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Research Article

Differential diurnal variations of anandamide and 2-arachidonoyl-glycerol levels in rat brain

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Abstract. The endogenous ligands of cannabinoid receptors, also known as endocannabinoids, have been implicated in many physiological and pathological processes of the central nervous system. Here we show that the levels of the two major endocannabinoids, anandamide and 2-arachidonoyl-glycerol (2-AG), in four areas of the rat brain, change dramatically between the light and dark phases of the day. While anandamide levels in the nucleus accumbens, pre-frontal cortex, striatum and hippocampus were significantly higher in the dark phase, the opposite was observed with 2-AG, whose levels were significantly higher during the light phase in all four regions. We found that the activity of the fatty acid amide hydrolase, which catalyzes the metabolism of anandamide, was significantly lower during the dark phase, thus providing a possible explaination for the increase in anandamide levels. However, the activities of monoacylglycerol lipase and diacylglycerol lipase, two of the possible enzymes catalyzing the degradation and biosynthesis of 2-AG, respectively, changed significantly only in the striatum. These data suggest that the levels of the two major endocannabinoids might be under the control of endogenous factors known to undergo diurnal variations, and underscore the different roles, suggested by previous studies, of anandamide and 2-AG in neurophysiological processes.

Key words. Cannabinoid; circadian; anandamide; 2-arachidonoylglycerol; FAAH.

The cannabinoid CB_1 receptor [1, 2] mediates many of the effects of one of the most abundant component of marijuana, Δ^9-tetrahydrocannabinol, and of its endogenous ligands, the endocannabinoids [3–5], which have been implicated in several physiological and pathological conditions of the central and peripheral nervous systems [see ref. 6 for a review]. While being altered in several neuromotor disorders [for a review see ref. 7], this signaling system appears to participate in the control of basic functions such as body temperature, food intake, sleep-wake cycles, hormone release and reproduction, particularly following stressful conditions [see refs 8–10 for reviews]. Many of these basic functions undergo diurnal variations, with dramatic changes occurring when passing from day to night and vice vers*a*, and are controlled by hormones and other mediators whose levels oscillate during the day. The tissue and blood levels of the endocannabinoids, and in particular N-arachidonoyl-ethanolamine (anandamide [3]), were found to be regulated by hormones, such as estrogens, progesterone, glucocorticoids and leptin [11–14]. In turn, plant, synthetic or endogenous cannabinoids appear to influence the release of corticosterone and gonadotropin-releasing hormones, corticosterone and adrenocorticotropic hormone (ACTH), by acting on the pituitary-hypothalamic-adrenal

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axis [15–17, and ref. 8 for a review], and of melatonin, by acting on the pineal gland [18]. As these hormones undergo circadian variations, endocannabinoid levels may also change with alternating light and dark phases, much in the way they peak in the female rat pituitary at puberty or during the estrous cycle [19, 20]. Some of the enzymes controlling the formation and degradation of anandamide and 2-arachidonoyl-glycerol (2-AG) also appear to be controlled by several hormones. In particular, the fatty acid amide hydrolase (FAAH [21]), which catalyzes the hydrolysis of anandamide, is up-regulated by progesterone and leptin [12, 13], and down-regulated by estrogens and glucocorticoids [14]. Changes in the activity of FAAH have been shown to lead to corresponding changes in anandamide levels [22, 23], and its genetic or pharmacological inactivation is associated with strong enhancement of anandamide brain levels in rodents [24, 25]. Likewise, inhibition of the activity of the monoacylglycerol lipase (MAGL) catalyzing 2-AG hydrolysis [26, 27] is accompanied by the enhancement of 2-AG levels in mouse macrophages [28]. We have recently identified and cloned two sn-1-diacylglycerol lipases (DAGLs) mostly responsible for 2-AG biosynthesis in the brain [29] and, during the preparation of this article, Okamoto and collaborators reported the purification and cloning of the enzyme catalyzing anandamide biosynthesis [30]. Although other lipases that could affect 2-AG levels, such as the lipoprotein lipase or the hormone-sensitive triacyglycerol-lipase, are known to undergo circadian rhythms [see for example ref. 31], no similar report has been published to date for the DAGL and MAGL responsible for 2-AG formation and inactivation, respectively.

In this study, we compared the levels of anandamide and 2-AG in four areas of the rat brain dissected from animals sacrificed either during the light or the dark phase of the day. Furthermore, in two of the four areas analyzed, we also assessed the activities of those enzymes catalyzing endocannabinoid biosynthesis or inactivation that have been identified to date, i.e. DAGL, MAGL and FAAH.

Materials and methods

Animals and tissue sampling

Male Sprague-Dawley rats (Charles River, Calco, Italy), weighing on arrival 200–250 g, were housed in groups of three per cage in a controlled environment, at constant temperature and humidity, on a 12-h light/dark cycle, with free access to food and water. After about 2 weeks of habituation, during which animals were daily handled to minimize a stressful response at the time of termination, rats were killed by decapitation without anesthesia at two different times: (i) during the light phase of the day (10.00 a.m.) and (ii) during the dark phase of the day (10.00 p.m.). Rat brains were quickly removed and the cerebral areas (striatum, nucleus accumbens, pre-frontal cortex, hippocampus) were obtained within 5 min by regional dissection on ice according to the method of Heffner and collaborators [32], and immediately frozen in liquid nitrogen to avoid the post mortem rise in the concentrations of long-chain N-acylethanolamines that starts approximately 30 min after sacrifice. Tissue were then stored at –80 °C until use. The experimental protocol was approved as required by Italian Governmental Decree No. 94/2000-A. All animal procedures met the guidelines of the European Community directives regulating animal research. The number of animals used and their suffering were kept to a minimum.

Endocannabinoid extraction, purification and quantification

Tissues (30–45 mg wet weight/data point) were dounce-homogenized with chloroform/methanol/ Tris-HCl 50 mM, pH 7.4 (1/1/1 by vol) containing 50 pmol of d_8-anandamide and 100 pmol of d_5-2-AG (Cayman Chemicals, Ann Arbor, Mich.) as internal standards. The lipid-containing organic phase was dried down, weighed and pre-purified by open-bed chromatography on silica gel, and analyzed by liquid chromatography-atmospheric pressure chemical ionisation-mass spectrometry (LC-APCI-MS) using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2010) quadrupole MS via a Shimadzu APCI interface. MS analyses were carried out in the selected ion monitoring (SIM) mode as described previously [11]. Temperature of the APCI source was 400 °C; the HPLC column was a Phenomenex (5 µm, 150 × 4.5 mm) reverse-phase column, eluted as described before [11]. Anandamide (retention time: 14.5 min) and 2-AG (retention time: 17.0 min) quasi-molecular ions were quantified by isotope dilution with the above-mentioned deuterated standards and their amounts in picomoles normalized per g of wet weight of tissue.

Enzyme assays

Due to the lengthy and time-consuming procedures, and to the requirement of at least 50 mg of wet weight tissue, for these assays only two of the three brain regions yielding the highest amounts of tissues were analyzed, i.e. the striatum and hippocampus. The tissues were homogenized in Tris-HCl buffer, pH 7.0, in a Dounce homogenizer. The homogenates were centrifuged at 4 °C sequentially at 800 g (5 min) and 10,000 g (25 min). The 10,000-g fraction was incubated at pH 7.0, 37 °C for 15 min (30 min in Tris-HCl buffer, pH 9.0, for FAAH activity), with different radiolabeled substrates (for DAGL activity, with sn-1-stearoyl-2-[^{14}C]-arachidonoyl-glycerol (Amersham, Little Chalfont, UK), 56.0 mCi/mmol, 100 µM; for MAGL activity, with synthetic 2-[^{3}H]arachidonoyl-glycerol, 1.0 mCi/mmol, 50 µM; for fatty acid amide

hydrolase activity, with synthetic arachidonoyl-[^{14}C]ethanolamide, 5.0 mCi/mmol, 25 μM). The concentrations of the substrates used were chosen to be higher than the apparent K_m values for each enzyme, determined in previous studies [21, 26, 27, 29]. After the incubation, lipids were extracted three times with 2 vol chloroform/methanol (2/1) and the extracts lyophilized under vacuum. For FAAH activity, 500 μl of the aqueous phase were counted with a β-counter to measure the [^{14}C]ethanolamine produced. For DAGL and MAGL activity, the extracts were fractionated by thin-layer chromatography on silica on polypropylene plates using chloroform/methanol/NH_4OH (85/15/0,1 by vol) as the eluting system. This allowed the measurement of [^{3}H]arachidonic acid produced from 2-[^{3}H]arachidonoyl-glycerol, in the case of MAGL, and of 2-[^{3}H]arachidonoyl-glycerol produced from *sn*-1-stearoyl-2-[^{3}H] arachidonoyl-glycerol, in the case of DAGL, as well as of the residual substrates in both cases [29]. Bands corresponding to each class of lipids (the R_f values of fatty acids, monoacylglycerols and diacylglycerols were 0.15, 0.25 and 0.65, respectively) were cut and their radioactivity counted with a β-counter [29].

Statistical analyses

Data from the two groups ('light' and 'dark') were compared by ANOVA followed by Bonferroni's test. The number of replicates for each experiments was three or four, and is shown in the respective figure legends.

Results and discussion

The key regulators of circadian rhythms are the suprachiasmatic nuclei (SCN), which add up to a region of the brain too small to be directly analyzed with the techniques currently used to determine endocannabinoid levels or the activity of endocannabinoid-biosynthesizing and -degrading enzymes. However, we know that the circadian activity of the SCN, via neuronal circuits involving other brain regions as well as the peripheral autonomous nervous system, and, by regulating the levels of several neuroendocrine mediators, results in oscillations of the activity of several central and peripheral tissues and organs [see ref. 33 for review]. The light-dark cycle is a major determinant of circadian activity, together with internal messages mostly related to the metabolic and energetic balance of the organism. Within the brain, the SCN are directly connected with other regions of the hypothalamus and with the paraventricular thalamic nuclei, the medial pre-frontal cortex, the lateral geniculate nucleus, the periaqueductal gray and the dorsal raphe nuclei. Through these projections, the perception of alternating light and dark phases transmitted to the SCN can be conveyed indirectly, via either multi-synaptic pathways or neuroendocrine modulation, to the pineal gland, hippocampus, amygdala, basal ganglia, limbic nuclei, frontal cortex and peripheral organs, thereby imposing circadian oscillations to motor, emotional, cognitive, sensory and endocrine functions [see ref. 34 for a review].

Within this scenario, with this first study on the possible circadian rhythm of the endocannabinoid system, we aimed at examining whether the production and degradation of the two major endocannabinoids, anandamide and 2-AG, in four key behavior-controlling, and CB_1 receptor-dense, regions of the rat brain, are subject to oscillations when passing from the light to the dark phase. We analyzed: (i) the hippocampus, a key area in the control of cognitive and mnemonic processes, (ii) the pre-frontal cortex, which is involved in cognitive and emotional behaviors and in the processing of sensory imputs, (iii) the dorsal striatum, a crucial region in the extra-pyramidal control of spontaneous activity and locomotion and (iv) the nucleus accumbens, which is at the center of the mesolimbic pathway controlling reward and translating motivation into action. The first observation that could be made from the data obtained here was that significant changes of opposing signs were found for anandamide and 2-AG tissue concentrations when passing from the light to the dark phase of the day (fig. 1). While the levels of anandamide increased dramatically in all four regions (fig. 1A), those of 2-AG were significantly decreased in the dark phase (fig. 1B). This difference in the sensitivity of the two major endocannabinoids to physiological or pathological processes in rats has been remarked in previous studies. Thus, for example, the pituitary concentrations of anandamide, but not 2-AG, undergo significant changes during estrus, or with the onset of puberty, in rats [19, 20], whereas short-term fasting seems to impart stronger changes in 2-AG than anandamide levels in the hypothalamus ad limbic forebrain [35]. In this latter area, in which the nucleus accumbens is also located, chronic treatment of rats with ethanol, nicotine and Δ^9-tetrahydrocannabinol leads to a specific increase only of anandamide levels [36, 37]. Quinpirole administration stimulates the release of anandamide, but not 2-AG, in the dorsal striatum [38]. Finally, a selective increase of anandamide levels was recently reported for the hippocampus of kainate-treated mice [39].

Interestingly, however, this is the first time that opposing effects on anandamide and 2-AG have been found, thus strongly suggesting that the two endocannabinoids have different, and not necessarily cannabinoid CB_1-receptor mediated, functions under physiopathological conditions, or at least within the circadian control of brain function. These possible separate functions might nevertheless be closely related, as suggested by our present finding that, among the four areas analyzed, the nucleus accumbens and the pre-frontal cortex demonstrated the smallest and greatest changes, respectively, for both endocannabi-

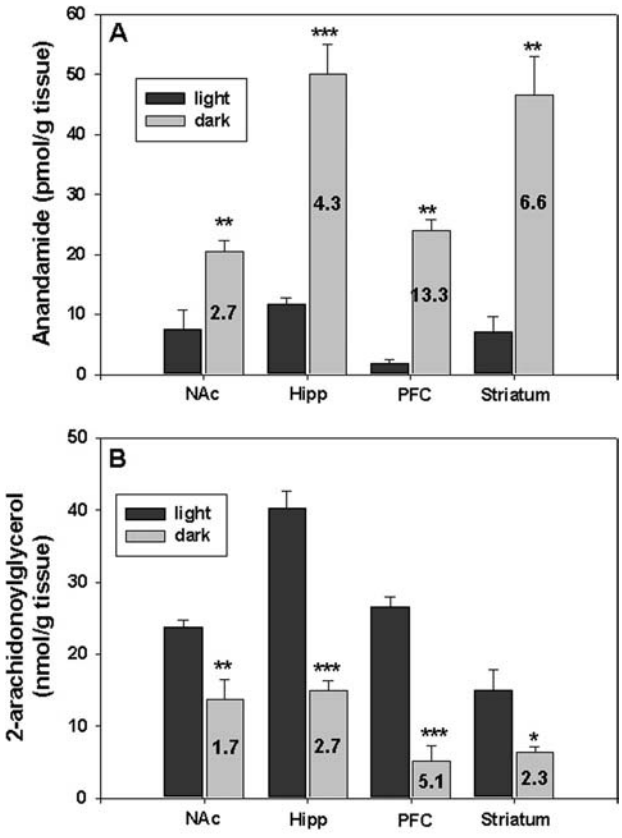

Figure 1. Concentrations of anandamide (in pmol/g tissue) (*A*) and 2-AG (in nmol/g tissue) (*B*) in four rat brain areas (NAc, nucleus accumbens; Hipp, hippocampus; PFC, pre-frontal cortex; striatum) during the two phases of the day (light and dark). Data are means ± SE of four determinations and were compared using ANOVA followed by Bonferroni's test. *p < 0.05; **p < 0.01; ***p < 0.001. The extent (fold) of the changes when passing from the light to the dark phase are shown on the dark-phase histograms.

noids. Indeed, as shown in figure 1, the extent of the changes followed the same ranking order (cortex > striatum = hippocampus > nucleus accumbens) for both anandamide and 2-AG. This similar ranking, which did not correlate with absolute endocannabinoid levels, suggests the existence of coordinated, up-stream regulatory events for the different biochemical mechanisms controlling the levels of the two compounds, although with opposite signs. One should note that different actions of the two endocannabinoids in mediating either depolarization-induced retrograde suppression of neurotransmission (for anandamide) or heterosynaptic retrograde signaling (for 2-AG) have been recently suggested [40]. Furthermore, unlike 2-AG, anandamide also acts on type 1 vanilloid receptors (TRPV1) at the level of both the peripheral and central nervous system [see ref. 41 for a review]. This property of anandamide may then result, in both the hippocampus and the substantia nigra, in effects on neurotransmitter release that are opposite to those exerted by 2-AG through CB_1 stimulation [42, 43]. Finally, by acting, again unlike 2-AG, on a yet-to-be-identified pharmacological target, anandamide inhibits glutamate release in principal neurons of the hippocampus, whereas 2-AG can exert a stimulatory effect by inhibiting GABA release via CB_1 receptors, which are most abundant on GABAergic hippocampal interneurons [44]. In summary, the opposing effects on anandamide and 2-AG brain tissue levels, through the involvement also of non-CB_1 receptors, might result in similar endocannabinoid-mediated modulatory actions on neurotransmitter release and, hence, on behavior. Alternatively, the effect of 2-AG, which is more abundant than anandamide, on cannabinoid receptors might predominate in some areas and for some behaviours. For example, reduced CB_1 receptor stimulation by 2-AG in the striatum during the night might explain in part why rats are more active in this phase of the day.

In the present study, we also addressed the issue of whether the circadian changes in the levels of endocannabinoid we observed were due to regulation of their biosynthesis or degradation. The activity and expression of the anandamide biosynthetic enzymes are likely to undergo diurnal variations since the levels of oleoylethanolamide, an anandamide congener produced from the same biosynthetic enzymes, are lower at night and higher during the day [45]. As we found changes in anandamide levels opposite to those reported for oleoylethanolamide, we hypothesized that the increased concentrations of the endocannabinoid found in the dark phase are not due to regulation of its biosynthesis, but rather to changes in its metabolism. Indeed, the anandamide-hydrolyzing enzyme, FAAH, is a strong factor in determining anandamide tissue levels, as shown by the dramatically higher concentrations of this endocannabinoid measured in the brain following pharmacological or genetic inactivation of the enzyme [24, 25]. In strong agreement with this hypothesis, we found that, in both areas where the activity of FAAH was assessed, i.e. the hippocampus and the striatum, the enzyme was significantly less active in the dark phase (table 1), thus possibly explaining the higher levels of anandamide in this brain structure. By contrast, in the case of 2-AG, we observed a decreased activity of both its biosynthesizing (DAGL) and metabolizing (MAGL) enzymes in the dark phase only in the striatum, which points to a decreased turnover of this endocannabinoid in this region and phase of the day. Since both activities were decreased by approximately 50%, this finding can explain the lower levels of 2-AG found in the striatum in the dark phase only if the effect of decreased biosynthesis predominates over that of decreased metabolism. On the other hand, in the hippocampus, the activity of neither DAGL nor MAGL underwent significant diurnal variations (table 1). This lack of effect points to the involvement of one or more biosynthetic enzymes other than DAGL [see ref. 46 for a review] as responsible for the circadian oscillation in 2-AG levels.

Table 1. Activities of FAAH, MAGL and DAGL in rat striatum and hippocampus during the two phases of the day (light and dark).

	Striatum			Hippocampus		
	FAAH	MAGL	DAGL	FAAH	MAGL	DAGL
Light	2.8 ± 0.2	5.9 ± 0.4	0.9 ± 0.10	1.5 ± 0.1	6.2 ± 0.3	0.5 ± 0.05
Dark	1.9 ± 0.1*	3.3 ± 0.3*	0.5 ± 0.03*	0.9 ± 0.05*	6.1 ± 0.4	0.5 ± 0.04

Data are means ± SD, n = 3, and are expressed as nmol min^{-1} mg $protein^{-1}$. *$p < 0.05$ by ANOVA followed by Bonferroni's test.

Indeed, other hormones that might regulate 2-AG levels in peripheral tissues, such as the hormone-sensitive triacylglycerol lipase, undergo diurnal variations [31]. At any rate, these findings (i) corroborate the hypothesis that the levels of two major endocannabinoids are differently regulated, thus possibly reflecting the differences in their molecular mode of actions and (ii) agree with previous findings [see ref. 38 for example] that the levels even of the same endocannabinoid can be regulated by the same stimulus (e.g. neuronal depolarization) in different ways in different brain areas.

In conclusion, we have described here for the first time diurnal changes in endocannabinoid levels in four important regions of the rat brain. These changes are likely due to corresponding alterations in either the metabolic (for anandamide) or biosynthetic (for 2-AG) enzymes, and suggest that the endocannabinoid system is under the control of, and may participate in, circadian rhythms, at least in the brain. This suggestion is strongly corroborated by the preliminary finding of significant changes in endocannabinoid levels in the rat pineal gland [18]. Finally, our data provide a striking example of the differential regulation of the two major endocannabinoids, thus supporting the hypothesis that anandamide and 2-AG may play different roles in central nervous system physiology and pathology.

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