

Circulating Endocannabinoids: From Whence Do They Come and Where are They Going?

Cecilia J Hillard^{*1}

¹Department of Pharmacology and Toxicology and Neuroscience Research Center, Medical College of Wisconsin, Milwaukee, WI, USA

The goal of this review is to summarize studies in which concentrations of circulating endocannabinoids in humans have been examined in relationship to physiological measurements and pathological status. The roles of endocannabinoids in the regulation of energy intake and storage have been well studied and the data obtained consistently support the hypothesis that endocannabinoid signaling is associated with increased consumption and storage of energy. Physical exercise mobilizes endocannabinoids, which could contribute to refilling of energy stores and also to the analgesic and mood-elevating effects of exercise. Circulating concentrations of 2-arachidonoylglycerol are very significantly circadian and dysregulated when sleep is disrupted. Other conditions under which circulating endocannabinoids are altered include inflammation and pain. A second important role for endocannabinoid signaling is to restore homeostasis following stress. Circulating endocannabinoids are stress-responsive and there is evidence that their concentrations are altered in disorders associated with excessive stress, including post-traumatic stress disorder. Although determination of circulating endocannabinoids can provide important information about the state of endocannabinoid signaling and thus allow for hypotheses to be defined and tested, the large number of physiological factors that contribute to their circulating concentrations makes it difficult to use them in isolation as a biomarker for a specific disorder.

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INTRODUCTION

Although the last several centuries have brought fundamental improvements in Western societies that have resulted in significant benefits to human health, these improvements have been accompanied by adverse changes in food intake, sleep patterns, and exposure to psychological stress that contribute to risk for obesity, diabetes, anxiety and depression. Accumulating evidence from preclinical studies demonstrates that the endocannabinoid signaling system is involved in all of these diseases and disorders; hyperactivity of endocannabinoid signaling contributes to excessive intake and storage of high-calorie foods, while endocannabinoid hypoactivity is a risk factor for the development of depression, anxiety and post-traumatic stress disorder. Millennia of human use of cannabis sativa, which contains the endocannabinoid mimetic Δ^9 -tetrahydrocannabinol (THC), and a brief period in which humans were treated with the endocannabinoid antagonist, rimonabant, provide support for the hypothesis that

endocannabinoid signaling is similarly involved in humans. Thus, better understanding of the endocannabinoid system in humans is an important step toward more sophisticated exploitation of this system to further improve human health.

Assessment of circulating endocannabinoids has been an important but sometimes frustrating approach to the assessment of endocannabinoid signaling in humans. The endocannabinoids are present in the human circulation and their concentrations are dynamic. The purpose of this review is to compile the available data that shed light on several important questions about endocannabinoids that are in the circulation, including: What physiological and pathophysiological factors regulate their concentrations? What are their sources? What are their physiological or pathological roles? Do relative concentrations of circulating endocannabinoids provide clinical information; in other words, are they useful biomarkers?

The available data indicate that circulating endocannabinoids come from multiple organs and tissues, including brain, muscle, adipose tissue and circulating cells. There are hints that endocannabinoids are 'placed' into the circulation to accomplish a specific purpose, as in the regulation of energy intake following exercise. In other cases, it seems more likely that endocannabinoids enter the circulation

*Correspondence: Dr CJ Hillard, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA, Tel: +1 414 955 8493, Fax: +1 414 955 6545, E-mail: chillard@mcw.edu
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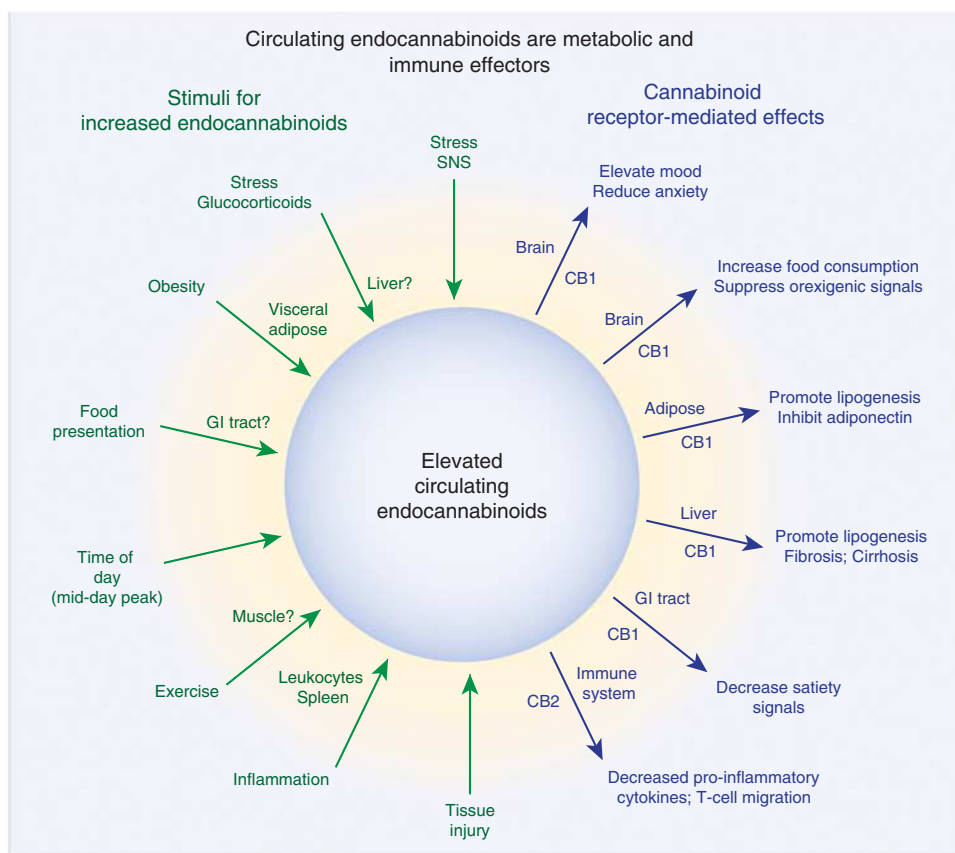


Figure 1. Schematic representing, on the left side, the stimuli and potential sources of endocannabinoids that are present in the circulation and, on the right side, the potential targets of endocannabinoids, together with the cannabinoid receptor subtype that is involved. These concepts are supported by the citations and discussions throughout the review.

by virtue of the fact that they are highly lipophilic and can equilibrate from organs and tissues into the circulation, in other words, are an indirect marker of tissue endocannabinoid tone. The available data also provide some hints and hypotheses regarding the targets of the circulating endocannabinoids, including the metabolic organs and the brain. The key sources and targets supported by data obtained from endocannabinoid measurements in human serum and plasma are shown in Figure 1.

The Endocannabinoids

The two most well-studied endocannabinoids (defined as endogenous ligands for cannabinoid receptors) are the arachidonic acid derivatives, *N*-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG). The endocannabinoids are found in all tissues, organs and bodily fluids thus far investigated. Both AEA and 2-AG are endogenous agonists of the canonical cannabinoid receptors, CB1R and CB2R, G protein coupled receptors that have a widespread distribution throughout the body. In addition to the CB receptors, both endocannabinoids can also bind and activate the vanilloid receptor (TRPV1) and AEA is an agonist for several subtypes of the peroxisome proliferator-activated receptor (PPAR) family.

The biosynthetic and catabolic pathways for both endocannabinoids have been studied and elucidated to varying degrees (Hillard, 2015). 2-AG is produced as a result of activation of phospholipase C (PLC) in cells that express diacylglycerol lipase. Diacylglycerol lipase converts the PLC product diacylglycerol to 2-acylglycerols, including 2-AG and 2-oleoylglycerol (2-OG). The concentration of 2-OG in human serum is typically 5–10 times greater than 2-AG. It has been suggested that 2-OG is an endogenous ligand for the orphan receptor GPR119, a receptor that plays an important role in coupling food intake to the release of incretin hormones (Syed *et al*, 2012).

2-AG is catabolized by hydrolysis of its ester bond, resulting in the formation of glycerol and free arachidonic acid. Several enzymes can accomplish this hydrolysis, including monoacylglycerol lipase (MAGL), alpha-beta hydrolase domain protein (ABHD)-6, and ABHD-12 (Blankman *et al*, 2007). Because it is metabolized to arachidonic acid by MAGL, 2-AG functions as a precursor for the formation of other arachidonic acid derivatives in the vasculature (Gauthier *et al*, 2005) and in the brain (Nomura *et al*, 2008; Viader *et al*, 2015). In the brain, MAGL activity has been shown to play a role in the regulation of neuroinflammation through this mechanism (Viader *et al*, 2015). 2-AG can also serve as a substrate for enzymes that metabolize free

arachidonic acid, including cyclooxygenase 2 (COX2) and some subtypes of lipoxygenase (Urquhart *et al*, 2015).

AEA and other *N*-acyl ethanolamines (NAEs) are synthesized from a low abundance phospholipid, *N*-acyl-phosphatidylethanolamine (NAPE), through several different pathways (Hillard, 2015). NAPE is synthesized via enzymatically mediated transfer of an acyl chain from the sn-1 position of a donor phosphatidylcholine to the amine of phosphatidylethanolamine (PE). The relative abundance of the NAEs formed reflects the relative abundance of the acyl chains found in the sn-1 position of the donor phospholipids; thus, concentrations of palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) are generally higher than AEA in biological samples, including serum and plasma. PEA and OEA exert anti-inflammatory and anorexigenic effects, respectively likely as a result of interactions with PPAR α (Guzman *et al*, 2004). The NAEs are catabolized by hydrolysis of the amide bond through the actions of fatty acid amide hydrolase (FAAH) (Cravatt *et al*, 1996) and *N*-acylethanolamine-hydrolyzing acid amidase (NAAA), which is found primarily in peripheral tissues (Ueda *et al*, 2001). AEA can also serve as a substrate for COX-2, and lipoxygenase and P450 enzymes that utilize arachidonic acid as a substrate (Urquhart *et al*, 2015).

Measurement of Endocannabinoids in the Circulation

Endocannabinoid concentrations in the circulation are typically measured in lipid extracts of serum or plasma obtained from venous blood using isotope-dilution, liquid chromatography-mass spectrometry. Although the endocannabinoids can be measured in both plasma and serum, there is evidence that AEA concentrations are higher in serum than in plasma (Lam *et al*, 2010) and 2-AG concentrations are more reliably measured in serum (Hillard *et al*, 2012). Known amounts of deuterated analogs of AEA and 2-AG are added to the serum or plasma samples at the beginning of the extraction process. These analogs are chemically indistinguishable from the naturally occurring molecules, yet have a mass that is 1 dalton higher for every deuterium atom that has been incorporated (usually 8) so can be differentiated from their endogenous counterparts in the mass spectral analyses. As a result, the recovery of the deuterated analog can be measured and provides an excellent estimate of recovery of the endogenous compounds through the extraction and separation procedures.

Blood cells can both release and inactivate AEA (Schmidt *et al*, 2006; Varga *et al*, 1998), thus the concentrations of AEA measured in serum or plasma are dependent upon the time between the blood draw and the separation of cells from serum or plasma (Vogeser *et al*, 2006). As a result, it is best practice to keep the time between blood draw and serum or plasma harvest as consistent as possible to minimize between sample differences in *ex vivo* generation or loss of AEA. AEA concentrations are stable in serum or plasma after it has been frozen (Jian *et al*, 2010). The arachidonic acid in 2-AG

readily migrates to the 1 position under most storage and extraction conditions (Rouzer *et al*, 2002). 2-AG and 1-AG migrate at different rates in the standard chromatography used to separate the endocannabinoids. Most methods assume that both 2-AG and 1-AG originate from biological 2-AG and add these peaks together to determine total 2-AG.

Several methods have been published for the extraction of endocannabinoids and related lipids from liquid matrices such as serum and plasma (Gouveia-Figueira and Nording, 2014). A liquid-liquid extraction method has been described in which the sample is mixed with methanol and chloroform with repeated vortexing (De Marchi *et al*, 2003). Acetonitrile can also be used as the organic solvent and sonication the method of mixing. Solid phase extraction using C18 columns of the samples that have been made 15% in ethanol, followed by elution with ethyl acetate is also an effective and reproducible method for endocannabinoid extraction from biological fluids (Hill *et al*, 2008c). A modification of this approach has been described that utilizes C18-containing pipet tips to reduce the solvent and time needed for extraction (Sergi *et al*, 2013).

Quantification of the deuterated and endogenous endocannabinoids in each sample is usually accomplished with high-pressure liquid chromatography (HPLC) utilizing a C18 column, followed by mass spectrometry. With the availability of quadrupole, tandem mass spectrometers, species identification and precision of measurement are enhanced because the abundance of daughter ions rather than parent ions are measured. A recent method described the use of on-line sample trapping and microflow liquid chromatography coupled to the use of a tandem mass spectrometer that allowed for detection of the endocannabinoids in 50 μ l of serum with the use of much less solvent than conventional methods (Kirkwood *et al*, 2016).

Serum AEA concentrations tend to fall between 1 and 5 nM while serum 2-AG concentrations range from 10–500 nM (see Table 2 in Hillard *et al* (2012)). Some of the likely reasons for the range in endocannabinoids in healthy individuals are discussed in the sections below, and include time of day and whether the individual has exercised or eaten recently. AEA can bind to the plasma protein, albumin (Giuffrida *et al*, 2000), although the equilibrium dissociation constant of bovine serum albumin for AEA is 55 nM at 37 °C (Bojesen and Hansen, 2003), suggesting that the majority of AEA in the circulation is not protein bound. Data regarding the binding of 2-AG to plasma proteins are not available; however, it is likely that its similarity to AEA and arachidonic acid would render it a low affinity ligand for albumin binding sites as well.

Endocannabinoids have been quantified in other biological samples obtained from humans, including saliva (Matias *et al*, 2012), hair (Krumbholz *et al*, 2013), semen (Amoako *et al*, 2010), breast milk (Lam *et al*, 2010), and amniotic fluid (Lam *et al*, 2010). Data from endocannabinoid measurements in these biological samples are far less frequent in the literature than measurements in the circulation, however, they could have important advantages. For example, measurement in the hair could provide a retrospective

picture of endocannabinoids and the saliva is easier to obtain than blood, particularly in a non-hospital setting.

CIRCULATING ENDOCANNABINOIDS AND METABOLIC REGULATION

The endocannabinoids play a very important role in the regulation of metabolism at systems and cellular levels. As was well stated in a recent review (Mazier *et al*, 2015): ‘The endocannabinoid system exerts regulatory control essentially on every aspect related to search, intake, metabolism and storage of calories...’. Activation of CB1R signaling by the endocannabinoids results in increased consumption of food. In the hypothalamus, tonic endocannabinoid-CB1R signaling functions to maintain food consumption and is suppressed by the homeostatic appetite regulator, leptin (Di Marzo *et al*, 2001). CB1 receptors are also present in hypothalamic nuclei that modulate energy homeostasis via interactions with ghrelin (Kola *et al*, 2008). In addition, activation of CB1R signaling enhances reward circuits through effects in the mesolimbic system (De Luca *et al*, 2012; Mahler *et al*, 2007; Melis *et al*, 2007), which potentiates hedonic (reward-driven) consumption of highly palatable foods in particular. Functional imaging of humans performing a reward-driven task following THC find significant attenuation of feedback to the reward circuits compared to placebo administration (van Hell *et al*, 2012). These studies suggest a particular role for CB1R signaling in feedback inhibition of reward seeking.

CB1R are also present in peripheral organs involved in energy metabolism, including adipose tissue, the endocrine pancreas, muscle and liver (Bermudez-Silva *et al*, 2012; Cota *et al*, 2006; Silvestri and Di Marzo, 2013). Increased CB1R signaling increases energy storage via stimulation of fat mass expansion, glucose uptake and adipogenesis in adipocytes (Vettor and Pagano, 2009); induction of lipogenesis in the liver (Osei-Hyiaman *et al*, 2005); and increased insulin secretion from the pancreas (Juan-Pico *et al*, 2006). CB1R signaling also increases processes that reduce energy utilization, including decreased brown fat thermogenesis (Verty *et al*, 2009) and glucose uptake into skeletal muscle (Liu *et al*, 2005). Thus, CB1R signaling is coordinated throughout the body to maximize intake and storage of energy. Clearly, this function of the endocannabinoid-CB1R system has great implications for obesity and other components of the metabolic syndrome in humans. In fact, a CB1R antagonist, rimonabant, was found to have significant, beneficial effects in humans to reduce body weight, improve lipid profiles and increase insulin sensitivity before it was removed from clinical use (Van Gaal *et al*, 2005). In addition, a recent study identified expression of the CB1R in the subfornical organ of the hypothalamus, which is increasing being recognized as a brain region that is responsive to changes in circulating signals of metabolic status (Hindmarch and Ferguson, 2016). This finding suggests that endocannabinoids in the circulation could

contribute to the information that is utilized by the brain to alter behavior and physiologic responses.

Circulating Endocannabinoids, Food Consumption and Obesity

The data discussed above suggest the hypothesis that circulating endocannabinoids coordinate CB1R roles in energy intake and storage among various tissues and organs. In this section, data regarding relationships between circulating endocannabinoids and food consumption, and the correlations among circulating endocannabinoids, body weight and measures of adiposity will be considered. Altogether, these findings support the hypotheses that endocannabinoid concentrations in the circulation are sensitive to several aspects of food consumption and dysregulated in obese and anorexic individuals.

The pattern of change in circulating AEA concentrations surrounding food consumption is consistent with its role as an orexigenic factor in homeostatic (hunger-driven) feeding. Presentation of food (prior to consumption) results in a significant increase in circulating AEA in normal weight (NW) and obese individuals (Gatta-Cherifi *et al*, 2012) followed by a small but reproducible fall in circulating concentrations of AEA over several hours following food consumption (Gatta-Cherifi *et al*, 2012; Monteleone *et al*, 2012, 2015). In NW individuals, the post-meal reduction in circulating AEA is independent of the hedonic value of the meal (Monteleone *et al*, 2012). Intravenous glucose administration also reduces circulating AEA for several hours following infusion in NW individuals (Di Marzo *et al*, 2009). Oral consumption of 250 ml of grape juice (41 g sugar) significantly elevated blood glucose levels and reduced circulating 2-AG but did not affect AEA concentrations (Feuerecker *et al*, 2012a), suggesting that there is a dose dependence to effects of sugars on endocannabinoids in the circulation. The suppressive effects of food and glucose on circulating AEA concentrations could be mediated by increased insulin, since exogenous infusion of insulin also reduces circulating AEA concentrations in the same time frame (Di Marzo *et al*, 2009). In agreement with this hypothesis, the effect of glucose to reduce circulating AEA was lost in insulin insensitive individuals (Di Marzo *et al*, 2009); however, another study found that a hyperinsulin/euglycemic clamp decreased circulating AEA concentrations in both insulin sensitive and insulin resistant obese individuals (Abdulnour *et al*, 2014). *In vitro* data demonstrate that normal but not insulin-resistant adipocytes respond to insulin with significant reductions in intracellular concentrations of both AEA and 2-AG (D'Eon *et al*, 2008).

The response of 2-AG to food is affected by its perceived hedonic value, suggesting that 2-AG is involved in reward-driven (hedonic) feeding. When NW individuals are presented with a favorite compared to a non-favored food, circulating 2-AG concentrations are significantly higher at food presentation, and during and after its consumption (Monteleone *et al*, 2012, 2015). Sleep deprivation

promotes increased hedonic eating, particularly in the later part of the day (Benedict *et al*, 2012; Nedeltcheva *et al*, 2009; St-Onge *et al*, 2011) and also increases circulating 2-AG concentrations at mid-day (Hanlon *et al*, 2016). These findings suggest that circulating 2-AG concentrations reflect activation of reward circuits in the brain and could be a mediator of hedonic eating.

A small study found that neither underweight nor weight-restored individuals with anorexia nervosa exhibited an increase in 2-AG in response to presentation or eating a favorite food (Monteleone *et al*, 2015). These data are consistent with hypotheses that dysregulated evaluation of rewarding stimuli occurs in anorexia (Keating *et al*, 2012) and that circulating 2-AG is related to perceived reward. On the other hand, circulating concentrations of AEA declined after favorite and non-favored food consumption in both subgroups of anorexic individuals, patterns that did not differ from NW individuals (Monteleone *et al*, 2015). These data are consistent with the notion that circulating AEA concentrations are involved in homeostatic and not hedonic feeding processes.

A recent study found that olfactory threshold discrimination identification capacity is significantly lower in obese women than NW and is inversely related to circulating 2-AG concentrations (Pastor *et al*, 2016). These findings are somewhat counterintuitive since preclinical studies suggest that CB1R signaling in the olfactory epithelium is associated with enhanced sensitivity to odors (Breunig *et al*, 2010) and THC exposure can enhance the sensory aspects of food consumption in humans (Brisbois *et al*, 2011). However, it is possible that the human data reflect downregulation of olfactory CB1 receptor signaling in response to high 2-AG concentrations.

Regardless of the motivation for overeating or the type of food consumed, excessive food intake over a prolonged period results in obesity. Food-induced changes in circulating endocannabinoids have been characterized in obese individuals to examine the hypothesis that high endocannabinoid tone is associated with obesity. The consumption-induced reduction in circulating AEA concentrations seen in NW individuals is blunted in insulin insensitive, obese individuals and the magnitude of the decrease in AEA following eating is significantly, inversely correlated with waist circumference (Gatta-Cherifi *et al*, 2012). Given that these individuals were insulin insensitive in addition to being obese, it is possible that loss of insulin sensitivity underlies both differences in waist circumference and the lack of AEA decrease following food consumption. Interestingly, the pre-meal increase in AEA is intact in obese individuals, suggesting that the mechanisms underlying the pre-meal increase and post-meal decrease in AEA are different. A second study also found that obese individuals exhibit a loss in the decline in AEA following food consumption, but this change occurs when favorite but not non-favored foods are consumed (Monteleone *et al*, 2016).

Multiple studies (Abdulnour *et al*, 2014; Bluher *et al*, 2006; Cote *et al*, 2007; Engeli *et al*, 2005; Fernandez-Aranda *et al*,

2014; Pastor *et al*, 2016) (but not all (Di Marzo *et al*, 2009)) have found that concentrations of 2-AG in blood harvested in the morning after an overnight fast are significantly higher in men and women with body mass index (BMI) values in the obese range compared to NW individuals. Although obese individuals with subcutaneous fat distributions also have elevated 2-AG compared to NW, those with a visceral fat distribution are higher still (Bluher *et al*, 2006). Furthermore, circulating concentrations of 2-AG are significantly, positively correlated with BMI, total body fat (particularly measures of visceral fat mass) (Bluher *et al*, 2006) and intra-abdominal adipose (IAA) tissue (Cote *et al*, 2007). One study also found a significant, positive correlation between BMI and circulating AEA concentrations in individuals with a range of BMI from 18 to 45 (Weis *et al*, 2010a). Studies that included only or mostly women found that AEA as well as 2-AG are significantly higher in obese than NW individuals (Engeli *et al*, 2005; Gatta-Cherifi *et al*, 2012). Conversely, a study in obese men with high amounts of IAA found that AEA concentrations in the morning are significantly lower than normal weight men or obese men with low IAA (Cote *et al*, 2007). It is possible that differences in the relative distributions of visceral and subcutaneous fat stores between men and women (Enzi *et al*, 1986) could underlie the sex differences observed in these studies. The one study that did not identify an increase in 2-AG in obese individuals studied a cohort without high visceral fat depots (Di Marzo *et al*, 2009), which further supports the visceral fat as the source of 2-AG in obesity. In one study (Abdulnour *et al*, 2014), insulin resistant obese had higher circulating 2-AG concentrations than insulin sensitive; however, the insulin resistant group also had a significantly higher BMI, which could underlie the higher circulating 2-AG. Taken together, these studies suggest that 2-AG enters the circulation from visceral, abdominal fat stores, so its concentration is highest in individuals with the highest amount of visceral fat. Given the roles of 2-AG to increase fat storage in adipose and liver (Mazier *et al*, 2015) and its ability to promote consumption of highly palatable foods through effects on the brain (Wei *et al*, 2016), these findings suggest that 2-AG-CB1 receptor signaling participates in a feed-forward cycle to promote further food consumption and increased body weight gain in the obese. One preclinical study has examined circulating endocannabinoid concentrations in mice made obese through long-term exposure to exogenous glucocorticoids and found that circulating AEA is nearly doubled in the treated, obese mice compared to controls (Bowles *et al*, 2015). 2-AG concentrations in the circulation were unchanged by the treatment, suggesting that obesity as a result of excess glucocorticoids is mediated by AEA rather than increased 2-AG.

Several studies have examined the expression of FAAH determined in adipose tissue biopsies in relationship to the circulating endocannabinoid concentrations. In postmenopausal women, adipose FAAH expression is reduced in obese women compared to normal weight; and is significantly, negatively correlated with circulating AEA (Engeli *et al*,

2005). Interestingly, the same study and another that included both men and women (Blüher *et al*, 2006) found that adipose FAAH expression is also significantly, negatively correlated with morning 2-AG concentrations. Although 2-AG can serve as a substrate for FAAH *in vitro* (Goparaju *et al*, 1998), preclinical studies in mice without FAAH expression show no changes in 2-AG content (Patel *et al*, 2005) which has led to the widely held assumption that FAAH does not catabolize 2-AG *in vivo*. Indeed, the correlation between adipose FAAH expression and circulating AEA is stronger than the correlation with circulating 2-AG (Engeli *et al*, 2005). It is possible that reduced FAAH expression is a causative factor, resulting in elevated AEA, which, in turn primes the system to increase caloric intake and fat storage through CB1 receptor activation. Adipose fat stores are increased, resulting in an increase in circulating 2-AG, leading to the feed-forward cycle described above. Another study did not find a significant relationship between adipose FAAH activity and BMI or any other measure of metabolic disorder, instead, found that BMI was significantly, positively correlated with adipose MAGL activity in obese without diabetes (Cable *et al*, 2014). The authors of this study concluded that the rates of catabolism of AEA and 2-AG in adipose do not contribute to their circulating concentrations.

Preclinical findings suggest that a source of circulating endocannabinoids is the small intestine (DiPatrizio *et al*, 2011). In particular, sham feeding of rats with a lipid meal results in increased endocannabinoid contents in the jejunum via a signal carried by the vagus nerve. Lipid feeding reduced the catabolism of both AEA and 2-AG, resulting in increased tissue contents. Although not studied, it is possible that these lipids enter the portal system and contribute to elevations in circulating concentrations. A recent paper reported that a peripherally restricted CB1R antagonist reduced hyperphagia in mice fed a Western diet (ie, high in carbohydrate and fat), which supports a role for peripheral CB1R signaling in hyperphagia (Argueta and DiPatrizio, 2017).

Although it is not an endocannabinoid *per se*, preclinical studies suggest the hypothesis that the NAE, OEA, also has important effects on satiety and feeding behavior. In particular, administration of OEA to mice reduces food intake and body weight gain, likely through PPAR- α in the GI tract (Fu *et al*, 2003) and or through actions in the area postrema (Romano *et al*, 2017). OEA is synthesized by enterocytes of the small intestine in response to fat intake (Fu *et al*, 2007). In accord with the preclinical findings, hypercholesterolemic patients treated with diets enriched in oleic acid exhibited significantly higher concentrations of plasma OEA concentrations and a significant, negative relationship between plasma OEA and percent body fat (Jones *et al*, 2014). These data, together with the finding that circulating OEA and AEA concentrations do not change in parallel in response to eating (Monteleone *et al*, 2016), support the hypothesis that circulating OEA concentrations are regulated by a mechanism that differs from that regulating concentrations of AEA.

Relationships of Endocannabinoid Concentrations with Time of Day and Sleep

Normalized serum 2-AG concentrations exhibit a significant circadian rhythm in humans under conditions of standardized sleep timing and caloric intake (Hanlon *et al*, 2015). The average low point (nadir) of 2-AG concentration occurred in this study at 0400 hours, 5 h after going to bed and 9 h after the last food consumption. 2-AG concentrations rose steadily from that point, followed by an extended period of maximal concentrations between 1200 and 1500 hours, a time period that encompassed the midday meal at 1400 hours. The return to lower, nocturnal 2-AG concentrations began in the late afternoon/early evening, with a small plateau in 2-AG concentrations occurring between 1700 and 2000 hours, which surrounded the evening meal at 1900 hours. A remarkable threefold change in circulating 2-AG concentration occurred between the nadir and acrophase.

A second study from the same group found that reduction of the number of hours of sleep to four, while maintaining the same sleep period mid-point, produced a significant increase in the amplitude and peak concentrations of 2-AG (Hanlon *et al*, 2016). In addition, the time of the acrophase was shifted significantly from 1230 to 1430 hours and the mean 2-AG concentrations between 1400 and 2100 hours trended ($p < 0.06$) higher. Thus, restricted sleep increases and extends the peak of 2-AG concentrations in the afternoon following sleep restriction. Interestingly, sleep restriction did not affect patterns of 2-AG in the morning and did not affect the nadir concentrations or timing. Instead, the effect of restricted sleep on 2-AG is delayed until later in the day.

In another study, 2-AG concentrations were also found to be elevated in healthy controls exposed to 4 days of restricted sleep, although the timing was different (Cedernaes *et al*, 2016). In that study, sleep was restricted to 4 h, but the period corresponded to the second half of the normal sleep period (ie, 0245–0700 hours). Under these conditions, 2-AG concentrations in the plasma are significantly elevated at 0830 following restricted sleep compared to normal sleep and are not different at 2030 hours. Midday 2-AG concentrations between normal and restricted sleep phases were not determined in this study. It is possible that the differences in timing of sleep in the restriction paradigm between this study and that by Hanlon (where the sleep midpoint was kept constant) (Hanlon *et al*, 2016) contributed to the difference in the time at which 2-AG was elevated. If this is the case, it suggests that sleep timing as well as number of hours regulate the chronicity of circulating 2-AG. In this regard, advancing the sleep schedule without changing the light-dark cycle has a moderate effect on the rhythms of melatonin and core body temperature (Danilenko *et al*, 2003), suggesting that sleep timing can function as a modulator of circadian rhythm. In any case, both studies identified increased circulating 2-AG following restricted sleep.

In a very small pilot study (five subjects) looking at three time points, AEA concentrations in the serum were found to

be higher upon waking (0730 hours) than late evening (2230 hours) (Vaughn *et al*, 2010). Furthermore, one night of complete sleep deprivation results in elevation of serum AEA concentrations later in the day. Both of these results suggest that AEA might have rhythms and relationships to sleep loss similar to those of 2-AG; however, more complete studies need to be carried out to confirm this conclusion.

These data place circulating endocannabinoids, particularly 2-AG, in the pantheon of molecules which alter metabolism and are circadian. In fact, 2-AG fills a unique role because it is an orexigenic mediator that exhibits its highest concentrations in midday and lowest during sleep. Neither the hormones controlled by the HPA axis nor adipokines and gut hormones reach peak concentrations in the middle of the normal feeding period (Copinschi *et al*, 2010).

As was pointed out in a commentary (Scheer, 2016) that accompanied (Hanlon *et al*, 2016), the increases in 2-AG and 2-OG began in the middle of the sleep episode, thus preceding breakfast, awakening and morning light exposure. The timing of food intake, transition from sleep to waking, and light/dark cycles are all important circadian regulators, but the increases in circulating 2-acylglycerols precede these events, so are not likely driven by them. Glucocorticoids also rise in the morning, and data obtained in rodents indicate that glucocorticoids can rapidly increase 2-AG synthesis in the hypothalamus (Hill *et al*, 2010). While morning changes in 2-AG paralleled the rise in cortisol, the nadir and acrophase for cortisol were 3–4 h earlier than for 2-AG (Hanlon *et al*, 2015). Thus, 2-AG concentrations appear to have an endogenous, circadian rhythm that differs significantly from those of other metabolic regulators.

Activation of CB1R signaling provides a highly efficacious orexigenic signal (Cota *et al*, 2006) and the timing of the peak in circulating 2-AG has been hypothesized to engage CB1Rs throughout the body to promote eating during the middle of the active period (Hanlon *et al*, 2015). In light of the argument presented above that circulating 2-AG is involved in hedonic rather than homeostatic eating, a more specific hypothesis is that midday 2-AG rise promotes food intake for storage, rather than for immediate use. It is interesting in this regard that insulin sensitivity exhibits a robust decrease from morning to evening in healthy human volunteers (Saad *et al*, 2012; Van Cauter *et al*, 1997) which is consistent with increased 2-AG-mediated CB1R signaling over that time period. Thus, elevation of 2-AG in situations that could have a significant effect on future metabolic needs, such as chronic loss of sleep, could protect against starvation by promoting an increase in fat stores. This hypothesis is supported by the findings that sleep deprivation both increased feelings of hunger and drive to eat high-calorie foods in the afternoon and increased and shifted the 2-AG acrophase to later times (Hanlon *et al*, 2016).

The mechanisms that underlie the circadian changes in 2-AG and the shift in pattern that occurs with sleep

deprivation are not known. However, given the fact that sleep deprivation is a significant stressor, it is possible that 2-AG concentrations are sustained as a result of stress-induced glucocorticoid elevation.

Circulating Endocannabinoids and Exercise

The hypothesis that circulating endocannabinoids coordinate a system-wide response to seek, consume and store energy leads logically to the hypothesis that increased energy utilization results in increased circulating endocannabinoids to replenish energy stores. In support of this hypothesis, multiple studies demonstrate that 30–90 min of moderate exercise increases circulating concentrations of AEA (Heyman *et al*, 2012b; Raichlen *et al*, 2012, 2013; Sparling *et al*, 2003) and other NAEs (Heyman *et al*, 2012b) immediately after the exercise period, although a recent study did not reveal an immediate increase in AEA (Cedernaes *et al*, 2016). AEA increases reached significance when heart rate was increased to about 75% of maximal (Raichlen *et al*, 2013); neither lower (ie, walking) nor near-maximal intensities of exercise affected AEA concentrations (Raichlen *et al*, 2012, 2013) suggesting a ‘U-shaped’ relationship between exercise and AEA mobilization. Well-trained healthy volunteers who participated in a ‘strenuous’ hike at altitude below 2100 meters and those who hiked and ascended approximately 2000 meters in altitude both exhibited significant increases in circulating AEA concentrations following the exercise period (Feuerecker *et al*, 2012b). Interestingly, hypoxic stress of exercise at high altitude appeared to potentiate the increase in AEA in this study. A preclinical study utilizing hepatic ischemia-reperfusion injury also found that 2-AG is increased in response to ischemia, which could reflect regulation of endocannabinoids by the state of oxygenation of tissues (Kurabayashi *et al*, 2005).

A few studies have examined chronic effects of physical exercise on baseline endocannabinoids. A study of NW and obese women found that moderate-vigorous physical activity measured over 6 days is positively correlated with circulating AEA concentrations (Fernandez-Aranda *et al*, 2014). Interestingly, high circulating AEA concentrations are also associated with high BMI. On the other hand, no differences in basal concentrations of AEA and 2-AG were found between active and sedentary NW men in another study (Gasperi *et al*, 2014).

The effects of exercise on circulating 2-AG concentrations have been less consistent. Studies that employed 30–90 min of sustained exercise and observed increases in AEA did not find an increase in 2-AG (Heyman *et al*, 2012b; Raichlen *et al*, 2012, 2013; Sparling *et al*, 2003). However, another study employing an exercise regimen known to induce exercise-induced hypoalgesia (3 min of isometric contractions of the forearm) found that 2-AG is increased very significantly, in addition to the NAEs (Koltyn *et al*, 2014). Another study found an increase in 2-AG measured in blood collected 15 min after a 30 min exercise period (Cedernaes

et al, 2016); however, the subjects in this study were not given food in the morning of the experiment, which could have resulted in a more physiologically stressful paradigm than exercise alone. Thus, the available data are most consistent with a hypothesis in which moderate levels of sustained (ie, more than 30 min) exercise increase AEA and other NAEs in the circulation while 2-AG increases in response to short bursts of activity, perhaps specifically in response to isometric contractions.

It is possible that the increased endocannabinoids in the circulation following exercise come from skeletal muscle. Messenger RNAs encoding NAPE-specific phospholipase D (Hutchins-Wiese *et al*, 2012), and diacylglycerol lipase (Crespillo *et al*, 2011) are present in skeletal muscle. PEA and another NAE, stearoylethanolamide, are increased by exercise in microdialysates of the trapezius muscle in individuals with chronic neck/shoulder pain (Ghafouri *et al*, 2013), providing additional support for the hypothesis that endocannabinoids can be produced in muscle. It is well established that exercise increases circulating glucocorticoid concentrations (Urhausen *et al*, 1995); and exogenous glucocorticoid treatment produces a rapid increase in brain AEA concentrations (Hill *et al*, 2010), suggesting a possible link between glucocorticoid receptor activation and AEA synthesis.

Endocannabinoid mobilization during exercise is very consistent with the role of CB1R signaling in the regulation of metabolism discussed above. Activation of CB1R in the liver and adipose move the system toward energy storage and thus can serve to both restrain energy use and contribute to recovery of energy stores following exercise (Pagotto *et al*, 2006). DiMarzo and colleagues have suggested an interesting hypothesis that AEA and other NAEs could produce positive metabolic effects locally in skeletal muscle, including enhanced glucose uptake, improved insulin action, and mitochondrial biogenesis via activation of PPAR γ (Heyman *et al*, 2012a). Although endocannabinoids can also act centrally to increase food consumption, a recent study found that exercise-induced increases in endocannabinoids were not correlated with feelings of subjective hunger (Cedernaes *et al*, 2016), suggesting that any metabolic role for the circulating endocannabinoids is likely to be more peripheral than central. A study in healthy cyclists demonstrated a significant, positive correlation between the increase in circulating AEA and an increase in the neurotrophic factor, BDNF, following a 90 min exercise period (Heyman *et al*, 2012b). Although the directionality of this relationship is not known, acute administration THC to healthy, non-cannabis-using humans also increases serum BDNF concentrations (D'Souza *et al*, 2009), suggesting that BDNF synthesis/release is evoked by CB1R activation. Preclinical studies in the CNS are also consistent with CB1R regulation of BDNF (Khaspekov *et al*, 2004; Marsicano *et al*, 2003). These data suggest that endocannabinoids could be the mediators of exercise-induced increases in BDNF (Szuhany *et al*, 2015) and, thus, of the hypothesized effects of exercise on cognitive function and mood (Phillips *et al*, 2014).

CIRCULATING ENDOCANNABINOIDS AND INFLAMMATION

Circulating endocannabinoids are significantly increased in several conditions in which there is systemic inflammation. For example, both 2-AG and AEA are folds-higher in the serum of patients with endotoxic shock than control serum (Wang *et al*, 2001). Circulating endocannabinoids are increased in patients with cirrhosis (Fernandez-Rodriguez *et al*, 2004); chronic hepatitis C infection (Patsenker *et al*, 2015); exposure to solar ultraviolet radiation (Felton *et al*, 2017); congestive heart failure (Weis *et al*, 2010b); and atherosclerosis (Maeda *et al*, 2009). Similarly, rats with acute pancreatitis also exhibit significantly elevated circulating AEA concentrations, particularly in animals with a severe, necrotizing pancreatitis (Matsuda *et al*, 2005). When activated, macrophages (Di Marzo *et al*, 1999; Varga *et al*, 1998), platelets (Varga *et al*, 1998), T and B cells (Sido *et al*, 2016) release endocannabinoids *ex vivo*, and so could be a source of circulating endocannabinoids. On the other hand, a recent study carried out in patients undergoing hematopoietic stem cell transplantation (HCT) suggests that bone marrow-derived immune cells *per se* may not be an important source of 2-AG (Knight *et al*, 2015). In particular, no change in 2-AG concentrations occurred between the period in which the HCT patient's immune system is ablated and after immune system reconstitution. Preclinical studies suggest two alternative hypotheses. First, it is possible that non-immune cells respond to inflammatory mediators with increased endocannabinoid release as has been shown in inflamed hepatocytes (Batkai *et al*, 2007). Second, inflammation results in a reduction in 2-AG catabolism by splenic carboxylesterase 2g in the spleen (Szafran *et al*, 2015), suggesting that the rise in 2-AG during inflammation could be the result of spillover from the spleen, which is enhanced as a result of decreased 2-AG hydrolysis in that tissue.

2-AG is an agonist of the CB2 receptor (Soethoudt *et al*, 2017), a GPCR that is expressed constitutively by human B and T cells; macrophages and, to a lesser extent, neutrophils (Galiegue *et al*, 1995). CB2R activation is associated with a reduction in proinflammatory cytokine release (Leleu-Chavain *et al*, 2013; Patsenker *et al*, 2015) and with reduced migration of T cells into the brain (Maresz *et al*, 2007). The findings that 2-AG can be elevated during inflammation and that it activates an anti-inflammatory receptor suggest the hypothesis that endocannabinoids function as a feedback system to dampen inflammation. A recent preclinical study highlights this relationship. In this study, antigen activation of macrophages and dendritic cells resulted in increased 2-AG and CB2R antagonist treatment increased antigen-specific immune responses in mice, suggesting that 2-AG/CB2R activation suppresses innate and adaptive immune responses in mice (Dotsey *et al*, 2017).

In contrast to these hypotheses, several studies have found significant, positive correlations between circulating 2-AG concentrations and concentrations of the pro-inflammatory cytokine interleukin-6 (IL-6) (Knight *et al*, 2015; Weis *et al*,

2010a), data that do not reconcile with this hypothesis. If 2-AG is anti-inflammatory even to a small extent, one would expect a negative relationship between IL-6 and 2-AG. In this regard, 2-AG potentiates LPS-stimulated IL-6 expression in a macrophage cell line (Marazzi *et al*, 2011). On the other hand, plasma concentrations of 2-AG and the cytokine, tissue necrosis factor alpha, were found to be significantly negatively correlated in another study (Koppel *et al*, 2009).

The relationships between 2-AG and cytokines in the circulation need further study as does the fundamental question of whether circulating endocannabinoids participate in regulation of inflammation in humans. In this regard, several of the conditions described above in which circulating endocannabinoids are elevated also result in inflammation, including acute bouts of exercise (Ruderman *et al*, 2006) and obesity (Marsland *et al*, 2010), data that support the possibility that there are connections between endocannabinoids and inflammation. Activation of CB1R signaling in the context of inflammation can be a contributing factor to fibrosis and other detrimental effects of chronic inflammation (Iyer *et al*, 2017). Circulating concentrations of AEA are elevated in patients with cirrhosis and their concentrations are positively correlated with negative indices of liver function (Caraceni *et al*, 2010).

CIRCULATING ENDOCANNABINOIDS AND STRESS

Exposure to physical and psychological stressors result in coordinated behavioral, endocrine and neuronal changes that increase chances for survival if an injury does happen. Acute stress responses include activation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. Stress exposure also produces consistent and reproducible increases in inflammation systemically and in the brain, likely as a downstream consequence of sympathetic activation (Dantzer *et al*, 2008). For example, acute psychological stress results in an immediate increase in natural killer T cells and interleukin 1beta (IL-1 β) (Yamakawa *et al*, 2009) and a delayed (90 min) increase in IL-6 (Steptoe *et al*, 2007). Sympathetic activation results in migration of monocytes and macrophages into the circulation and triggers release of IL-1 β and other cytokines (Yamakawa *et al*, 2009).

Preclinical data support the hypotheses that endocannabinoid signaling is altered by stress and mediates or modulates stress responses (Hillard, 2014). Evidence obtained in brain demonstrate that 2-AG mobilization can occur downstream of glucocorticoid receptor activation (Di *et al*, 2005) while AEA is elevated by corticotropin releasing hormone receptor activation, secondary to inhibition of FAAH (Gray *et al*, 2015). Endocannabinoid/CB1R signaling inhibits the activation of the HPA axis by stress (Hill *et al*, 2009a) and enhances recovery following stress offset (Hill *et al*, 2011).

CB1R are present on sympathetic nerve terminals and their activation results in sympathomimetic effects in

preclinical studies (Ishac *et al*, 1996; Pfitzer *et al*, 2005). However, AEA infused intra-arterially in the human forearm failed to affect blood flow (Movahed *et al*, 2005), suggesting that CB1R activation may not affect release from sympathetic nerve terminals in healthy humans. Preclinical studies demonstrate that CB1R signaling in the nucleus tractus solitarius (NTS) potentiates the sympathoinhibitory responses to activation of the baroreceptor reflex (Chen *et al*, 2010; Seagard *et al*, 2004, 2005). On the other hand, CB1R activation in the periaqueductal gray (PAG) increases activity of the SNS (Dean, 2011).

Effects of Acute Stress on Circulating Endocannabinoids

Activation of the SNS as a result of inducing postural hypotension with a head up tilt is accompanied by a significant increase in 2-AG concentrations (Schroeder *et al*, 2009). However, associations between 2-AG concentrations and measures of sympathetic activation were not found (Schroeder *et al*, 2009). In the same study, circulating concentrations of AEA were significantly, positively correlated with the ability of patients to tolerate the procedure without fainting (Schroeder *et al*, 2009). This result suggests a positive relationship between AEA and SNS activity.

Vestibular activation of the SNS triggers nausea and vomiting (Balaban, 1999); and parabolic flight maneuvers that trigger motion sickness produce an immediate increase in circulating AEA concentrations and a delayed (recovery phase) increase in 2-AG (Chouker *et al*, 2010). Increases in both 2-AG and AEA concentrations are associated with less motion sickness, which is consistent with preclinical and human data that exogenous activation of CB1R signaling reduces nausea and vomiting (Sticht *et al*, 2015).

Hyperemesis is a significant physiological stress and is accompanied by increases in both SNS and HPA axis activation. Cyclic vomiting syndrome (CVS) is a functional disorder characterized by relapsing and remitting bouts of prolonged nausea and vomiting. Since endocannabinoids are elevated during stress and can reduce nausea and vomiting, circulating endocannabinoids were compared between sick (active vomiting) and well phases in individuals with CVS (Venkatesan *et al*, 2016). Concentrations of both AEA and 2-AG are elevated in the sick compared to well phases, but the increases did not reach statistical significance ($p = 0.09$ for both). Concentrations of both PEA and OEA were significantly increased and positively correlated with the amount of distress. No significant associations between circulating endocannabinoid concentrations and circulating cortisol or salivary amylase measured at the same time were found in this study.

Acute exposure to psychological stress also elevates circulating endocannabinoid concentrations. A commonly used procedure to evoke psychological stress in humans in a laboratory setting is the Trier Social Stress Test (TSST). There are various forms of the TSST, but most include

public speaking and solving difficult arithmetic problems (Allen *et al*, 2014). The TSST results in activation of both the SNS and HPA axis as well as an increase in perceived anxiety. Administration of a seventeen-minute version of the TSST resulted in a significant increase in 2-AG concentrations immediately afterwards that returned to basal concentrations 30 min after the end of the stress (Hill *et al*, 2009b). Serum AEA concentrations were not significantly different from baseline at either time point. However, a second study found that a shorter stress period (5 min) produced no significant change in 2-AG concentrations, but significantly increased circulating AEA concentrations immediately after the stress (Dlugos *et al*, 2012). Interestingly, this study also found preliminary evidence of differences in the endocannabinoid response among males and females in each phase of their menstrual cycles, with males exhibiting a greater increase from baseline than females, particularly females in the follicular phase. This study also found preliminary evidence of differences in the endocannabinoid response to stress between those of African and Caucasian descent. The change in PEA from baseline to after stress was significantly, positively correlated with the change in cortisol measured in the same samples; the change in AEA concentration was not significantly associated with cortisol.

A study in which imagery of a previous stressful experience was administered found that AEA concentrations were not changed from baseline but were significantly lower than baseline 75 min after the end of the stress (Mangieri *et al*, 2009), which is reminiscent of the effects of food consumption on circulating AEA concentrations discussed above.

Thus, while these studies all demonstrate that circulating endocannabinoid concentrations are sensitive to acute, psychological stress, they also indicate that the relationships are complex, time-dependent, and affected by sex. Circulating concentrations of both AEA and 2-AG can be affected by acute stress, but studies of associations with the primary stress effector systems, the HPA axis and SNS, do not consistently find evidence of a direct relationship between activation of these systems and concentrations of endocannabinoids in the circulation. However, preclinical studies indicate that the function of elevated endocannabinoids during stress is to buffer the stress response and hasten return to homeostasis through inhibition of the HPA stress response in particular. Thus, if endocannabinoids in the circulation are both downstream and upstream of the HPA axis, the relationships between endocannabinoid and cortisol concentrations would likely be nonlinear and dependent upon timing and individual responsivity of both systems. It is also possible that changes in endocannabinoid concentrations occur with a time delay, so that concentrations of cortisol or measures of SNS are not related when examined at the same point in time. Models that allow for these complexities to be assessed are needed to answer this question.

Circulating Endocannabinoids and Chronic SNS or HPA Axis Activation

Although there is little evidence that circulating endocannabinoids are associated with any measure of SNS activation at baseline, several studies suggest that AEA is associated with SNS-mediated physiological effects in various pathological conditions. For example, AEA concentrations are higher in individuals with sleep apnea and are strongly, positively correlated with both systolic and diastolic blood pressure, independent of BMI and insulin resistance (Engeli *et al*, 2012). Interestingly, depressed women also exhibit a significant, positive correlation of circulating AEA concentrations and diastolic blood pressure, with a trend ($p < 0.07$) toward a significant association with systolic pressure as well (Ho *et al*, 2012). Intravenous injections of AEA in experimental animals reduce blood pressure (Ho and Gardiner, 2009), suggesting that the relationship underlying the correlation between circulating AEA concentrations and blood pressure is that elevated blood pressure results in increased AEA. Indeed, increased blood pressure in rats produces an elevation of AEA concentrations in the NTS (Seagard *et al*, 2004). On the other hand, circulating concentrations of AEA are significantly reduced in individuals with preeclampsia, a condition associated with hypertension in late pregnancy (Molvarec *et al*, 2015), suggesting that increased blood pressure alone is not sufficient to elevate circulating AEA concentrations.

An interesting recent study examined circulating endocannabinoid concentrations in individuals who were isolated from family and friends for more than 500 days in a study simulating a trip to Mars (Yi *et al*, 2016). Individuals in this condition exhibited significant reductions in positive emotions and increased SNS activation. Circulating concentrations of 2-AG but not AEA were significantly reduced at the end of this significant, chronic stress. 2-AG concentrations were significantly, positively correlated with EEG alpha activity (which was suppressed by the isolation) and significantly, negatively correlated with circulating cortisol. These very interesting findings suggest that 2-AG is brain-derived and driven by excitatory signaling, which is consistent with considerable data that glutamate increases 2-AG synthesis (Hillard, 2015). These findings are also consistent with the role of 2-AG to reduce HPA axis activation (Hillard *et al*, 2016). It is interesting that circulating 2-AG concentrations and measures of positive emotion were not associated in this study, given the evidence below that 2-AG has been found in other studies to be negatively associated with symptoms of depression.

CIRCULATING ENDOCANNABINOIDS AND REWARD, ANXIETY AND COGNITION

As has been discussed in preceding sections, CB1R signaling is involved in regulation of mood; modulates reward seeking behavior and hedonia; and is involved in behavioral responses to threat and stress. Some of the earliest studies

in which circulating endocannabinoids were measured examined their relationships to indices of mood, anxiety and perception. These studies have the underlying assumption that circulating concentrations are affected by synaptic endocannabinoid tone, particularly in limbic brain regions. However, the only preclinical study that has tested this directly disputes this assumption (Hill *et al*, 2008a). In this study, rats exposed to chronic, unpredictable stress exposure had reduced endocannabinoid concentrations throughout the limbic regions of the brain, while plasma concentrations of AEA and 2-AG were significantly increased. However, the data that have come from human studies have largely found correlations between circulating endocannabinoids and measures of mood and anxiety in particular, suggesting that CNS function is somehow translated into alterations in circulating endocannabinoids.

Endocannabinoids and Anxiety

Several studies have found that circulating AEA concentrations are inversely related to both cognitive and somatic measures of anxiety (Dlugos *et al*, 2012; Hill *et al*, 2008c). In accordance with these data, carriers of FAAH C385A have significantly increased circulating AEA concentrations, and anxiety evoked by guided imagery of their trauma decayed more quickly than those homozygous for C at this position (Spagnolo *et al*, 2016). However, circulating endocannabinoids were not altered by touch massage, a procedure that produced a significant reduction in feelings of anxiety, although concentrations of PEA were significantly increased by this procedure (Lindgren *et al*, 2015).

Circulating endocannabinoids are altered in individuals diagnosed with post-traumatic stress disorder (PTSD). In particular, circulating concentrations of 2-AG are significantly lower in individuals with PTSD that occurred as a result of the World Trade Center attacks (Hill *et al*, 2013). The 2-AG decrease remained significant after correction for sex, alcohol use, and depression. Significantly lower circulating 2-AG concentrations without a difference in AEA were also found in a study of individuals with PTSD as a result of childhood sexual abuse compared to controls (Schaefer *et al*, 2014). On the other hand, hair OEA concentrations were significantly reduced in war survivors suffering from PTSD compared to survivors without the diagnosis and strongly, negatively correlated with symptom severity (Wilker *et al*, 2016). It is possible that measurement in the hair provides a more stable and reliable measurement, particularly for a chronic disorder, than a single time point measurement in the blood.

A few observations from these studies shed light on the possible relationships between circulating endocannabinoids and symptoms of PTSD. Although no group effects were identified, AEA concentrations were significantly, negatively associated with the number of intrusive symptoms in those with chronic PTSD (Hill *et al*, 2013). A study of alcoholics with PTSD found that the FAAH C385A SNP is associated with reduced hyperarousal (Spagnolo *et al*, 2016).

On the other hand, a study of war refugees with average scores of greater than 90 on the clinical administered PTSD scale (CAPS) and on average more than eight episodes of trauma exhibited significantly increased concentrations of both 2-AG and AEA compared to trauma-exposed individuals without PTSD and controls (Hauer *et al*, 2013). The contrast between these data and the other studies suggests that the amount of distress, not just the diagnosis of PTSD, could be an important determinant of endocannabinoid regulation.

Endocannabinoids, Reward and Depression

As was discussed above, increased circulating 2-AG concentrations are associated with enhanced hedonic food consumption, perhaps as a result of CB1R-mediated activation of reward seeking. Exercise has reported beneficial effects on mood, feelings of vigor, and anxiety, which are effects mediated by the CNS. Exercise-induced increases in AEA are significantly positively correlated with increased positive affect on the Positive and Negative Affect Schedule (PANAS) in one study (Raichlen *et al*, 2012), suggesting that recruitment of AEA-mediated signaling could contribute to the rewarding or reinforcing effects of exercise. Exercise-induced increases in AEA are positively correlated with circulating concentrations of BDNF, which is also associated with elevation of mood (Heyman *et al*, 2012b).

Several studies have examined the acute effects of drugs that are associated with increased pleasurable feelings on circulating endocannabinoid concentrations. Consumption of 250 ml of red wine with low sugar content resulted in reductions in AEA and 2-AG at 20 and 45 min after consumption, respectively (Feuerecker *et al*, 2012a). Healthy volunteers receiving an intravenous dose of 0.1 mg/kg THC responded with small increases in both 2-AG and AEA in the plasma at 30 min, followed by marked reductions in both 5 h after the THC administration (Thieme *et al*, 2014). The effects of THC are particularly interesting as they suggest that the endocannabinoid system is regulated by cannabinoid receptor ligand concentrations, so that endocannabinoid concentrations are reduced in the face of exogenous agonist consumption.

Guided imagery procedures that produce alcohol desire and craving in social drinkers increase circulating concentrations of AEA, which were positively correlated with alcohol craving and increased heart rate (Mangieri *et al*, 2009). Importantly, the relationship between AEA and alcohol craving is lost in recently abstinent alcoholics, suggesting that endocannabinoid signaling within the reward circuit is dysregulated during abstinence. Baseline concentrations of AEA in the circulation were found to be significantly lower in abstinent alcoholics than social drinkers as well.

Interestingly, circulating AEA concentrations are also significantly lower in runners who meet criteria for exercise dependence compared to runners who carry out similar amounts of activity but do not exhibit exercise dependence

(Fergusson and Boden, 2008). Individuals who met criteria for exercise dependence exhibit both greater mood disturbance and significantly lower basal levels of AEA when deprived of exercise than runners who do not exhibit exercise dependence (Antunes *et al*, 2016). While these data suggest deficient endocannabinoid signaling could contribute to dysphoria during abstinence, resumption of exercise resulted in elevation of mood without an increase in circulating AEA, which does not support this hypothesis. A study that investigated a large number of NAEs and monoacylglycerols (MAGs) in plasma from abstinent cocaine addicts found NAEs were increased while MAGs were decreased (Pavon *et al*, 2013). However, neither AEA nor 2-AG were affected specifically in this study.

Several studies have found that circulating concentrations of both 2-AG and AEA are significantly altered in women diagnosed with depression. 2-AG concentrations were significantly lower in those newly diagnosed with major depression compared to controls with a significant, negative relationship between circulating 2-AG and the duration of depressive symptoms (Hill *et al*, 2008c), which suggests the hypothesis that endocannabinoid deficiency contributes to depressive symptoms. Interestingly, AEA concentrations were increased in women diagnosed with minor depression, which the authors argued could be a compensatory mechanism to maintain elevated mood. In a second study of depressed women, baseline concentrations of both AEA and 2-AG were significantly lower than in controls (Hill *et al*, 2009b). Interestingly, 2-AG mobilization in response to the TSST was not different between control and depressed women (Hill *et al*, 2009b), suggesting that the mechanisms involved in the endocannabinoid response to acute stress and their role in depression are different. A study of individuals undergoing HCT found a significant, negative association between circulating 2-AG concentrations and depressive symptoms (Knight *et al*, 2015). On the other hand, a study of patients with osteoarthritis found that circulating 2-AG concentrations were significantly, positively associated with depression scores on the Hospital Anxiety and Depression Scale and negatively correlated with memory performance scores (La Porta *et al*, 2014). More work needs to be done to determine whether circulating endocannabinoid concentrations are related to specific depression symptoms or clusters of symptoms.

Endocannabinoids and cognition

Cannabis use in humans is associated with impaired executive function, including reduced behavioral flexibility (Ramaekers *et al*, 2006). A study in 150 healthy women showed a significant, negative correlation between 2-AG and cognitive flexibility performance assessed using the Wisconsin Card Sorting Test (WCST) (Fagundo *et al*, 2013), which is consistent with the hypothesis that excessive CB1R activation, likely in the prefrontal cortex, reduces executive functioning. On the other hand, the same study found a positive correlation between circulating AEA concentrations

and behavioral flexibility in both the WCST and the Iowa Gambling Task.

The roles of cannabinoids and endocannabinoid signaling in memory formation, retrieval and extinction have been studied extensively in preclinical models (Kruk-Slomka *et al*, 2016). While it is clear that memory is regulated by CB1R signaling, there is considerable variability in pharmacological studies that are likely related to dose, timing and differences in model systems. As noted above, circulating 2-AG concentrations are significantly, negatively correlated with memory in individuals with osteoporosis (La Porta *et al*, 2014). On the other hand, plasma 2-AG concentrations are significantly higher in individuals diagnosed with Alzheimer's Disease (AD) compared to matched controls and are positively correlated with memory and attention in those with AD (Altamura *et al*, 2015). Yet another study found no differences in circulating endocannabinoid concentrations between patients with AD and controls (Koppel *et al*, 2009). The general anesthetic, propofol, has efficacy as a FAAH inhibitor and elevates brain AEA concentrations in mice (Patel *et al*, 2003). In rats, propofol treatment after training in an inhibitory avoidance task significantly increased latencies 48 h later, which is consistent with stronger memory consolidation (Hauer *et al*, 2011). This effect of propofol was blocked by rimonabant, consistent with a CB1R-mediated enhancement of memory. However, humans tend to exhibit amnesia with propofol treatment (Pryor *et al*, 2015), and humans do not exhibit an increase in circulating AEA after propofol treatment (Jarzinski *et al*, 2012; Schelling *et al*, 2006) so whether this mechanism is operative remains to be determined.

Cognitive deficits are a core symptom of schizophrenia and the degree of cognitive deficiency is a primary factor in functional impairment (Green *et al*, 2000). There is evidence that chronic, heavy use of cannabis is associated with some of the cognitive deficits in schizophrenia (Solowij and Michie, 2007), which suggests the hypothesis that endocannabinoid signaling is dysregulated in schizophrenia. Indeed, concentrations of AEA in the cerebrospinal fluid (CSF) of first episode schizophrenics are very significantly higher than control (Leweke *et al*, 1999). But contrary to the hypothesis that dysregulated endocannabinoid signaling contributes to disease symptoms, CSF concentrations of AEA are negatively correlated with psychotic symptoms in unmedicated patients (Giuffrida *et al*, 2004). Similarly, individuals in the initial, prodromal state of psychosis with the lowest CSF AEA concentrations had the highest risk for converting to a full psychotic episode (Koethe *et al*, 2009). These findings suggest the hypothesis that elevation of CSF AEA reflects a beneficial recruitment of CNS AEA signaling. No differences in circulating AEA were detected between schizophrenics and controls in which robust differences in CSF concentrations were found (Leweke *et al*, 2007) and in another study (Desfosses *et al*, 2012). However, other studies have found increased circulating AEA concentrations in schizophrenics compared to controls (De Marchi *et al*, 2003; Potvin *et al*, 2008). Serum AEA concentrations were increased in

response to treatment with cannabidiol, which also alleviated the symptoms of schizophrenia (Leweke *et al*, 2012). Circulating AEA and 2-AG concentrations are significantly higher in patients diagnosed with borderline personality disorder, a psychiatric disorder that has some of the positive symptoms of schizophrenia, than controls (Schaefer *et al*, 2014).

CIRCULATING ENDOCANNABINOIDS AND PAIN

Preclinical and human studies support the overall hypothesis that CB1R and CB2R activation both result in reduced pain perception (Piomelli *et al*, 2014). In addition, data are emerging that PEA is anti-inflammatory and analgesic (Mattace Raso *et al*, 2014). On the other hand, there are mechanisms by which both 2-AG and AEA could increase pain sensation. 2-AG has been hypothesized to serve as a precursor for arachidonic acid (Kozak *et al*, 2000) and is converted by COX-2 to oxygenated products that increase pain perception (Hu *et al*, 2008); and AEA activates pro-nociceptive TRPV1 channels (Zygmunt *et al*, 1999). Thus, endocannabinoids have the potential to increase or decrease the perception of pain, depending upon the mechanism of action.

Increases in circulating AEA concentrations occur in patients with chronic pain syndromes, such as fibromyalgia (Kaufmann *et al*, 2008) and complex regional pain syndrome (Kaufmann *et al*, 2009) when compared to controls without pain. A relatively common single nucleotide polymorphism in the gene for FAAH that results in elevated circulating concentrations of AEA (rs324420) (Sipe *et al*, 2010) is associated with lower sensitivity to cold pain (Cajanus *et al*, 2016), suggesting that higher circulating AEA concentrations seen in chronic pain could reduce pain perception.

Increases in circulating 2-AG concentrations have also been reported in chronic pain, including neuromyelitis optica (NMO) (Pellkofer *et al*, 2013) and osteoarthritis (La Porta *et al*, 2015). In NMO, 2-AG is negatively correlated with mechanical pain thresholds, suggesting an analgesic role (Pellkofer *et al*, 2013). However, in osteoarthritis, 2-AG is positively correlated with knee pain, suggesting that it contributes to pain perception (La Porta *et al*, 2015).

In a dietary intervention study in chronic headache sufferers, increased omega-3 and reduced omega-6 fatty acid consumption was used to alter the circulating concentrations of AEA and 2-AG, which were both reduced by this intervention (Ramsden *et al*, 2015). Circulating concentrations of AEA and 2-AG were both significantly, positively correlated with the numbers of daily severe headaches, suggesting roles in the etiology of headache. Interestingly, this dietary intervention produces very significant increases in the ethanolamide and 2-acylglycerol of docosahexaenoic acid (DHA), both of which were inversely correlated with the number of severe daily headaches.

Exercise reduces the perception of pain, termed exercise-induced hypoalgesia (EIH), which occurs through both

opioid and non-opioid mechanisms (Koltyn, 2000). In an early review, Dietrich and McDaniel speculated that endocannabinoids could be responsible for non-opioid EIH (Dietrich and McDaniel, 2004). In support of this hypothesis, Koltyn and colleagues demonstrated that brief periods of isometric exercise produce non-opioid-dependent EIH and a very significant increase in circulating 2-AG concentrations (Koltyn *et al*, 2014). Interestingly, correlational analyses did not support a significant relationship between circulating 2-AG (or AEA) concentrations and any measure of pain responsivity. However, CB1R are densely expressed on peripheral nerve terminals of A-delta and C primary afferents innervating skeletal muscle (Hohmann and Herkenham, 1999), so it is possible that the endocannabinoids increased by muscle activity act locally to produce EIH. This could explain the lack of correlation between endocannabinoids in the circulation and EIH.

SUMMARY AND FUTURE STUDIES

While our current state of understanding of the role of the endocannabinoid system in humans has been enhanced by measurement of circulating endocannabinoids, many of the studies have examined only a few individuals and only single time points. Recent studies showing that 2-AG is significantly circadian and that there are differences in the response to stress among men and women and, potentially, among races indicate that large, comprehensive studies that include a diverse population of individuals will provide the best data. Careful consideration of the time of sampling, and better yet, determination of endocannabinoids at multiple time points or after a stress or exercise challenge could provide important information that cannot be obtained at a single time point.

An important question that is particularly related to the studies of the relationships between circulating endocannabinoids and psychiatric disorders is whether circulating endocannabinoids are a biomarker for brain endocannabinoid signaling. Aside from the correlations and associations described above, the available data suggest that brain concentrations are not related to peripheral concentrations in a straightforward manner. For example, CSF AEA concentrations are increased multifold without consistent changes in their circulating concentrations, which indicates that overflow might not be consistent, particularly from the brain (Jumpertz *et al*, 2011). In addition, a preclinical study that examined both brain and peripheral endocannabinoids found no relationship between brain and plasma concentrations of 2-AG or AEA in chronically stressed rats (Hill *et al*, 2008b). It is possible that CNS function is translated into circulating endocannabinoids through changes in neuronal or endocrine outputs that drive alterations in endocannabinoid synthesis or degradation in peripheral tissues.

In my opinion, it is time for preclinical studies, where the relationships among circulating endocannabinoids and tissues can be determined, to catch up to the data obtained in

humans. Preclinical models can help us to understand the extent to which circulating concentrations of endocannabinoids are related to brain concentrations; the degree to which circulating concentrations represent signaling pools; and, most importantly, whether or not circulating endocannabinoid concentrations can function as biomarkers of endocannabinoid signaling status. Currently, the answers to these fundamental questions are unknown.

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