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Sex and hormonal cycle differences in rat brain levels of pain-related cannabimimetic lipid mediators

Heather B. Bradshaw, Neta Rimmerman, Jocelyn F. Krey, and J. Michael Walker

Department of Psychological and Brain Sciences, Indiana University, Bloomington, Indiana

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Bradshaw, Heather B., Neta Rimmerman, Jocelyn F. Krey, and J. Michael Walker. Sex and hormonal cycle differences in rat brain levels of pain-related cannabimimetic lipid mediators. *Am J Physiol Regul Integr Comp Physiol* 291: R349–R358, 2006. First published March 23, 2006; doi:10.1152/ajpregu.00933.2005.—One important function of endocannabinoids and related lipid mediators in mammalian central nervous system is modulation of pain. Evidence obtained during the last decade shows that altered levels of these compounds in the brain accompany decreases in pain sensitivity. Such changes, if sexually dimorphic, could account for sex differences in pain and differences that occur during different phases of the hormonal cycle in females. To examine this possibility, we measured the levels of the pain-modulatory lipids anandamide, 2-arachidonoyl glycerol, N-arachidonoyl glycine, N-arachidonoyl gamma amino butyric acid, and N-arachidonoyl dopamine in seven different brain areas (pituitary, hypothalamus, thalamus, striatum, midbrain, hippocampus, and cerebellum) in male rats, and in female rats at five different points in the estrous cycle. The cerebellum did not demonstrate a change in endocannabinoid production across the estrous cycle, whereas all other areas tested showed significant differences in at least one of the compounds measured. These changes in levels occurred predominantly within the 36-h time period surrounding ovulation and behavioral estrus. Differences between males and females were measured as either estrous cycle-independent (all estrous cycles combined) or cycle-dependent (comparisons of males to each estrous cycle). In cycle-independent analyses, small sex differences were observed in the pituitary, hypothalamus, cerebellum, and striatum, whereas no differences were observed in the thalamus, midbrain, and hippocampus. In cycle-dependent analyses, the hypothalamus and pituitary showed largest sex differences followed by the striatum, midbrain, and hippocampus, whereas no sex differences were measured in thalamus and cerebellum. These data provide a basis for investigations into how differences in sex and hormonal status play a role in mechanisms regulating endocannabinoid production and pain.

estrous; anandamide; 2-arachidonoyl glycerol; N-arachidonoyl glycine

ENDOGENOUS CANNABINOIDS are involved in a myriad of physiological processes, including immune function, feeding regulation, vascular tone, memory, mood, reproduction, and pain (for reviews, see Refs. 29, 31, 34, 35, 44–46). Sex differences in the cannabinoid signaling system have been reported, these being limited mainly to examinations of sex- and cycle-related differences in the efficacy of the exogenous cannabinoid delta-9 tetrahydrocannabinol (THC; reviewed in Ref. 6). The antinoceptive effects of THC were significantly greater in female rats vs. male rats in a model of acute peripheral pain (39). Prenatal THC exposure produced a sex-dependent effect on proenkephalin mRNA levels in several brain regions in which females had significantly higher expression than males (28). Also, the cannabinoid receptor agonist CP55 940 produced greater antinociception in peripubertal female rats compared with male rats (34). Sex and hormonal cycle differences in brain cannabinoid receptor densities and affinities were also observed (32).

Elucidating the mechanism of action of endogenous cannabinoids on pain and other systems has centered on understanding the endogenous ligands arachidonoyl ethanolamine (AEA; Ref. 9) and 2-arachidonoyl glycerol (2-AG; Refs. 25 and 38) in relation to the known cannabinoid receptors, CB1 (7) and CB2 (19). Cannabinoid receptors have been localized in the central nervous system, which contains primarily CB1 (14, 27, 40, but see 42), and the immune system, which contains primarily CB2 (14). Other endogenous compounds that are structurally similar to AEA or 2-AG, and are active in classical cannabinoid tetrad tests (hypothermia, catalepsy, locomotion, and nociception; Ref. 22), or bind to cannabinoid receptors are becoming recognized as members of the family of cannabimimetic lipid mediators, more simply known as endocannabinoids. As outlined in a recent review (5), many of these compounds are likely involved in the neurophysiology of pain. For instance, N-arachidonoyl glycine (NAGly) and N-arachidonoyl gamma amino butyric acid (NAGABA) have been shown to be antinociceptive and anti-inflammatory (16) and N-arachidonoyl dopamine (NADA) has been shown to cause hyperalgesia and bind to CB1 and TRPV1 receptors (17).

The study of endocannabinoid formation in the midbrain has implications for understanding the neurophysiology of pain. An increase in release of anandamide in the periaqueductal gray (PAG) was shown to be associated with CB1-mediated analgesia induced by stimulation of the dorsal and lateral PAG (46). More recently, anandamide and 2-AG production in the dorsal midbrain was shown to be associated with stress-induced analgesia (15). These studies demonstrated that changes in endocannabinoid levels lead to concomitant changes in pain sensitivity. Therefore, changes in the formation of endocannabinoids in the brain as a result of sex or hormonal cycle may account for some of the sex and hormonal cycle differences observed in various pain conditions.

Address for reprint requests and other correspondence: J. M. Walker, Dept. of Psychological and Brain Sciences, Indiana Univ., 1101 East 10th St., Bloomington IN 47405 (e-mail: walkerjm@indiana.edu).

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Pain is defined as a sensory, emotional, and motivational experience. Therefore, it is unsurprising that activation of nociceptors recruits multiple areas of the brain involved in these experiences. Even though the midbrain has been identified as a key component in the descending modulation of pain, this extremely complex perception likely does not follow one specific pathway. As a first step toward examining the role of the endogenous cannabinoid system in sex differences in pain, we measured the levels of five endocannabinoids in seven brain regions in male rats and in female rats at five time points in the hormonal cycle using liquid chromatography/tandem mass spectrometry (LC/MS/MS). The five time points in the 4-day cycling female were early metestrus, early diestrus, early proestrus, late proestrus/early behavioral estrus, and late behavioral estrus. These times were chosen to examine the times of most dynamic endocrine and behavioral changes as outlined in a recent article stating the strategies and methods for research on the study of sex and cycle differences (2).

EXPERIMENTAL METHODS

Materials

[$^2H_8$]-Anandamide, and 2-AG were purchased from Cayman Chemical (Ann Arbor, MI). NAGly, NAGABA, and NADA were purchased from Biomol (Plymouth Meeting, PA). HPLC-grade water, methanol, and acetonitrile were purchased from VWR International (Plainview, NY). HPLC-grade acetic acid and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO).

Animals

Age-matched and littermate female Sprague-Dawley rats at five points of estrous cycle ($n = 7$ per group) and male Sprague-Dawley rats ($n = 7$), 10–14 wk of age (females weighing 250–300 g, males weighing 400–450 g) were maintained on a 12:12-h light-dark cycle (0700 light, 1900 dark). Food and water were available ad libitum. Determination of the stage of the estrous cycle was assessed by daily vaginal smears, which were obtained during the first 2 h of the light phase on each day for at least 2 wk before tissue collection. Additionally, male rats were handled in a manner similar to that of females during vaginal smears to control for any stress effects associated with handling. Tissue was collected only in rats for which vaginal smears indicated that the rat exhibited three consecutive regular 4-day cycles. Rats with more or less than a 4-day cycle were not used in this study.

Tissue Collection

Animals were euthanized by decapitation at 1000 for the light cycle points and 2200 for the dark cycle time point. On any given test day, rats at different points in the estrous cycle and males were randomly selected (e.g., one in metestrus, one in diestrus, one in proestrus, and one male) for tissue collection. Brains were dissected with forceps on an ice-cold metal dissection plate into the following regions: hypothalamus, pituitary, striatum, thalamus, hippocampus, midbrain, and cerebellum. Each of the seven tissues was placed in preweighed microcentrifuge tubes, then weighed, and flash frozen in liquid nitrogen to be stored at −80°C until used for lipid extraction and analysis. Each dissection was performed by the same experimenter to ensure continuity. The entire procedure took ~10 min, which is significantly less than the amount of time required for post mortem generation of 2-AG and anandamide (20).

Compound Extraction From Brain Tissue

All tissues from the same brain area were processed on the same day. Tissues were removed from the −80°C freezer and 20 volumes of 1:1 mixture of cold methanol and acetonitrile were added to each tissue and kept on ice. The compounds being measured are structurally similar, as they have an arachidonic acid backbone, and they have been shown to act similarly during methanol extraction and purification. Therefore, all compounds were extracted and measured with a procedure optimized for these arachidonic acid conjugates. Because of this optimization, we did not measure the many other acyl ethanolamides also currently under study such as palmitoyl ethanolamide. [$^2H_8$]-Anandamide (100 pmol in 10 μl) was added to the methanol/

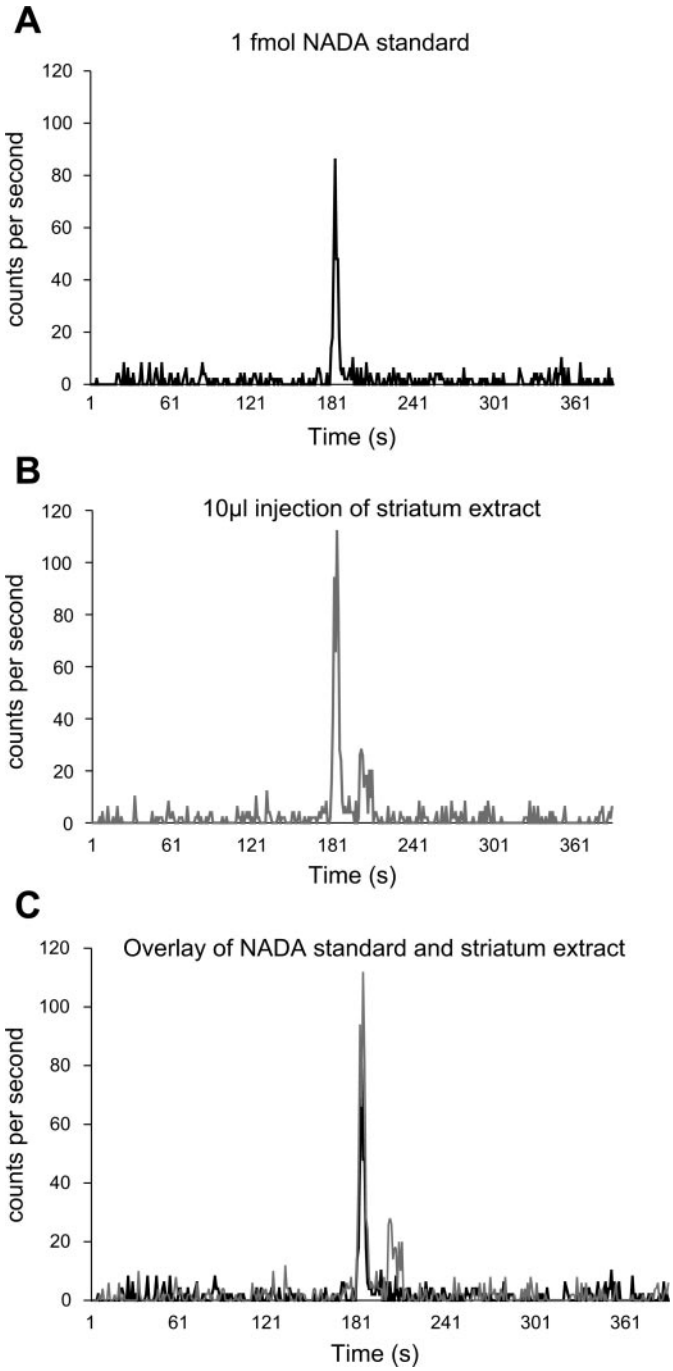

Fig. 1. Chromatograms of N-arachidonoyl dopamine (NADA). *A*: 1 fmol standard of NADA using the LC/MS/MS multiple reactions monitoring (MRM) method and organic gradient outlined in EXPERIMENTAL METHODS. *B*: 10 μl injection of brain extract from striatum using the same NADA LC/MS/MS MRM method *C*: overlay of chromatograms from *A* and *B*.

Table 1. *Brain region tissue weights*

Brain Regions	Weights, g					
	Male	M	D	P	NP	E
PIT	**0.016±0.0008**	0.020±0.0017	0.018±0.0009	0.019±0.0016	0.020±0.0015	0.020±0.0020
HYP	**0.031±0.0024**	0.026±0.0021	0.0231±0.0020	0.030±0.0025	0.023±0.0022	0.027±0.0017
THAL	0.159±0.005	0.154±0.008	0.155±0.005	0.150±0.005	0.156±0.007	0.146±0.006
HIPP	0.171±0.006	0.158±0.005	0.159±0.008	0.160±0.005	0.161±0.004	0.155±0.007
STR	**0.096±0.004**	0.078±0.003	0.084±0.007	0.089±0.005	0.085±0.005	0.087±0.004
MB	0.135±0.005	0.126±0.004	0.130±0.004	0.130±0.004	0.137±0.004	0.129±0.006
CER	**0.302±0.013**	0.264±0.006	0.254±0.009	0.270±0.009	0.273±0.006	0.266±0.007

These data are averages of tissue weights from each region dissected and examined for endocannabinoid production ($n = 7$ per group). Data are shown as means ± SE. Values in bold denote significance; $P \leq 0.05$. The pituitary (PIT) in males weighed significantly less than metestrus (M) and night proestrus (NP) females. The hypothalamus (HYP) in males and proestrus (P) females weighed significantly greater than diestrus (D) and NP stages. The cerebellum (CER) in males weighed significantly greater than females in all estrous stages. E, estrus; THAL, thalamus; HIPP, hippocampus; STR, striatum; MB, midbrain.

acetonitrile-tissue sample and used as an internal standard to track the recovery of the test compounds. The samples were maintained on ice and homogenized (sonication for pituitary and hypothalamus samples; polytron for all others). The homogenates were centrifuged for 20 min at 40,000 g and 24°C. Supernatants were collected and transferred to polypropylene 15-ml or 50-ml centrifuge tubes (VWR) and HPLC-grade water was added to each sample to create a 70/30 (water/organic) solution. Analytes were extracted from the supernatant using C18 solid-phase extraction columns (100 mg for pituitary and hypothalamus, 500 mg for striatum, thalamus, hippocampus, midbrain, and cerebellum; Varian, Harbor City, CA). Each 100 mg column was conditioned with 2.5 ml methanol and 2 ml water, whereas, 500-mg columns were conditioned with 5.0 ml methanol and 2.5 ml water followed by loading of the water/supernatant solution. Columns were then washed with 2 ml water and 1.5 ml 55% methanol. Compounds were eluted with 1.75 ml methanol. Each elution was vortexed at maximum speed for 1 min before mass spectrometric analysis.

LC/MS/MS Analysis and Quantification

Samples were analyzed by LC/MS/MS. Rapid separation was obtained using 10-μl injections of analyte (Agilent 1100 series autosampler, Palo Alto, CA) onto a Zorbax eclipse XDB 2.1X 50-mm reversed-phase column. Gradient elution (200 μl/min) was formed under pressure on a pair of Shimadzu 10AdVP pumps (Columbia, MD). Mass spectrometric analysis was performed with an Applied Biosystems/MDS Sciex (Foster City, CA) API3000 triple quadrupole mass spectrometer using electrospray ionization. Levels of each compound were analyzed by multiple reactions monitoring (MRM) on the LC/MS/MS system. In MRM mode, detection of each compound is based on fragmentation of the precursor ion $[M+H]^+$ or $[M\text{-}H]^+$ to yield a prominent product ion. Mass spectrometric conditions were optimized for each compound using direct flow injection of synthetic standards of each compound. The molecular ion and fragment for each compound measured were as follows for positive ion mode: 2-AG 379.3→287.3; AEA 348.3→287.3; for negative ion mode: NAGly 360.3→74.2; NADA 348.4→123.1; and NAGABA 388.3→102.1.

Data Analysis

The data were analyzed as male values compared with all female values combined (cycle-independent sex differences), male values compared with individual estrous cycle time points (cycle-dependent sex differences), and comparisons among all estrous cycle time points. Each of these comparisons was statistically analyzed using SPSS software. Statistical differences were determined using ANOVA with post hoc Fisher's LSD using a 95% confidence interval for the mean. Data are presented as means ± SE of the means where $P \leq 0.05$ was considered statistically significant.

RESULTS

Calibration curves based upon synthetic standards were used in combination with isotope dilution methods to calculate the amount of analyte in each sample. Figure 1 illustrates a chromatogram of a 1-fmol NADA standard, a chromatogram of striatum extract using the NADA MRM method from a female in night proestrus, and a combined chromatogram of both. The matching retention time and the isolation of both the precursor ion and a specific fragment ion by the MRM scan provide high confidence that the material being measured in the tissue extract is the compound of interest. Table 1 illustrates the average weights of the dissected brain regions, illustrating the relatively low degree of variability among the groups. Because sex and estrous stage differences were found in the

Table 2. *Cycle-independent sex differences of endocannabinoid production in seven brain regions*

	Sex	PIT	HYP	THAL	CER	STR	HIPP	MB
2-AG	M	**11.55±1.5**	**18.40±1.2**	8.72±0.7	**16.60±0.7**	4.26±0.3	2.66±0.2	13.77±0.5
	F	20.92±0.8	22.60±0.8	8.77±0.2	14.54±0.4	4.70±0.2	2.77±0.1	14.77±0.6
AEA	M	11.46±1.7	9.60±0.6	18.18±1.0	23.82±1.3	26.49±1.8	11.70±0.6	15.46±1.2
	F	10.31±0.6	11.3±0.7	18.03±0.4	24.31±0.5	29.10±0.9	10.86±0.3	16.26±0.4
NAGly	M	17.91±2.0	7.17±0.6	12.32±0.6	13.70±0.6	16.06±0.9	12.02±0.4	13.27±1.6
	F	23.91±1.4	9.36±0.6	13.71±0.4	16.20±0.6	17.80±0.4	13.13±0.4	14.97±0.5
NAGABA	M	nd	8.70±0.7	10.71±0.3	11.60±0.9	**10.75±0.7**	6.51±0.3	9.96±1.1
	F	nd	10.52±0.6	12.18±0.4	12.00±0.5	12.85±0.3	6.11±0.2	10.05±0.3
NADA	M	nd	nd	nd	nd	4.47±0.2	nd	nd
	F	nd	nd	nd	nd	4.92±0.2	nd	nd

Data are moles per gram tissue and are shown as means ± SE. Values in bold denote significance; $P \leq 0.05$. nd, not detected; M, Male; F, Female; 2-AG, 2-arachidonyl glycerol; AEA, arachidonyl ethanolamine; NAGly, N-arachidonoyl glycine; NAGABA, N-arachidonoyl gamma amino butyric acid; NADA, N-arachidonoyl dopamine.

wet weights of the tissues (Table 1), all compounds were analyzed as moles per gram tissue.

In all of the tissues examined, 2-AG was found in ~1,000 times higher levels than any of the other compounds. The levels of NAGABA were below our detection limit in pituitary, and the levels of NADA were below our detection limit in all brain regions except striatum. However, in a subset of samples the eluent was dried-down, pooled, and reconstituted (male rats only). In these samples, NADA was also found in picomoles per gram in hippocampus, and femtomoles per gram in cerebellum, thalamus, brainstem, and midbrain.

Cycle-Independent Sex Differences

To examine overall sex differences in brain endocannabinoid levels, data from each of the five estrous cycle time points were combined and compared with males. Table 2 illustrates these values. Levels of 2-AG were found to be significantly lower in males in hypothalamus and pituitary yet significantly higher in cerebellum. NAGABA levels in the striatum were significantly lower in males.

Pituitary

Estrous cycle differences. The levels of 2-AG measured in pituitary were significantly higher in proestrus (P) compared with night proestrus (NP)/early behavioral estrus and morning/late behavioral estrus (E) (Fig. 2*A*), whereas AEA levels were significantly higher in P only, compared with metestrus (M; Fig. 2*B*). Conversely, levels of NAGly in the pituitary were significantly lower in NP compared with all estrous stages (Fig. 2*C*).

Cycle-dependent sex differences. Levels of 2-AG in males were significantly lower in pituitary compared with females in all stages of estrous (Fig. 2*A*). Pituitary levels of NAGly in males were significantly lower compared with females in diestrus (D), P, E, and M (Fig. 2*C*). No cycle-dependent sex differences in levels of pituitary AEA were observed (Fig. 2*B*).

Hypothalamus

Estrous cycle differences. AEA levels were significantly higher in D compared with all other stages of the cycle (Fig. 3*B*). Levels of 2-AG in hypothalamus were significantly higher in D compared with P and M (Fig. 3*A*). Similarly, levels of NAGABA were significantly higher in D compared with M (Fig. 3*D*). Conversely, NAGly levels were significantly lower in E compared with D, P, and NP (Fig. 3*C*).

Cycle-dependent sex differences. Levels of 2-AG in males were significantly lower in hypothalamus compared with females in D, NP, and E (Fig. 3*A*). Similarly, levels of AEA and NAGABA in males were significantly lower in hypothalamus compared with females in D (Fig. 3*B*). No cycle-dependent sex differences were measurable in levels of NAGly (Fig. 3*C*).

Thalamus

Estrous cycle differences. Levels of 2-AG in thalamus were significantly higher in NP compared with P (Fig. 4*A*). No estrous cycle differences were measurable for AEA, NAGly, or NAGABA. (Fig. 4, *B*, *C*, and *D*).

Cycle-dependent sex differences. No cycle-dependent sex differences in the levels of 2-AG, AEA, NAGly, or NAGABA were measurable (Fig. 4, *A–D*).

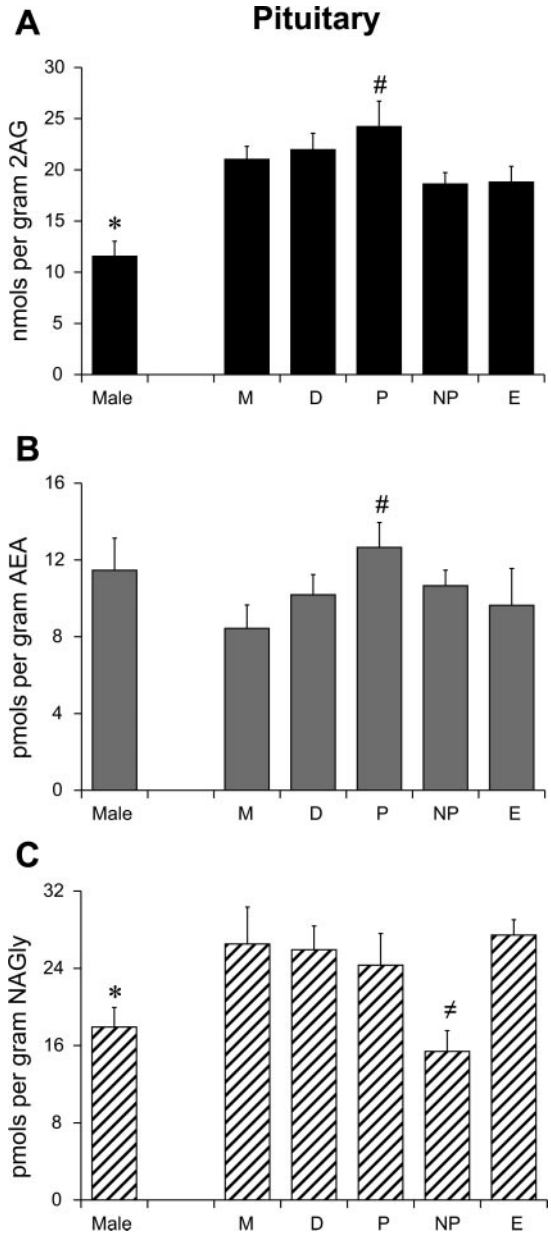

Fig. 2. Endocannabinoid production in pituitary. *A*: levels of 2-arachidonoyl glycerol (2-AG) production: #$P \leq 0.05$ proestrus (P) compared with night proestrus (NP) and estrus (E); *$P \leq 0.05$ males compared with metestrus (M), diestrus (D), P, NP, and E. *B*: levels of arachidonoyl ethanolamine (AEA) production: #$P \leq 0.05$ P compared with M. *C*: levels of N-arachidonoyl glycine (NAGly) production: ≠$P \leq 0.05$ NP compared with M, D, P, and E; *$P \leq 0.05$ males compared with M, D, P, and E. Data are shown as means ± SE.

Cerebellum

Estrous cycle differences. No estrous cycle differences in 2-AG, AEA, NAGly, or NAGABA were observed (Fig. 5, *A–D*).

Cycle-dependent Sex differences. No cycle-dependent sex differences in 2-AG, AEA, NAGly, or NAGABA were observed (Fig. 5*A–D*).

Striatum

Estrous cycle differences. The levels of NADA were significantly lower in P compared with D (Fig. 6*E*). No estrous cycle

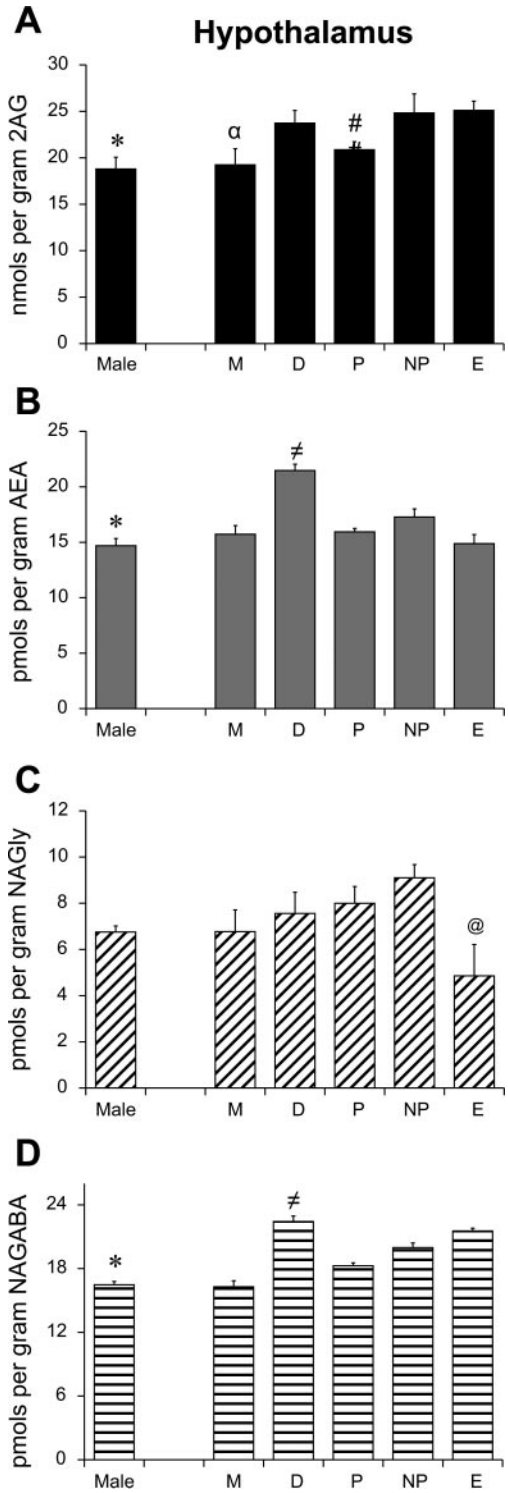

Fig. 3. Endocannabinoid production in hypothalamus. *A*: levels of 2-AG production: $\alpha = P \leq 0.05$ M compared with D, NP, and E; $\#P \leq 0.05$ P compared with E; $*P \leq 0.05$ males compared with D, NP, and E. *B*: levels of AEA production: $\neq P \leq 0.05$ D compared with M, P, and E; $*P \leq 0.05$ males compared with D. C: levels of NAGly production: $@P \leq 0.05$ E compared with D, P, and NP. *D*: Levels of N-arachidonoyl gamma amino butyric acid (NAGABA) production: $\neq P \leq 0.05$ D compared with M; $*P \leq 0.05$ in males compared with D. Data are shown as means ± SE.

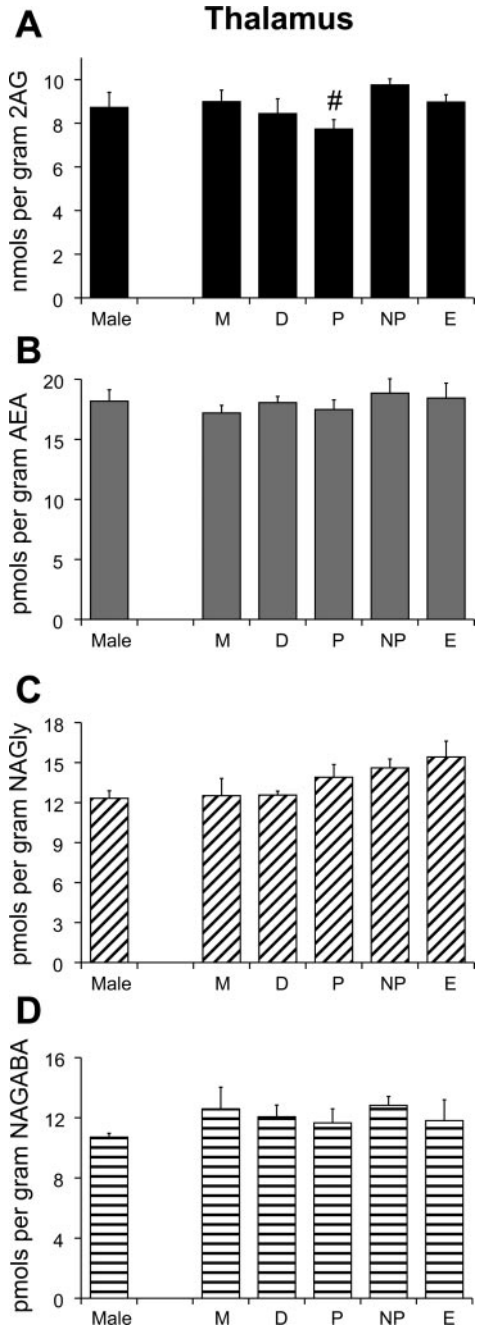

Fig. 4. Endocannabinoid production in thalamus. *A*: levels of 2-AG production: $\#P \leq 0.05$ P compared with NP *B*: levels of AEA production *C*: levels of NAGly production. *D*: levels of NAGABA production. Data are shown as means ± SE.

differences in 2-AG, AEA, NAGly, or NAGABA were measurable (Fig. 6*A*–*D*).

Cycle-dependent sex differences. The levels of NAGly in males were significantly lower in striatum compared with females in D and NP (Fig. 6*C*). Likewise, NAGABA levels in males were significantly lower compared with females in M, D, NP, and E (Fig. 6*D*). No cycle-dependent sex differences in 2-AG, AEA, or NADA were measurable (Fig. 6, *A*, *B*, and *E*).

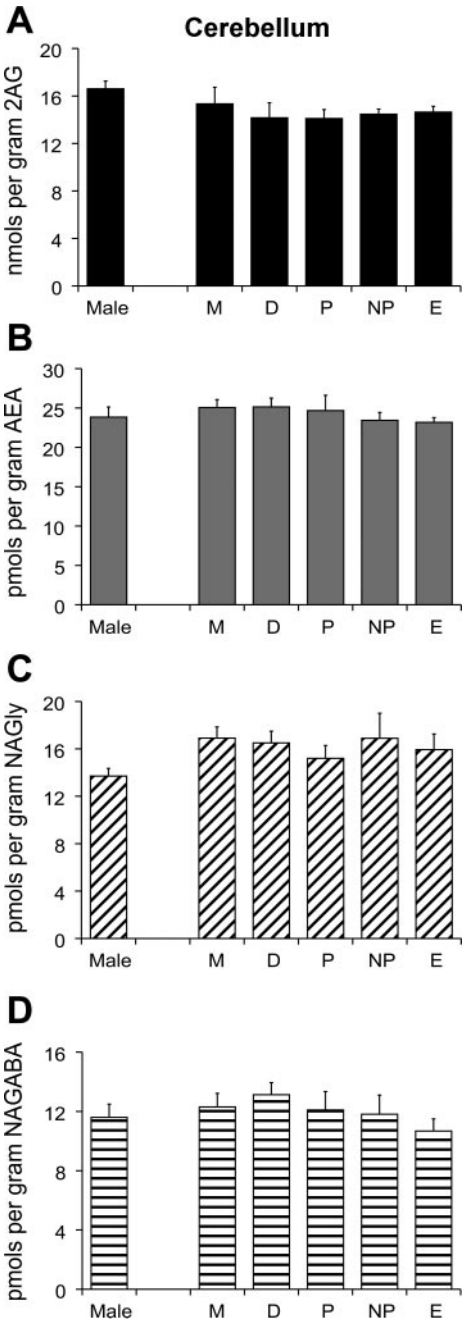

Fig. 5. Endocannabinoid production in cerebellum. *A*: levels of 2-AG production. *B*: levels of AEA production. *C*: levels of NAGly production *D*: levels of NAGABA production. See RESULTS for details. Data are shown as means ± SE.

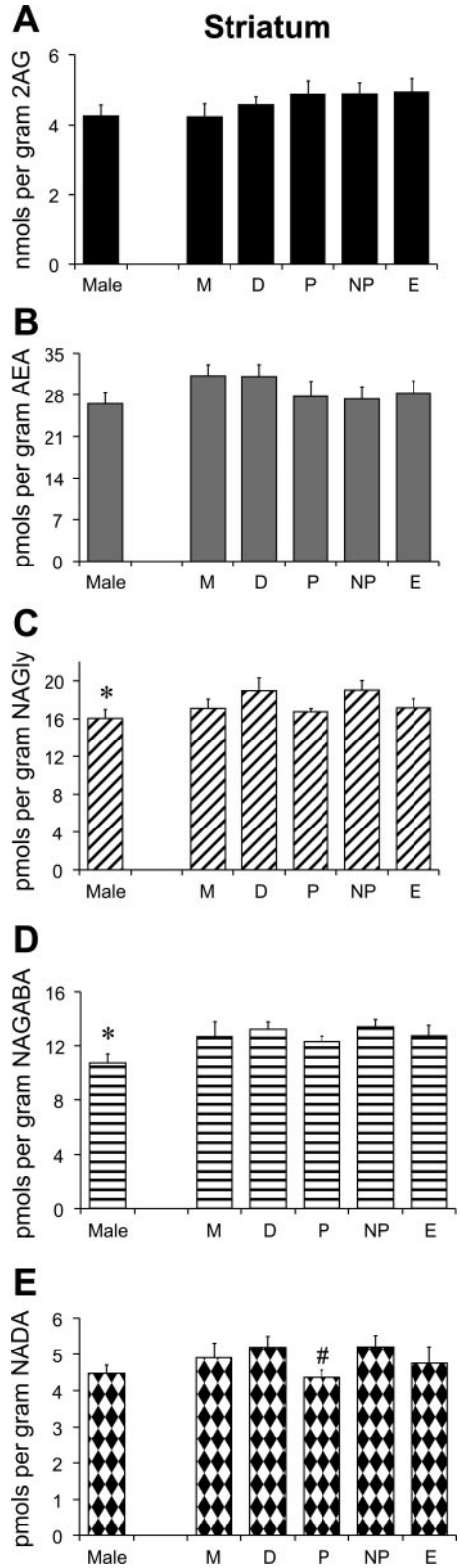

Fig. 6. Endocannabinoid production in striatum. *A*: levels of 2-AG production. *B*: levels of AEA production. *C*: levels of NAGly production: *$P \leq 0.05$ males compared with D and NP. *D*: levels of NAGABA production: *$P \leq 0.05$ males compared with D and NP. *E*: Levels of NADA production: #$P \leq 0.05$ P compared with D. Data are shown as means ± SE.

Hippocampus

Estrous cycle differences. Levels of 2-AG and NAGABA in hippocampus were significantly lower in P compared with NP (Fig. 7, *A* and *D*). Similarly, AEA levels were significantly lower in P compared with NP and E (Fig. 7*B*), and NAGly levels in hippocampus were significantly lower in P compared with NP, E, and M (Fig. 7*C*).

Cycle-dependent sex differences. Levels of AEA in males were significantly higher in hippocampus compared with females in P (Fig. 7*B*). There were no measurable sex

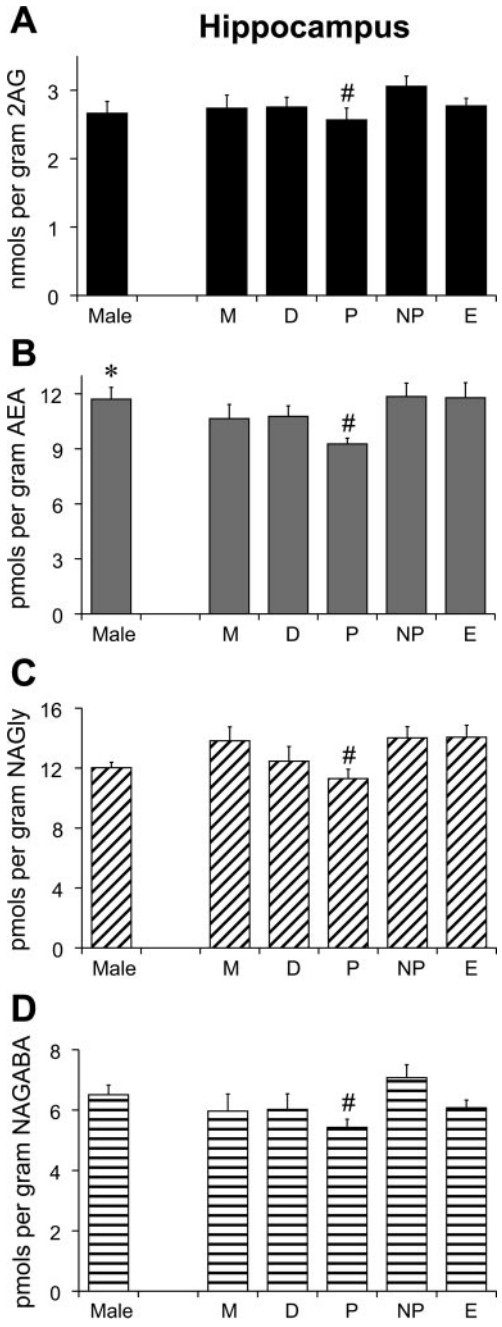

Fig. 7. Endocannabinoid production in hippocampus. *A*: levels of 2-AG production: #$P \leq 0.05$ P compared with NP. *B*: levels of AEA production: #$P \leq 0.05$ P compared with NP and E: *$P \leq 0.05$ males compared with P. *C*: levels of NAGly production: #$P \leq 0.05$ P compared with M, NP, and E. *D*: levels of NAGABA production: #$P \leq 0.05$ P compared with NP. Data are shown as means ± SE.

differences in 2-AG, NAGly, or NAGABA (Fig. 7, *A*, *C*, and *D*).

Midbrain

Estrous cycle differences. Levels of 2-AG in midbrain were significantly higher in NP compared with P, E, M, and D (Fig. 8*A*). Similarly, levels of AEA in midbrain were significantly higher in NP compared with P, E, and M (Fig. 8*B*). There were no measurable differences in NAGly and NAGABA across the estrous cycle (Fig. 8, *C* and *D*).

Cycle-dependent sex differences. 2-AG levels in midbrain in males were significantly lower than females in NP (Fig. 8*A*). Likewise, AEA levels were significantly lower in males compared with females in NP. There were no measurable cycle-dependent differences in NAGly and NAGABA levels between males and females (Fig. 8, *C* and *D*).

Tables 3 and 4 summarize the differences in the five endocannabinoid levels measured here in all seven brain areas as a

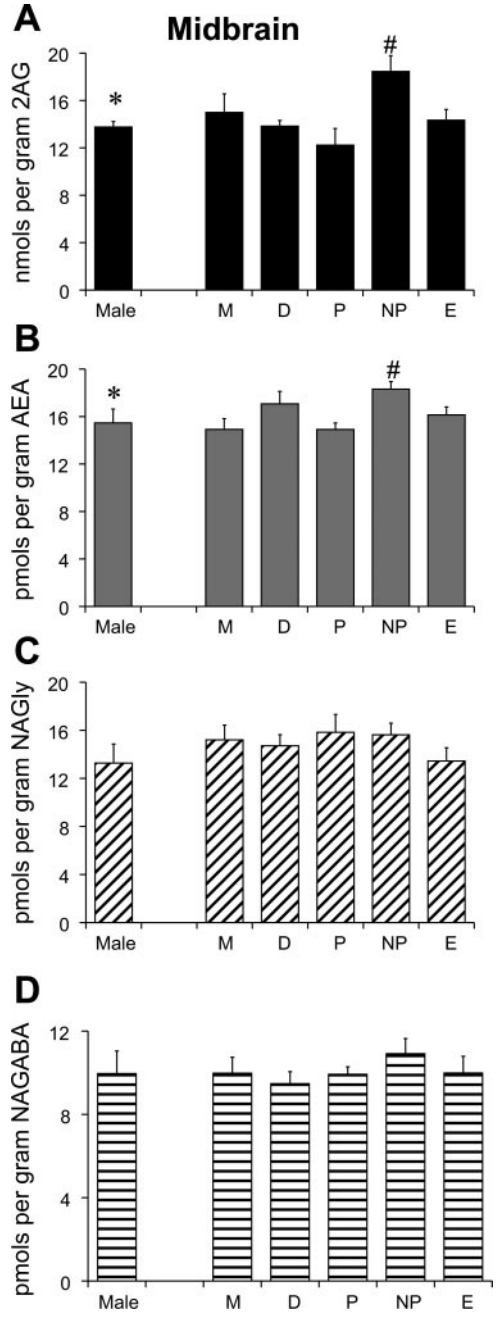

Fig. 8. Endocannabinoid production in midbrain. *A*: levels of 2-AG production: #$P \leq 0.05$ NP compared with M, D, P, and E, *$P \leq 0.05$ males compared with NP. *B*: levels of AEA production: #$P \leq 0.05$ NP compared with M and P, *$P \leq 0.05$ males compared with NP. *C*: levels of NAGly production. *D*: levels of NAGABA production. Data are shown as means ± SE.

Table 3. *Summary of estrous cycle differences of endocannabinoid production in seven brain regions*

Estrous Cycle Differences	AEA	2-AG	NAGly	NAGABA	NADA
Pituitary	P > M	P > NP, E	NP < P,E,D,M		
Hypothalamus	D > P,E,M	M < D,NP,E P < E	E < D,P,NP	D > M	
Thalamus		P < NP			
Hippocampus	P < NP, E	P < NP	P < NP,E,M	P < NP	
Striatum					P < D
Midbrain	NP > P,M	NP > P,E,M,D			
Cerebellum					

The < and > signs indicate significant differences; $P \leq 0.05$. See Figs. 2–8 and results for more details.

function of estrous cycle and cycle-dependent sex differences, respectively.

DISCUSSION

The levels of five endocannabinoids in brain vary as a function of sex and hormonal cycle (M, D, P, NP, and E). Hormonal cycle differences in endocannabinoid production center primarily on the period surrounding ovulation and receptivity (early proestrus, late proestrus/early behavioral estrus, and late behavioral estrus). In examining the cycle-independent comparisons of male vs. female levels of endogenous cannabinoids, we observed few differences, with the predominant finding being the different levels of 2-AG. However, when these comparisons were made in a cycle-dependent manner, an array of differences were observed that were linked to the variations within the estrous cycle.

The levels of endocannabinoids measured here are equivalent to those observed in previous studies that measured nanomolar amounts of 2-AG (4) and picomolar amounts of anandamide (4, 10, and 37), NAGly (16), and NADA (17). In those studies that correlated behavioral changes to changes in midbrain anandamide and 2-AG production, the change in production levels were between 20 and 40% (17, 45). Significant differences in production levels of endocannabinoids observed here were between 16 and 53%. Given that these changes were measured in relatively large regional dissections of tissue, this suggests a functional relevance of the regulation of these compounds by changes in the hormonal milieu.

The possible relationships between the hormonal and endocannabinoid systems and their synergistic roles in brain neurophysiology have yet to be fully elucidated. Although there are some data addressing potential hormonal regulatory roles for anandamide and 2-AG production, there are no data on hormonal relationships with NAGly, NAGABA, and NADA. Therefore, our discussion on how these data may relate to our current understanding of the field will primarily focus on anandamide and 2-AG. The implications for these data for further investigations into the mechanisms underlying sex differences in pain are multifaceted and will likely vary depending on the brain area of study.

Pituitary

In the anterior pituitary, CB1 receptor density is higher in males than in females during diestrus (13). Here, we found that the levels of endocannabinoids are significantly higher in proestrus compared with other points in the cycle (~24 h later). It is difficult to adequately compare the two results because in our study the two lobes of the pituitary were combined. Both findings do, however, suggest a neuroendocrine regulatory role for the pituitary endocannabinoid system. Although the pituitary has a myriad of functions, one of its neuroendocrine products involved in stress and pain is prolactin (1, 18, 21, 30). AEA delivered to the cerebral ventricles was shown to significantly decrease prolactin release in male rats and initiate a significant increase in prolactin release in estrogen-primed ovariectomized females (36). Although the mechanisms of prolactin and its influence on pain are not fully understood (12, 18, 26, 30), the regulatory interactions between prolactin, endocannabinoids, and hormonal milieu may prove to be important in understanding the mechanisms of pain associated with prolactin release in certain pain disorders.

Hypothalamus

Rodríguez de Fonseca et al. (32) showed that the densities of cannabinoid receptors in the hypothalamus were the highest in diestrus, decreasing through the cycle and becoming significantly lower in estrus. A complementary pattern was observed in levels of AEA, 2-AG, and NAGABA (i.e., the levels were significantly higher in diestrus compared with other stages of

Table 4. *Summary of cycle-dependent sex differences of endocannabinoid production in 7 brain regions*

Cycle-Dependent Sex Differences	AEA	2-AG	NAGly	NAGABA	NADA
Pituitary		ML < all	ML < M,D,P,E		
Hypothalamus	ML < D	ML < D,NP,E		ML < D	
Thalamus					
Hippocampus	ML > P				
Striatum			ML < D,NP	ML < D,NP,E	
Midbrain	ML < NP	ML < NP			
Cerebellum					

The < and > signs indicate significant differences; $P \leq 0.05$. See Figs. 2–8 and results for more detail.

the estrous cycle). Additionally, we show here that there is a significant overall sex difference in the level of hypothalamic 2-AG, whereas, Rodríguez de Fonseca and colleagues showed no sex difference in receptor density (32). However, that study did not compare males to females at different stages of the cycle, so we cannot compare and contrast those data to the current findings. Although one may postulate many functional outcomes consequent to these differences, one avenue to explore is the etiology of headache. A recent review of the neurobiology of primary headaches targets the hypothalamus as one of the key brain areas involved (9). Given that there are significant sex differences in primary headaches (3), this may be a fruitful area of exploration to understand the regulatory factors among these neurophysiological systems.

Hippocampus

Changes in dendritic spine type and density as a function of sex and hormonal status have been demonstrated by many researchers (24, 48). These changes in spine density occur at the same time as the changes in endocannabinoid levels that we observed here. Additionally, endocannabinoids have been implicated in a myriad of hippocampal cellular events, including those associated with pain processing (43; for a review, see Ref. 11). The data here provide a basis to examine the role of changes in hippocampal dendritic spines associated with sex and hormonal differences, endocannabinoids, and the interactions of these systems in the context of pain.

Midbrain

The production of the endocannabinoids anandamide and 2-AG in the midbrain in response to noxious stimuli and stress demonstrated a direct relationship between endocannabinoid production and suppression of pain (46, 15). The significant increases in the levels of both anandamide and 2-AG in the midbrain during night proestrus/behavioral estrus may be related to the behavioral changes that occur during that time period. Martinez-Gomez et al. (23) showed significant diurnal variations with longer tail-flick latencies at the beginning of the dark phase (the time of sampling in this study). This effect was most pronounced in diestrus and proestrus, suggesting a change in physiology with the dark phase that was cycle dependent. In addition, Rodríguez de Fonseca et al. (32) showed an overall sex difference in cannabinoid receptor density in which males had higher levels in the midbrain, yet they did not observe a significant cycle difference in the three time points they measured (which did not include late proestrus). They did observe, however, a significant increase in receptor density in ovariectomized females that had progesterone replacement. Night proestrus/behavioral estrus is the time point in the cycle in which progesterone is the highest. The high levels of AEA and 2-AG at a time in the cycle when progesterone is elevated suggests a hormonal regulatory role for the midbrain endocannabinoid system.

The changes in midbrain AEA and 2-AG observed in the current study could, however, be due to a diurnal change in midbrain AEA and 2-AG. One group using GC-MS showed dramatic diurnal differences in 2-AG and AEA in male rat brain (42), finding that 2-AG increased two- to fourfold, whereas AEA decreased two- to fourfold in all areas of the brain during the dark phase. Although we did measure increases in 2-AG during the dark phase, we did not measure any decreases in AEA production levels in this study. These differences are likely due to significant differences in experimental conditions between these studies, including but not limited to the differences between GC/MS and LC/MS/MS, their use of pooled samples versus our use of individual samples and sex differences.

In conclusion, this study was undertaken to provide a baseline from which to develop hypotheses on sex differences and pain, and the results shown here have provided that framework. The knowledge that endocannabinoid levels are regulated differently between the sexes and that the majority of these differences are dependent on changes across the estrous cycle sets the stage for future experiments aimed at elucidating the relationships between endocannabinoids, hormonal milieu, and pain.

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