Psychoneuroendocrinology (2009) xxx, xxx–xxx

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SHORT COMMUNICATION

Circulating endocannabinoids and *N*-acyl ethanolamines are differentially regulated in major depression and following exposure to social stress

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Received 1 October 2008; received in revised form 12 January 2009; accepted 17 March 2009

KEYWORDS
Cannabinoid;
Stress;
Depression;
Metabolic;
Sympathetic;
Antidepressant

Summary Central endocannabinoid signaling is known to be responsive to stressful stimuli; however, there is no research to date characterizing the effects of stress on peripheral endocannabinoid content. The current study examined serum content of the endocannabinoid ligands *N*-arachidonylethanolamide (anandamide; AEA) and 2-arachidonoylglycerol (2-AG), and the non-cannabinoid *N*-acyl ethanolamine (NAE) molecules palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) under basal conditions, immediately following the Trier Social Stress Test (TSST), and 30 min thereafter, in 15 medication-free women diagnosed with major depression, and 15 healthy matched controls. Basal serum concentrations of AEA and 2-AG, but not PEA or OEA, were significantly reduced in women with major depression relative to matched controls, indicating a deficit in peripheral endocannabinoid activity. Immediately following the TSST, serum 2-AG concentrations were increased compared to baseline; serum AEA concentration was unchanged at this time point. Serum concentrations of PEA and OEA were significantly lower than baseline 30 min following the cessation of the TSST. The magnitude of these responses did not differ between depressed and control subjects. These are the first data to demonstrate that the peripheral endocannabinoid/NAE system is responsive to exposure to stress.

1. Introduction

In mammals, the primary effector systems of the stress response are glucocorticoid and catecholamine hormones, and secretion of these molecules from the adrenal glands into the blood in response to stressful stimuli results in rapid and delayed alterations in cardiovascular, immune and metabolic functions. These changes, in turn, promote survival by directing energy flow to systems directly responsible for dealing with the threat at hand while suppressing other systems competing for energy resources (Charmandari et al., 2005).

The endocannabinoid system, which exists in both the brain and the periphery, is both a regulator and effector of the stress response (Gorzalka et al., 2008; Steiner and Wotjak, 2008). The endocannabinoid system is composed of two G-protein coupled receptors (CB_1 and CB_2; Howlett, 2002)

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0306-4530/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.psyneuen.2009.03.013

and two arachidonate-derived ligands (Bisogno, 2008), arachidonylethanolamide (anandamide; AEA) and 2-arachidonoylglycerol (2-AG). AEA is one of a family of *N*-acyl ethanolamines (NAE); other members of this family are palmitoylethanolamide (PEA) and oleoylethanolamide (OEA; Matias et al., 2007). Neither PEA nor OEA activates the cannabinoid receptors, but they share catabolic, and some metabolic, pathways with AEA (Matias et al., 2007); both PEA and OEA have been found to be agonists to peroxisome proliferator-activated-receptor alpha (PPAR-α; Lo Verme et al., 2005; Fu et al., 2003). Preclinical studies have demonstrated that the contents of the endocannabinoids in limbic and hindbrain regions are regulated by a variety of stressful stimuli (Gorzalka et al., 2008). These changes in endocannabinoid activity dampen or promote recovery of the hormonal stress response since endocannabinoid signaling negatively modulates the sensitivity and output of the hypothalamic–pituitary–adrenal (HPA) axis (Gorzalka et al., 2008; Steiner and Wotjak, 2008).

Thus, while there are significant data that endocannabinoid signaling attenuates stress-induced neuronal signaling in the brain (Gorzalka et al., 2008; Steiner and Wotjak, 2008), little is known about the role of endocannabinoid signaling in the peripheral response to stress. Both AEA and 2-AG are present in the peripheral circulation of humans and recent studies have demonstrated that their concentrations correlate with emotional variables (Hill et al., 2008). While the unequivocal source of circulating endocannabinoids is unknown, there is evidence that adipocytes, endothelial cells and macrophages, as well as visceral organs, such as the liver and intestines, all possess the ability to synthesize and release endocannabinoids into the blood (Randall, 2007; Matias et al., 2006, 2007). Functionally, peripheral endocannabinoids, as well as non-cannabinoid NAEs, are known to modulate metabolic (adipogenesis, blood glucose levels), cardiovascular (vasoconstriction and blood pressure) and immune (production of pro- and anti-inflammatory molecules) processes (Mackie and Stella, 2006; Matias et al., 2006, 2007; Fu et al., 2003; Lo Verme et al., 2005). Moreover, all of these physiological processes are coordinately affected by exposure to stress. Accordingly, the current experiment was designed to examine the effects of social stress on circulating endocannabinoid and NAE contents. This experiment was performed in both medication-free patients diagnosed with major depression and healthy matched controls, as we demonstrated previously, in an independent population, that serum endocannabinoid content is significantly reduced in women with major depression (Hill et al., 2008) and physiological responses to stress related to metabolic and inflammatory processes are altered in individuals suffering from depressive illness (Miller et al., 2005; Pace et al., 2007).

2. Methods

2.1. Subjects

The subjects were 30 women who were part of a larger project investigating immune responses to acute stress in depression and were recruited as described previously (Miller et al., 2005). To qualify for the study, depressed subjects had to meet diagnostic criteria for a current major depressive episode ($N = 15$) according to DSM-IV (1994). Diagnoses were made by trained interviewers using the Depression Interview and Structured Hamilton (Freedland et al., 2002). Subjects with a history or current diagnosis of co-morbid psychotic, eating, alcohol, substance abuse (other than nicotine dependence), or anxiety (other than generalized anxiety disorder) disorders were excluded. These co-morbidities were diagnosed using modules from the diagnostic interview schedule (Robins et al., 1981) and the primary care evaluation of mental disorders (Spitzer et al., 1994). It should be noted that no subjects were on any prescribed medication regimen in the past 6 months *including* antidepressants except for oral contraceptives (the exclusion of which would have compromised recruitment). Control subjects were matched to a depressed subject in age and ethnicity; had to have a lifetime history free of medical and psychiatric illness; and scored <5 on the 10-item Center for Epidemiologic Studies Depression Scale (Radloff, 1977).

2.2. Trier Social Stress Test (TSST)

Subjects visited the laboratory twice. During the first session they provided written informed consent and underwent structured psychiatric and medical history interviews to determine eligibility. One week later subjects returned to the laboratory. They arrived between 08:00 h and 10:00 h following an overnight fasting period during which food, alcohol, and caffeine were avoided. Subjects were seated in a comfortable chair and, after a 10-min adaptation period, had three blood pressure readings collected at 2-min intervals (Dinamap Pro 100; Critikon Corp., Tampa, FL). Height, weight, and waist/hip circumference data were then collected.

Subjects were reseated; a butterfly needle was placed in the antecubital region of the non-dominant arm; and subjects relaxed quietly for 30 min as they acclimated to the presence of the needle. Baseline blood samples (10 ml) were collected at the end of this period into serum separating tubes. A 17-min acute stressor was then administered. The stressor consisted of a 17-min mock job interview [for specific details see Miller et al., 2005] that was modeled after the Trier Social Stress Test (Kirschbaum et al., 1995). Blood samples were collected immediately after the conclusion of the stressor (17 min in duration). Subjects spent the next 30 min sitting quietly alone, and blood was drawn at the end of the period to assess recovery. On completing the protocol, subjects were paid $150. These procedures were approved by the Institutional Review Board of Washington University.

2.3. Endocannabinoid and NAE extraction from serum

Following collection, blood samples settled and were centrifuged within 60 min of collection, at $1000 \times g$ for 25 min, after which serum was collected and stored at $-70\ ^{\circ}C$ until analysis. Lipid extraction from serum was performed as previously detailed (Hill et al., 2008). Contents of AEA, 2-AG, PEA and OEA were quantified by using atmospheric pressure, chemical ionization liquid chromatography/mass spectrometry (LC-APCI-MS) as described previously (Hill

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et al., 2008). During the extraction process, one subject from the control group had her sample compromised and it was thus excluded from analyses.

Glucose was measured using standard glucose oxidase techniques on a Hitachi 747 instrument (Kyowa Medex) and both leptin and insulin concentrations were determined with a commercially available immunoassay (Linco Corp., St. Louis, MO).

2.4. Statistics

Both demographic variables and basal serum endocannabinoid/NAE levels between the two populations were compared using an independent *t*-test. Following basal analysis, stress and recovery measurements were normalized to the percent magnitude change relative to the basal concentrations. The data from the TSST were analyzed using a repeated measures analysis of variance (ANOVA) with stress (across the basal, stress and recovery phases) representing the within-subjects variable and depression diagnosis (major depression or healthy control) representing the between subjects variable. Post hoc analysis was performed using a Tukey's test. Significance was defined as a p value less than 0.05.

3. Results

For individuals with major depression and their matched controls, there were no significant differences in age [t (27) = 0.69, p = 0.50; control 25.9 ± 6.0 years old vs. depressed 24.5 ± 4.5 years old], race [t (27) = 0.24, p = 0.89; both groups consisted of eight Caucasians, six African-Americans and one Asian subject; the sample which was compromised during extraction was from an African-American control subject], weight [t (27) = 0.17, p = 0.87; control 157.7 ± 49.1 lbs vs. depressed 154.8 ± 42.3 lbs], tobacco use [t (27) = 0.54, p = 0.62; control smoked 11.0 ± 11.2 cigarettes per days vs. depressed smoked 6.5 ± 0.7 cigarettes per day], contraceptive use [t (27) = 0.17, p = 0.86; control 50.0 ± 51.9% of subjects were on contraception vs. depressed 46.7 ± 51.6% of subjects were on contraception], body-mass index [BMI; t (27) = 0.37, p = 0.72; control 25.9 ± 6.5 vs. depressed 26.9 ± 8.0] or alcohol consumption [t (27) = 0.46, p = 0.65; control drank 3.6 ± 6.6 alcoholic drinks per week vs. depressed drank 2.6 ± 5.2 alcoholic drinks per week]. The individuals diagnosed with major depression exhibited a mean Hamilton score of 18.9 ± 1.3, and were on average experiencing only their second episode of major depression. Physiologically, there was also no difference between depressed women and healthy controls in their resting blood levels of glucose [t (27) = 0.97, p = 0.34; control 76.3 ± 9.1 vs. depressed 79.3 ± 7.4], insulin [t (27) = 0.09, p = 0.94; control 8.7 ± 4.4 vs. depressed 8.9 ± 5.4] or leptin [t (27) = 0.62, p = 0.54; control 19.6 ± 3.0 vs. depressed 16.9 ± 10.5]. None of the demographic or physiological data in the sample population correlated with AEA, 2-AG, PEA or OEA, except for insulin which exhibited a significant correlation with OEA levels (r = 0.45, p = 0.04).

Under basal conditions, serum concentrations of both AEA [t (27) = 2.06, $p < 0.05$; Figure 1] and 2-AG [t (27) = 2.40, $p < 0.03$; Figure 1] were significantly reduced in depressed women relative to healthy, matched controls. Neither PEA [t (27) = 0.10, $p > 0.05$; Figure 1] nor OEA [t (27) = 0.67, $p > 0.05$; Figure 1] serum concentrations differed between

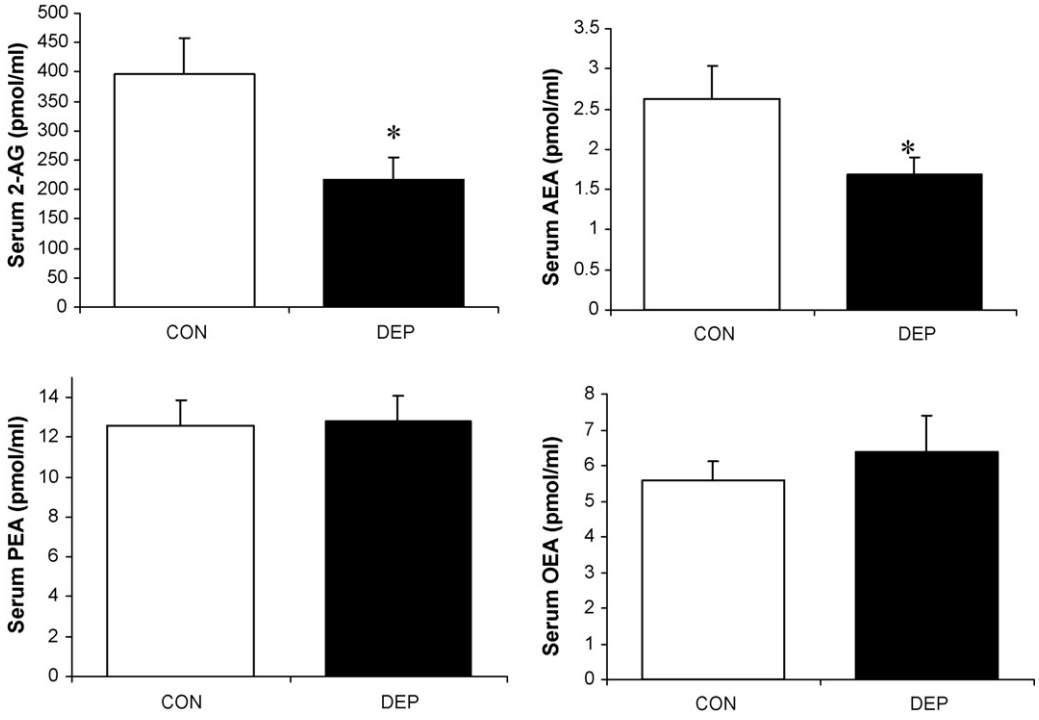

Figure 1 Serum 2-arachidonoylglycerol (2-AG), anandamide (AEA), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) content under basal conditions in women diagnosed with major depression (DEP) or healthy, matched controls (CON). Data are presented as mean ± SEM. Significant differences ($p < 0.05$) are denoted by (*).

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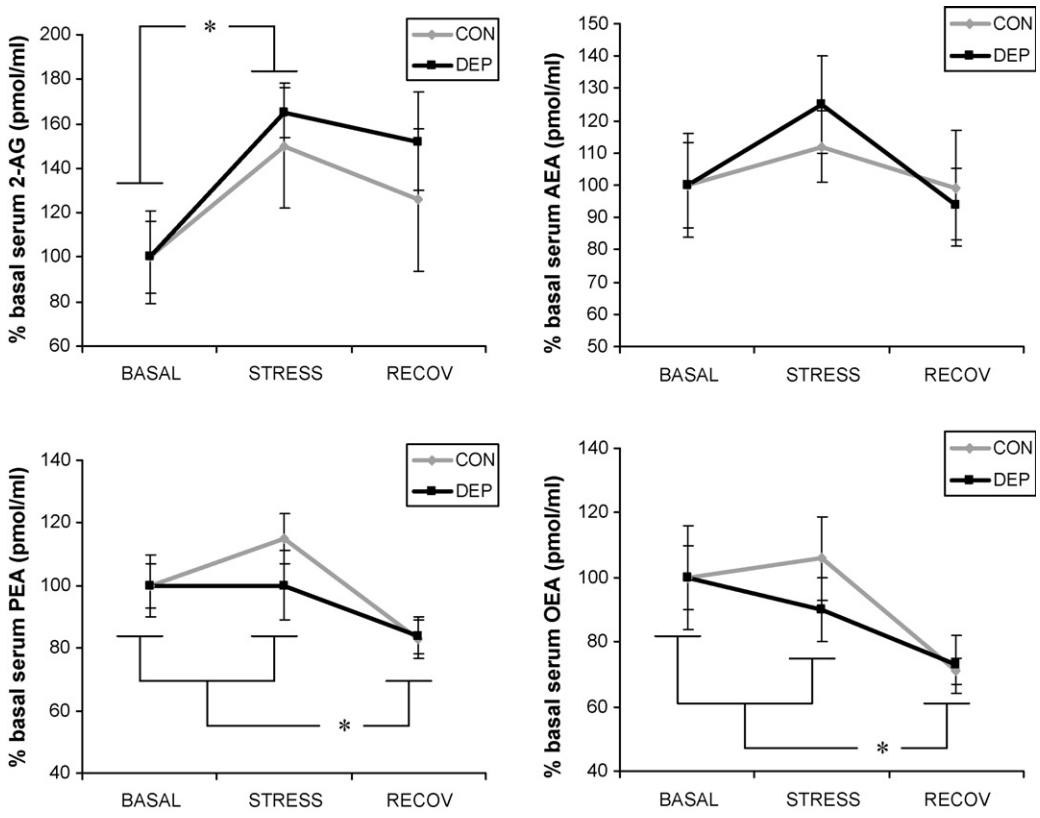

Figure 2 The change, relative to baseline, in serum 2-arachidonoylglycerol (2-AG), anandamide (AEA), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) content in response to a 17 min session of the Trier Social Stress Test, followed by a 30 min recovery period, in women diagnosed with major depression (DEP) or healthy, matched controls (CON). Data are presented as mean ± SEM. Significant differences ($p < 0.05$) are denoted by (*).

groups. The significant reduction of circulating AEA and 2-AG in major depression was not affected when we statistically controlled for cigarette smoking, oral contraception, alcohol consumption, BMI and weight.

There was a significant effect of stress exposure on serum 2-AG [F (2, 54) = 3.35, $p < 0.05$; Figure 2] contents, with post hoc analysis demonstrating that the serum 2-AG concentration was significantly increased relative to basal levels immediately after the offset of the stressor ($p = 0.04$); however, 30 min after stress cessation, 2-AG was no longer significantly elevated relative to basal serum concentration ($p = 0.17$). There was no interaction between depression diagnosis and stress exposure on the changes in serum 2-AG [F (2, 54) = 0.12, $p > 0.05$].

There was no significant effect of stress on serum AEA [F (2, 54) = 1.24, $p > 0.05$] nor was there a significant interaction between depression diagnosis and stress on serum AEA in women [F (2, 54) = 0.19, $p > 0.05$; Figure 2].

There was a significant effect of stress on serum PEA concentration [F (2, 54) = 4.45, $p < 0.02$; Figure 2]. Post hoc analysis revealed that serum PEA concentration was reduced 30 min after cessation of stress relative to both the basal concentration ($p < 0.05$) and the concentration immediately following exposure to stress ($p < 0.02$). There was no significant interaction between depression diagnosis and stress [F (2, 54) = 0.60, $p > 0.05$].

The changes in serum OEA concentrations in response to stress were similar to those of PEA; there was a significant effect of stress on serum OEA [F (2, 54) = 3.72, $p < 0.04$; Figure 2]. This effect was driven by a significant reduction in serum OEA 30 min after stress cessation relative to its concentrations at baseline ($p < 0.02$) and immediately following stress ($p = 0.05$). There was no interaction between depression diagnosis and stress on serum OEA [F (2, 54) = 0.44, $p > 0.05$].

4. Discussion

These data demonstrate that exposure of humans to an acute social stressor results in altered circulating concentrations of the endocannabinoids, AEA and 2-AG, as well as two other NAEs, PEA and OEA. In particular, stress exposure evoked a significant increase in circulating 2-AG concentration in women immediately following administration of the TSST, while both PEA and OEA exhibited a significant decline during the stress recovery phase. The diagnosis of depression had no bearing on the magnitude of alteration in endocannabinoid/NAE content in response to stress. As mentioned, the source of circulating endocannabinoids is not well characterized, but adipocytes, endothelial cells and macrophages, as well as visceral organs, such as the liver and intestines, all possess the ability to synthesize and release endocannabinoids into the blood (Randall, 2007; Matias et al., 2006, 2007).

These data reiterate the potential importance of the endocannabinoid system in affective illness (Hill and Gorzalka, 2005) and replicate previous findings of a reduction in

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circulating endocannabinoid content in an independent population diagnosed with major depression (Hill et al., 2008). While all of AEA, PEA and OEA are NAE molecules, neither PEA nor OEA was altered in depressed women, despite a reduction in AEA. The biosynthesis of AEA can occur via three independent pathways that have been documented to date (Bisogno, 2008). In this regard, Simon and Cravatt (2008) have outlined a scheme that results in the preferential synthesis of long chain, polyunsaturated NAEs, including AEA, compared to shorter, saturated or mono-unsaturated family members such as PEA or OEA. Perhaps this synthetic pathway of AEA is disrupted in depressed women, leading to a preferential reduction in AEA, but not PEA or OEA. The mechanism by which 2-AG levels are reduced in major depression remains to be determined.

Given the physiological role of peripheral endocannabinoid signaling (Randall, 2007; Mackie and Stella, 2006; Matias et al., 2006, 2007), it seems reasonable to speculate that this reduction in circulating endocannabinoid content in depression may be associated with increased rates of inflammation, cardiovascular disease and autoimmune dysfunction seen in this disease (Pace et al., 2007); however, this remains to be experimentally determined. Endocannabinoid signaling is also known to influence mood and emotion, such that impairments in endocannabinoid signaling can produce depressive-like and anxiety-like symptoms in rodents (Hill and Gorzalka, 2005) and administration of CB_1 receptor antagonists to humans has been found to increase indices of depression and anxiety (Christensen et al., 2007; Hill and Gorzalka, 2009). Accordingly, the deficit in circulating endocannabinoids documented in individuals with major depression may contribute to the emotional sequelae associated with this disease.

The mechanism by which stress induces 2-AG release is unknown. Preclinical studies indicate that central endocannabinoid content is regulated by glucocorticoid hormones which are released in response to stress (for review see Gorzalka et al., 2008). However, while not presented herein (for data see Miller et al., 2005), women in the current study were exposed to the TSST in the morning, when cortisol is at its diurnal peak, which in turn occluded the detection of an increase in salivary cortisol following stress exposure (Miller et al., 2005). Thus, the increase in 2-AG seen following stress cannot be explained by an increase in glucocorticoid signaling. In addition to increases in glucocorticoids, stress exposure also induces sympathetic nervous system activation resulting in a very rapid release of catecholamines into the circulation, which, among other targets, binds to alpha adrenergic receptors in the vasculature as well as many peripheral organs (Charmandari et al., 2005). Activation of α-adrenoreceptors couples to activation of phospholipase C through Gq heterotrimeric G-proteins, which is the initiating step in 2-AG biosynthesis (Bisogno, 2008). Given that CB_1 receptors are present on sympathetic nerve terminals and their activation by endocannabinoids results in inhibition of norepinephrine release (Ishac et al., 1996), it is our working hypothesis that the stress-induced increase in serum 2-AG concentrations is driven by sympathetic activation, which functions to suppress further sympathetic activity by inhibition of norepinephrine release. As a result, 2-AG may function as a buffer against the sympathetic response to stress, and could also promote or hasten the reinstatement of homeostasis following stress. This hypothesis is in accord with the inhibitory or stress-buffering role that endocannabinoids play in the regulation of the HPA axis (Gorzalka et al., 2008; Steiner and Wotjak, 2008), and suggests that endocannabinoids may be an integral component of stress recovery, both centrally and peripherally.

The decline in both PEA and OEA that was documented in the stress recovery phase could bear relevance to changes in inflammatory parameters that occur during exposure to stress. Through activation of PPAR-α, both PEA and OEA are able to reduce inflammation and the expression of pro-inflammatory cytokines, such as interleukin-6 (Lo Verme et al., 2005; Berdyshev et al., 1997). Exposure to the TSST has been found to evoke a delayed increase in inflammatory molecules, such as interleukin-6 (Pace et al., 2006). Accordingly, exposure to stress could increase inflammatory markers by down-regulating the circulating content of the endogenous anti-inflammatory molecules PEA and OEA. Mechanistically, given the coordinated decline in both PEA and OEA, the most parsimonious explanation is that NAE catabolism is accelerated by stress; however, this hypothesis cannot account for why AEA levels do not decline in a fashion similar to PEA and OEA, given that these three molecules all share the same catabolic pathway.

In addition to measures of inflammation, future research should examine if this stress-induced shift in the endocannabinoid/NAE signaling network may be involved in other physiological changes evoked by stress exposure. Given the role of peripheral endocannabinoid/NAE signaling in the regulation of appetite and metabolism (Matias et al., 2006, 2007), it is of particular interest to determine if there is a putative role of endocannabinoid signaling in the induction of stress-induced "comfort feeding", fat accumulation and, potentially, obesity and metabolic syndrome.

Role of funding source

This research was supported by operating grants from the American Heart Association and the Canadian Institutes of Health Research (CIHR) to GEM; a Young Investigator Award from NARSAD and a Michael Smith Foundation for Health Research (MSFHR) Scholar Award to GEM; partial funding was provided by Research for a Healthier Tomorrow, a component of the Advancing a Healthier Wisconsin endowment at the Medical College of Wisconsin (CJH); operating grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and CIHR to BBG; and a MSFHR postgraduate trainee award and a NSERC Canadian Graduate Scholarship to MNH. None of these institutions had any further role in study design, collection, analysis or interpretation of the data nor in the decision to submit this paper for publication.

Conflict of interest

Drs. Hill, Miller, Carrier, Gorzalka, Hillard report no biomedical financial interests or potential conflicts of interest.

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