in a non-photic manner and affect light input as well (Challet, 2007).

The widespread distribution of the CB_1 receptor in the brain offers support for multiple possibilities by which ligands of this receptor could alter the response of the SCN to light input and could modulate clock outputs (Figure 3). CB_1 receptors are present in the hamster (Sanford *et al.*, 2008) and mouse SCN (Wittmann *et al.*, 2007). Therefore, CB agonists could directly influence glutamate and/or GABA neurotransmission within the SCN. In support of this possibility, Sanford *et al.* (2008) have recently reported that CB_1 receptor activation inhibits the phase advance in activity patterns produced by a light pulse administered to hamsters held in total darkness. CB_1 receptor antagonists had no effect alone on the light-induced phase advance, but completely blocked the agonist effect. Because CB_1 receptors are present in the hamster SCN, these data are consistent with a role for CBs to inhibit input to the SCN from the RHT. However, these data could also reflect CB_1 receptor inhibition of SCN outputs (Figure 3).

CB_1 receptors are found in the dorsal and median raphe nuclei of the hamster (Sanford *et al.*, 2008), and in the raphe nuclei of rats and mice (Moldrich and Wenger, 2000; Haring *et al.*, 2007). Serotonergic agents are capable of inhibiting light-induced phase shifts in a manner similar to the effect of CB_1 receptor agonists discussed above (Weber *et al.*, 1998; Gannon and Millan, 2006). There is evidence that the raphe–hypothalamic projection is affected by ECS. Treatment of rats with the CB_1 receptor agonist CP55940 increases hypothalamic levels of serotonin (5HT) (Arevalo *et al.*, 2001). Pharmacological inhibition of FAAH increases brain AEA concentrations and the firing rate of serotonergic dorsal raphe neurons via CB_1 receptor activation (Gobbi *et al.*, 2005). The ECS could interact with the serotonergic system in the dorsal raphe nucleus (DRN) in part through mediating the orexin modulation of glutamatergic synaptic transmission to DRN 5HT neurons (Haj-Dahmane and Shen, 2005). The orexins are neuropeptides involved in sleep–wakefulness, feeding and reward (Matsuki and Sakurai, 2008; Shioda *et al.*, 2008; Mieda

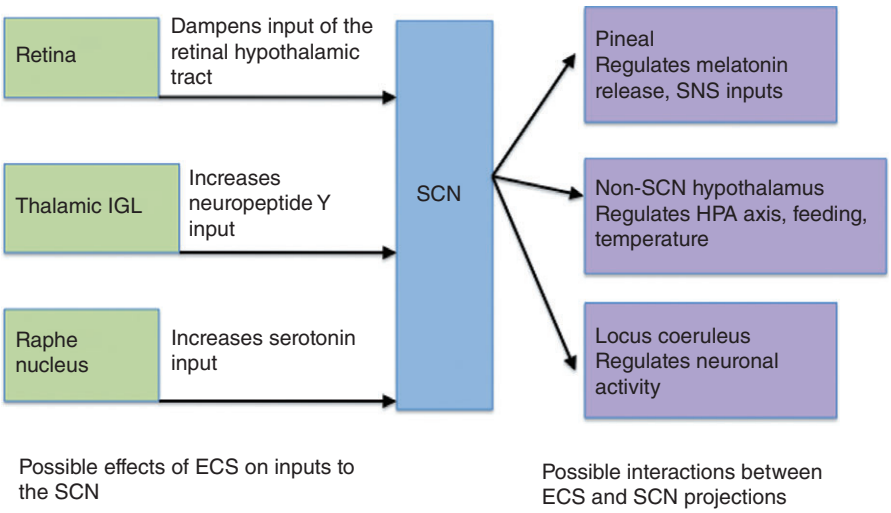

Figure 3 Possible mechanisms by which endocannabinoid/CB_1 receptor signalling can alter inputs to the SCN and projections from the SCN. See the text for details and references.

and Sakurai, 2009). Orexin B acts on DRN serotonergic neurons to stimulate the synthesis and release of 2-AG (Haj-Dahmane and Shen, 2005). In addition, the CB_1 and orexin receptors can form dimers that influence the localization and signalling of both receptors (Ellis *et al.*, 2006).

Neuropeptide Y (NPY) is released in the SCN from projections originating in the IGL (Harrington *et al.*, 1985; Harrington, 1997), and is thought to convey non-photic cues to the SCN and inhibit light-induced adjustments of the circadian rhythm (Biello, 1995; Maywood *et al.*, 2002). Like the CB_1 receptor agonist, NPY agonists inhibit light-induced phase shifts (Huhman and Albers, 1994; Weber and Rea, 1997; Yannielli and Harrington, 2000; 2001). CB_1 receptor agonists increase while antagonists decrease resting and evoked NPY release in rat hypothalamic explants (Gamber *et al.*, 2005). This mechanism is consistent with the effect of CB_1 receptor agonists to inhibit the light-induced phase shift. The presence of CB_1 immunoreactivity in the hamster IGL (Sanford *et al.*, 2008) supports the possibility that CB_1 agonists inhibit light-induced phase shifts through increasing IGL–SCN afferent activity.

ECS components are present in the pineal (Koch *et al.*, 2008), a brain region that receives multi-synaptic relays originating in the SCN, and synthesizes and releases melatonin. Within the pineal gland, CB_1 receptors are present in both pinealocytes and on the terminals of sympathetic afferents to the gland. Both FAAH and NAPE-PLD are also expressed in pinealocytes. Interestingly, NAPE-PLD is also present in sympathetic terminals, which leads to the possibility that an NAE could be synthesized and released as a co-transmitter with norepinephrine. The function of ECS within the pineal has not been studied; however, Δ^9-tetrahydrocannabinol (THC) and other plant-derived CBs reduce melatonin synthesis in the pineal via a non-CB_1 receptor-dependent mechanism (Koch *et al.*, 2006).

ECS and sleep patterns

There is considerable evidence that ECS affects the organization of sleep. Acute administration of THC causes a decrease in rapid eye movement sleep (REMS) in rabbits (Fujimori and Himwich, 1973), cats (Wallach and Gershon, 1973) and humans (Pivik *et al.*, 1972; Freemon *et al.*, 1974), and increases slow wave sleep (SWS) in rabbits (Fujimori and Himwich, 1973), humans (Pivik *et al.*, 1972) and rats (Moreton and Davis, 1973). However, chronic administration has been found to decrease SWS with inconsistent effects on REMS in humans, cats and squirrel monkeys (Barratt and Adams, 1973; Pranikoff *et al.*, 1973; Barratt *et al.*, 1974; Adams and Barratt, 1975). These findings suggest that tolerance or some other form of adaptation occurs when THC is administered chronically.

More recent studies have explored the involvement of ECS in the control of the sleep/wake cycle. Available data indicate that ECS maintains and/or promotes the sleep state (Murillo-Rodriguez, 2008a). In particular, treatment of rats with a CB_1 receptor antagonist 4 h after lights on increases the time spent in wakefulness and decreases time spent in both SWS and REMS during the subsequent 4 h (Santucci *et al.*, 1996). Conversely, injection of AEA i.c.v. or into the pedunculopontine tegmental nucleus at onset of light caused a decrease in wakefulness and an increase in SWS and REMS (Murillo-Rodriguez *et al.*, 1998). The effect of AEA was blocked by both rimonabant and a PLC inhibitor (Murillo-Rodriguez *et al.*, 2001). Inhibition of AEA clearance with AM404 also decreased wakefulness and increased sleep, although these effects were only partially sensitive to rimonabant (Murillo-Rodriguez *et al.*, 2008). Further evidence in support of a role for the ECS in the regulation of sleep comes from studies demonstrating that CB_1 receptor density is significantly increased in the rat pons during the rebound phase following sleep deprivation (Martinez-Vargas *et al.*, 2003). These data suggest that increased ECS, at the level of the receptor, could be involved in the homeostatic recovery of sleep following deprivation.

CBs and the circadian rhythm of temperature

There is considerable evidence that exogenously administered CB_1 receptor agonists affect body temperature regulation. At

'normal' ambient temperatures, THC produces a very significant hypothermia (in the range of 5°C reduction in rectal temperature) (Martin *et al*., 1991) that is completely abolished by co-administration of a CB_1 receptor antagonist (Hillard *et al*., 1999). However, studies in which ambient temperature is a variable indicate that mice become poikilothermic when treated with THC (Bloom and Kiernan, 1980). More recently, investigators have utilized intra-hypothalamic injection of direct and indirect agonists of the CB_1 receptor to study the effects of ECS on thermoregulation. The available results are contradictory, demonstrating that agonists both increase (Fraga *et al*., 2009) and decrease core body temperature (Benamar *et al*., 2009) in a CB_1 receptor-dependent manner.

Most mammals exhibit a daily circadian rhythm in body temperature, which is higher during the active phase than the inactive phase (Kelly, 2006). This rhythm is regulated by cyclical release of melatonin by the pineal gland; a higher melatonin release during the inactive phase correlates with and likely drives lower body temperature (Cagnacci *et al*., 1997). The hypothermic effect of THC is greatest in the second half of the inactive phase, less in the first half of the inactive phase and least in the active phase (Abel, 1973). This suggests that THC could function to potentiate the secretion of melatonin. Interestingly, there is some functional evidence that THC inhibits noradrenergic increases in melatonin synthesis, perhaps via inhibition of norepinephrine release from sympathetic terminals in the pineal (Koch *et al*., 2006; 2008), a result opposite of the outlined expectation.

Brain temperature also exhibits a circadian rhythm (Aschoff *et al*., 1973). Exogenous administration of CBs disrupts the circadian rhythm of brain temperature. Daily injection of rats with THC for 1 week caused the circadian variation in brain temperature to be diminished (Perron *et al*., 2001). Further, there was a gradual lengthening of cycle duration such that by the end of the treatment period, the brain temperature rhythm of THC-injected animals was 12 h out of phase relative to vehicle-injected controls. The out-of-phase cycling continued without change during a recovery week. In contrast to the marked effect on brain temperature, the circadian rhythm of abdominal temperature was not significantly affected by THC. The finding that the presence of exogenous CB_1 receptor agonist administered at a consistent time of day entrains brain temperature suggests that ECS is an important regulator of this process. Whether the ECS is involved in entrainment of the rhythms of other physiological processes is not known, but is an important question.

ECS and circadian regulation of the HPA axis

There is a pronounced circadian rhythm to the secretions of corticotropin-releasing hormone, corticotropin (ACTH) and glucocorticoids that is controlled by the SCN (Moore and Eichler, 1972). There is also considerable evidence that ECS regulates the activation of the HPA axis by stress (Patel *et al*., 2004), and is required for normal glucocorticoid-mediated feedback on the HPA axis (Di *et al*., 2003; Cota *et al*., 2007). Studies using CB_1 receptor null mice indicate that the fundamental rhythm of circulating glucocorticoids is intact in the global absence of ECS (Cota *et al*., 2007). However, relative to wild-type mice, CB_1 receptor null mice exhibit significantly higher circulating glucocorticoid concentrations at the onset of the active phase (Cota *et al*., 2007). These data are consistent with a role for the ECS to negatively regulate HPA axis activation, likely as a downstream mediator of glucocorticoid receptor activation (Di *et al*., 2003).

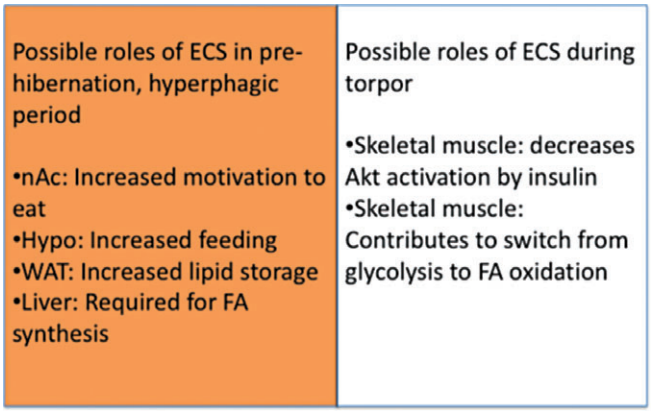

Figure 4 Possible mechanisms by which endocannabinoid signalling (ECS) could contribute to the feeding and metabolic changes that occur during the late-summer hyperphagic period and during the period of hibernation. See the text for details and references.

Hibernation is an extreme example of seasonal rhythm

Hibernation includes regulated decreases in body temperature (T_b), heart rate, respiration and metabolic rate (Zucker, 2001; Carey *et al*., 2003b; Geiser, 2004). However, hibernation is not just a winter phenomenon as it also requires 'preparation' for the winter food shortages through excess caloric intake during the summer months. Thus, hibernating species exhibit dramatic shifts in feeding behaviour and fat storage, resulting in significant changes in body weight through the year (Boswell *et al*., 1994; Dark, 2005). Moreover, hibernators do not exhibit a continual state of torpor, but rather hibernation consists of cycles of torpor (minimal T_b) and arousal (euthermia). These minimal and maximal states of body temperature are separated by distinct transition periods called entry into torpor and arousal, respectively (see figure 4 in Carey *et al*., 2003a).

The duration of day-time sunlight (photoperiod) synchronizes the circannual rhythms of hibernation, but torpor induction requires both a short photoperiod and low environmental temperature (Heldmaier *et al*., 1989). This combined need could relate to the animal's thermogenic capacity, as short photoperiod and cold together maximally increase brown adipose tissue mass, expression of mitochondrial uncoupling protein and lipolytic enzymes and sympathetic innervation (Cannon and Nedergaard, 1985).

The body mass cycle is regulated by a circannual clock (Lee and Zucker, 1991; Zucker, 2001). Like circadian rhythms, the seasonal rhythm will 'free run' with a period of 100 days in the absence of natural changes in the duration of sunlight and temperature. Return to natural conditions resets the

period to nearly a year, suggesting that, like the circadian clock, the circannual clock is entrained by environmental light and temperature.

Circannual cycle and feeding behaviour

The circannual hibernation cycle is characterized by shifts from normal feeding to hyperphagia to hypophagia. A key mediator of the circannual cycle of feeding behaviour is the anorexigenic hormone, leptin (Scarpace and Zhang, 2009). In black bears, circulating leptin increases in late summer, remains high during hibernation and decreases in spring (Donahue *et al*., 2006). Increased concentrations of leptin during hibernation are consistent with the profound hypophagia that occurs during hibernation, and decreased concentrations of leptin in spring likely allow for spring feeding. Leptin infusion in arctic ground squirrels following emergence from hibernation prevents hyperphagia (Boyer *et al*., 1997). The occurrence of hyperphagia in late summer despite high leptin concentrations in black bears (Donahue *et al*., 2006) could reflect resistance to the effects of leptin. Consistent with this hypothesis, brown bats become leptin resistant during the period of maximal fat deposition (Kronfeld-Schor *et al*., 2000).

ECS and feeding behaviour

ECS regulates orexigenic drive in reward-related brain regions and in the primary orexigenic nuclei of the hypothalamus. Recent evidence demonstrates that food consumption is characterized by learned habits and can be motivated by reinforcers (Volkow and Wise, 2005). There is clear evidence that ECS modulates the rewarding properties of food (Kirkham *et al*., 2002). CB_1 receptors are present in several primary relay nuclei of the reward pathway, including the prefrontal cortex (Eggan and Lewis, 2006) and the nucleus accumbens (Lupica and Riegel, 2005). Injection of 2-AG into the shell of the nucleus accumbens results in increased food intake (Kirkham *et al*., 2002).

The drive to eat is also regulated by the hypothalamus, and ECS occurs at multiple sites within the hypothalamic circuits involved in metabolic regulation (Cota *et al*., 2006). 2-AG levels in the hypothalamus are increased during fasting, and decreased as the animals are re-fed (Hanus *et al*., 2003), indicating that ECS in this region is recruited by changes in feeding status. Furthermore, injection of AEA into the ventromedial hypothalamus of satiated rats results in CB_1 receptor-mediated hyperphagia (Jamshidi and Taylor, 2001), suggesting that increased ECS over-rides normal satiety signals to induce inappropriate food consumption. In the context of the cycle of hibernation, this mechanism could be beneficial during the late-summer hyperphagic period (Figure 4).

A milestone in our understanding of the role of ECS in the regulation of satiety came from studies of Di Marzo and Kunos demonstrating that ECS is an important effector of leptin in the hypothalamus. In particular, leptin reduces hypothalamic contents of AEA and 2-AG in normal mice, and mice deficient in leptin signalling are obese and hyperphagic, and have elevated hypothalamic endocannabinoid contents (Di Marzo *et al*., 2001). Blockade of the CB_1 receptor in these mice results in decreased food intake, indicating that at least part of the anorexigenic effect of leptin is due to decreased ECS. Recent evidence also suggests that increased CB_1 receptor activity is involved in the orexigenic effects of NPY (Gamber *et al*., 2005) and ghrelin (Tucci *et al*., 2004). There is evidence that loss of leptin-induced inhibition of ECS could contribute to some forms of leptin resistance (Osei-Hyiaman *et al*., 2008), an observation that is interesting in light of the hypothesis presented above that late-summer hyperphagia in bears could be an example of leptin resistance. Overall, the tight relationship between leptin and ECS in the hypothalamus, together with the data discussed above that leptin is a critical mediator of the transitions between late summer and hibernation, and between hibernation and spring feeding, suggests the hypothesis that ECS is involved in the regulation of feeding in animals with a circannual cycle.

Circannual cycles of lipid and carbohydrate metabolism

Increased body mass in hibernators is largely due to increased lipid storage in white adipose tissues (WATs), and it is hypothesized that this process has a set point that varies circannually (Davis, 1976; Mrosovsky and Faust, 1985; Dark, 2005). Consistent with this hypothesis, limiting the foraging time of ground squirrels leads to decreased lean body mass, but WAT fat stores are conserved (Bachman, 1994).

Hibernators also exhibit dramatic shifts in cellular sources of metabolic fuel. During active periods, both carbohydrates and lipids are used (Buck and Barnes, 2000; Squire *et al*., 2003). Conversely, during torpor, metabolic needs are met exclusively by oxidation of fatty acids (FAs). Key molecular switches in cellular metabolism include the kinase Akt (Miyamoto *et al*., 2009). Activation of Akt by insulin receptor signalling increases utilization of carbohydrates as cellular energy sources. Consistent with a shift to lipolysis during hibernation, torpor is associated with significant decreases in Akt activity (Cai *et al*., 2004; Abnous *et al*., 2008). Recent data in human skeletal muscle suggest that CB_1 receptor activation, perhaps in response to AEA released from adipocytes, negatively regulates insulin stimulation of Akt activation (Eckardt *et al*., 2009). These data lead to the notion that adipose-derived endocannabinoids could modulate the switch between carbohydrate and lipid utilization during torpor (Figure 4).

Another key determinant of the cellular energy source is pyruvate dehydrogenase kinase 4 (PDK4) (Roche and Hiromasa, 2007). PDK4 inactivates pyruvate dehydrogenase, which leads to decreased glycolysis and increased FA oxidation. PDK4 expression is increased during hibernation (Andrews *et al*., 1998; Buck *et al*., 2002), and likely contributes to decreased pyruvate dehydrogenase activity observed in heart and kidney of hibernating animals (Brooks and Storey, 1992). Interestingly, PDK4 expression appears to be tonically maintained by CB_1 receptor activation in skeletal muscle cells from both lean and obese humans (Cavuoto *et al*., 2007).

ECS and lipid storage

There is clear evidence that ECS signalling occurs in adipose tissue and functions to regulate energy homeostasis. In particular, CB_1 receptor blockade results in enhanced lipolysis in WAT through stimulation of enzymes involved in beta-oxidation and the tricarboxylic acid cycle; increases energy expenditure in adipose tissue via futile cycle induction; and up-regulates expression of glucose transporter type 4, resulting in improved glucose utilization (Jbilo *et al*., 2005). Furthermore, CB_1 receptor blockade results in a restoration of 'lean' adipocyte morphology in cells taken from obese animals. Adipocytes also function as endocrine cells, releasing the adipokines adiponectin and visfatin. Adiponectin is an insulin-sensitizing hormone with anti-inflammatory properties (Kadowaki and Yamauchi, 2005). Visfatin is a recently discovered adipokine capable of activating the insulin receptor and thought to promote obesity (Marra and Bertolani, 2009). CB_1 receptor activation in WAT inhibits the secretion of adiponectin and increases the secretion of visfatin, an effect that will favour insulin resistance and weight gain (Perwitz *et al*., 2006). Taken together, these data suggest that high ECS in WAT is associated with lipid storage, and more significantly, could be required for lipid storage to occur. In the context of hibernation, these findings suggest the hypothesis that adipocyte ECS would be high during the late summer and very low during the hibernation phase (Figure 4).

ECS in the liver is also emerging as an important player in metabolic regulation. Hepatic FA synthesis requires intact ECS as a CB_1 receptor antagonist inhibited both basal hepatic FA synthesis, as well as high-fat diet-induced hepatic steatosis (Osei-Hyiaman *et al*., 2005). Activation of the CB_1 receptor in hepatocytes results in increased expression of several genes involved in *de novo* synthesis of FAs, including the lipogenic transcription factor, SREBP-1c (Osei-Hyiaman *et al*., 2005). Hepatocyte CB_1 receptor activation also results in inhibition of AMP kinase (Kola *et al*., 2005). Recent studies utilizing mice with cell-specific deletions of the CB_1 receptor in hepatocytes strongly suggest that many of the effects of ECS on metabolism are mediated by the liver (Osei-Hyiaman *et al*., 2008). In particular, these mice became obese when fed a high-fat diet, but did not develop hepatic steatosis, insulin resistance or leptin resistance to the degree of wild-type controls. As in the WAT, high ECS tone in the liver is consistent with the pre-hibernation phase in which metabolic fuel is preserved and stored for later use (Figure 4).

Changes in cellular lipid composition during hibernation

WAT and cellular phospholipids from hibernating animals are relatively rich in polyunsaturated FAs (PUFAs), and the increased lipid fluidity that results is thought to benefit the animal at lower T_b. Evidence suggests that the FA composition of lipids in hibernating animals has a profound effect on torpor bouts, with significantly longer bouts in animals on PUFA-rich diets (Florant *et al*., 1993; Geiser *et al*., 1994; Dark, 2005). Hibernating animals also appear to have regulatory mechanisms to retain PUFAs (Cochet *et al*., 1999). These regulatory mechanisms include preferential oxidation of saturated FA and scavenging of MAGs that contain PUFA. Central to the latter process is the enzyme monoacylglycerol acetyltransferase (MGAT). MGAT is highly expressed during hibernation (Mostafa *et al*., 1993; Xia *et al*., 1993). By preferentially acylating monoacylglycerols that contain PUFAs, MGAT facilitates their continued storage in triacylglycerides (TAGs). Ground squirrels also exhibit significantly lower activity of cytosolic phospholipase A_2 activity during hibernation (Woods and Storey, 2007), a change that would both reduce the generation of oxidation products of arachidonic acid and preserve phospholipid arachidonate.

The endocannabinoids are arachidonate derivatives that target the CB receptors. In addition, several studies suggest that they could function as arachidonate donors, in particular under conditions in which arachidonic acid is shuttled from cell to cell (Pratt *et al*., 1998; Gauthier *et al*., 2005). Therefore, the concept that PUFAs such as arachidonic acid are handled differently during hibernation could have interesting implications for ECS. Among many other possibilities, perhaps a reduction in the availability of arachidonic acid for signalling purposes results in reduced ECS, which in turn contributes to the switch from fat storage to fat mobilization during hibernation.

Changes in circulating concentrations of *N*-acylethanolamines during hibernation

Despite the intriguing overlaps between ECS function and hibernation, to our knowledge, there are no published reports on the role or regulation of the ECS in hibernators. As a first step in addressing this question, plasma lipids from summer-active (SA) and torpid (T) *Marmota monax* (common names are groundhog and woodchuck) were analysed using LC–MS for the two endocannabinoids and several related lipids (Figure 5). Interestingly, concentrations of 2-AG could not be detected in plasma from any of the *M. monax*. This is remark-

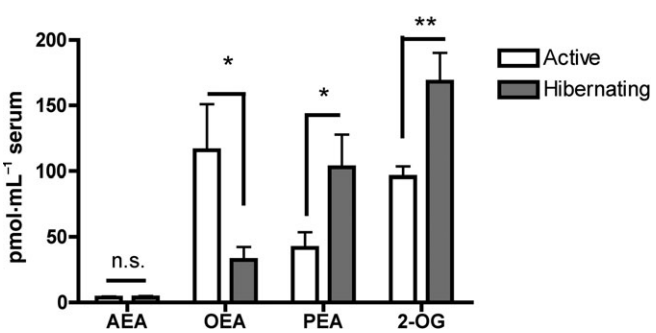

Figure 5 Endocannabinoid and family members were determined in plasma from *Marmota monax* during the summer active period (active) and during a torpor bout in the hibernation period (hibernating). Plasma samples were purchased from Northeastern Wildlife (Harrison, ID, USA); the samples were from both male and female animals, and similar proportions of each sex were represented in each time period. Each bar is the mean of three to five plasma samples; vertical bars represent SEM. Statistical comparisons between summer and hibernating groups for each lipid were made using unpaired *t*-tests; $*P < 0.05$; $**P < 0.01$.

able in light of the nM concentrations of 2-AG measured in plasma and/or serum from humans (Hill *et al*., 2008; 2009). In contrast, AEA was detectable in *M. monax* plasma at concentrations of 2–7 nM, which is approximately threefold greater than its concentration in human serum. The concentration of AEA was not different in plasma from *M. monax* in the SA (3.67 ± 0.98 pmol·mL^{-1}) and T (4.00 ± 0.98 pmol·mL^{-1}). In contrast, plasma concentrations of palmitoylethanolamide (PEA) and 2-oleoylglycerol (2-OG) increased during torpor, whereas concentrations of oleoylethanolamide (OEA) decreased. PEA has prominent anti-inflammatory properties and could contribute to suppression of immune function during hibernation. OEA activates the nuclear receptor PPARα, which plays important roles in lipid metabolism and thermoregulation in hibernation (Carey *et al*., 2003a; Ishida, 2009). OEA has been hypothesized to enter the circulation from metabolism of dietary fat and to function as a satiety factor (Fu *et al*., 2008). Its reduced concentration in the plasma of hibernating (thus, not feeding) *M. monax* is consistent with this notion. The function of 2-OG has not yet been elucidated.

Could the pattern of NAEs reflect hibernation-associated needs for maintaining high PUFA levels in hibernators discussed above? We speculate that 2-AG concentrations could be very low because high activity of MGAT preferentially promotes conversion of 2-AG to TAG. Additionally, perhaps increased concentrations of PEA, a saturated FA-containing NAE, and decreased concentrations of OEA, a monounsaturated FA-containing NAE, indicate preferential metabolism of *N*-acylPEs containing unsaturated FA. Thus, the pattern of plasma NAEs could be both the result of, as well as the modulator of, unique hibernation-associated biological processes. The roles of the endocannabinoids and their structural cousins in hibernation remain a rich area for future research.

Summary and concluding remarks

Many physiological functions are regulated by circadian rhythms, and data are accumulating that dysregulation of circadian rhythms or a mismatch between circadian rhythmicity as occurs in modern human society contribute to human diseases (Takahashi *et al*., 2008). Bipolar disorder and depression are serious human psychiatric disorders for which circadian dysregulation is a contributing or causative factor. For example, Clock mutant mice exhibit a behavioural phenotype that includes many components of mania: hyperactivity in a novel environment, decreased sleep, risk taking behaviour and reduced anxiety (Roybal *et al*., 2007). Epidemiological studies demonstrate that bipolar patients have a 20–40% lifetime likelihood for abusing *Cannabis sativa*, compared to 6% in the general US population (Regier *et al*., 1990). *Cannabis* use increases the number or duration of manic episodes, and chronic exposure of humans to *Cannabis* correlates with an increased incidence of bipolar disorder (Strakowski and DelBello, 2000). *Cannabis* consumption in humans is associated with increased likelihood of developing bipolar disorder; perhaps interactions of THC with circadian rhythms contribute to this mechanism.

Although the study of hibernation seems distant from human biology, there are several likely sites of interaction. For example, enhanced understanding of the processes that protect organs during hibernation could increase organ preservation strategies for transplantation, or during organ failure in diseased humans. On the other hand, understanding the molecular mechanisms involved in hyperphagia and nutrient storage that occur in the period preceding hibernation could shed light on the mechanisms of human obesity.

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Conflicts of interest

The authors declare no conflicts of interest.

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