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The hypothalamic levels of the endocannabinoid, anandamide, peak immediately before the onset of puberty in female rats

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Abstract

Several data suggest that the endogenous cannabinoid system plays a role in neuroendocrine regulation in adult individuals, although the information on its involvement in peri-pubertal processes is scarce. In the present study, we have examined the ontogeny (from postnatal day [PND] 5 up to adulthood) of hypothalamic and anterior pituitary contents of anandamide (arachidonyl-ethanolamide, AEA). We observed that the content of AEA in the hypothalamus was low at PND5, PND15 and PND25, but it markedly increased (approximately 3-fold) immediately before the puberty (on the day of 1st proestrus), to return to intermediate values immediately after the vaginal opening (day of 2nd proestrus) and, eventually, adulthood. By contrast, no consistent differences were observed in AEA levels in the anterior pituitary. These results demonstrate the occurrence of a parallelism between the peri-pubertal events and a rise in the hypothalamic content of AEA immediately before the puberty, which might indicate that this endocannabinoid may be involved in the onset of puberty in rats.

Keywords: Cannabinoid; Hypothalamus; Puberty; Rat

Introduction

Cannabinoids are compounds found in marijuana. The isolation of the psychoactive component of the marijuana plant (Cannabis sativa L.), Δ^9tetrahydrocannabinol (THC) (1) led to

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studies on the pharmacology of the cannabinoids. The demonstration of the stereoselectivity of THC action and of specific binding for a very potent cannabinoid agonist (2) indicated that the drug acted via a receptor protein. Radioligand studies carried out using these agonists confirmed the presence of cannabinoid binding sites in the central nervous system (CNS) (3) and subsequently the CB_1 cannabinoid receptor was cloned (4). The distribution of cannabinoid binding sites in the CNS was shown to be consistent with the pharmacological properties of THC and other psychotropic cannabinoids (5). It has been shown that the CB_1 receptor may either inhibit or stimulate adenylate cyclase through G*i* or G*s* proteins (6).

The identification of cannabinoid receptors suggested the existence of an endogenous cannabinoid agonist. Mechoulam's group isolated, from porcine brain extracts, an eicosanoid, arachidonyl ethanolamide, which was named anandamide (AEA) and was identified as one of the endogenous, specific ligands of cannabinoid receptors (7).

It has been shown that exogenous and endogenous cannabinoids markedly modify the regulation of anterior pituitary hormone secretion (8). Cannabinoid receptors are sparsely distributed in the hypothalamus (9), although their activation produces important neuroendocrine effects (see for review in 8) and may imply a role of the endogenous cannabinoid system in the fine tuning of neuroendocrine functions. However, despite initial considerations about their exclusive action via the hypothalamic factors involved in the control of anterior pituitary hormone secretion (10), recent studies have demonstrated the presence of elements of the endocannabinoid system also in the hypophysis, thus supporting an additional role of the endocannabinoid system in neuroendocrine regulation by direct pituitary actions (11). Gonzalez et al (12) demonstrated the presence of CB_1 receptor mRNA transcripts in the pituitary by using *in situ* hybridization. They also found measurable amounts of endogenous cannabinoids in the anterior pituitary (12). Hence, the neuroendocrine action of AEA was suggested to be mediated mainly through the activation of cannabinoid receptors which are present in both the hypothalamus and pituitary. However, no direct evidence exists concerning the cellular sources and targets of AEA (13). It is possible that, like a hormone, AEA activates receptors that are remote from its site of synthesis/release (14). Another possibility is that AEA acts as a local neuromodulator, as postulated elsewhere (13), and that it intervenes in hypothalamic-pituitary signaling.

It was postulated that the endogenous cannabinoid system has all its elements developed at early age and maybe atypically distributed in the developing brain compared with the adult one (15). This suggests that the role of these atypically located CB_1 receptors and of the endocannabinoids is related to CNS development, and thus also to the development of neuroendocrine regulatory centers.

The onset of puberty is followed by important hormonal changes in reproductory organs. The vaginal opening, which is the sign of puberty in female rats, usually occurs on the day after the first pre-ovulatory surge of gonadotropins (16). It was suggested that AEA might play a role in the regulation of peri-pubertal events, although still little evidence exists in support of this hypothesis. The plant-derived cannabinoid, THC, when administered before puberty caused a 2 day delay of vaginal opening and post-puberal alterations in the ovarian cycle (17). Therefore, in the present study we tried to explore the role of AEA in the onset of puberty by measuring the amounts of this endocannabinoid in the hypothalamus and pituitary of female rats at different stages of sexual and hormonal maturation.

Methods

Animals

Female Sprague-Dawley rats were used in this study. The animals were maintained under controlled conditions of temperature (20 ± 2°C) and light (on 0500 h, off 1900 h) and were provided with water and food (rat pellets) ad lib. All experimental procedures conformed to the European (and local) Animal Care Guides. The animals were quickly sacrificed by decapitation at the age of postnatal (PN) day 5, 15, 25, first day of proestrus (prepuberal, PE1), second day of proestrus (postpuberal, PE2) and at the age of PN 250 (no more cycling), respectively. Table 1 shows the number of animals per group.

To determine PE1 the vaginal membrane was artificially opened at the age of PN 30–31 and vaginal smears taken. In another group vaginal smears were taken after the vaginal membrane opening (first estrus, PN 38).

Sample collections

After sacrifice pituitaries and hypothalami were quickly removed at air conditioned room temperature (20–21°C) and within a period not longer than 1 min in each rat and immediately frozen on dry ice to avoid artificial rises in AEA levels which occur at >20°C and only after 1 hour from dissection (18). The samples (Table 1) were pooled to get a sufficient tissue weight to allow the detection of AEA (> 0.3 g for the hypothalamus and > 0.05 g for the pituitary) for each individual measurement and homogenized in 5 vol of chloroform/methanol (2:1) and subjected to lipid extraction, as described below.

Analysis of endogenous cannabinoid levels by GC/MS

As mentioned above, pooled pituitaries or hypothalami were homogenized in 5 vol. of chloroform/methanol (2:1) and subjected to lipid extraction. Great care was taken to extract the tissues and lyophilize the extracts in order to avoid losses of material. Homogenates were centrifuged at 13000 g for 16 min (4°C), the supernatants collected and the precipitates resuspended in 5 vol. of chloroform/methanol (2:1) and again centrifuged. This procedure was repeated three times more. Afterwards, the supernatants collected in the 5 consecutive centrifugations for each pool of tissues were mixed and the organic solvents evaporated in a speed-vac. Lyophilized samples were then stored frozen at -80°C under nitrogen atmosphere until analyzed. Lyophilized extracts were resuspended in chloroform/methanol 99:1 by vol.

Table 1
Number of animals/group

Group	Number of animals
Postnatal day 5	207
Postnatal day 15	230
Postnatal day 25	100
1st proestrus	77
2nd proestrus	78
Postnatal day 250	20

and 1 nmol of d_8-AEA added to the solution. The deuterated standard was synthesized from d_8 arachidonic acid and ethanolamine as described in Devane et al. (7). The solutions were then purified by open bed chromatography on silica as described in Fontana et al. (19). Fractions eluted with chloroform/methanol 9:1 by vol. (containing AEA) were collected and the excess solvent evaporated with a rotating evaporator. The former fractions were further fractionated by normal phase high pressure liquid chromatography (NP-HPLC) carried out using a semi-preparative silica column (Spherisorb S5W, Phase Sep, Queensferry, CLWYD, U.K.) eluted with a 40 min linear gradient from 9:1 to 8:2 (by vol.) of n-hexane/2-propanol (flow rate=2ml/min). These elution conditions allow the separation of 1(3)- and 2-acyl-glycerols (retention time of 18 and 20 min, respectively) from N-acylethanolamines (retention time=26–27 min). NP-HPLC fractions from 24 to 28 min were pooled, the solvent evaporated in a speed-vac, and the components derivatized with 20 μl N-methyl-N-trimethylsilyl-trifluoroacetamide + 1% trimethylchlorosylane for 2 hours at room temperature and analyzed by GC-MS carried out under conditions described previously (12) and allowing the separations of N-acylethanolamines with different fatty acid chains. MS detection was carried out in the selected ion monitoring mode using m/z values of 427 and 419 (molecular ions for deuterated and undeuterated AEA), and 412 and 404 (loss of 15 mass units from deuterated and undeuterated AEA). The area ratios between signals of deuterated and undeuterated AEA varied linearly with varying amounts of undeuterated AEA (20 pmols–20 nmol). AEA levels in unknown samples were therefore calculated on the basis of their area ratios with the internal deuterated standard signal areas.

Statistics

Statistical analysis was carried out by ANOVA and/or Student-Newman-Keuls Multiple Comparison test, and $p < 0.05$ was regarded as significant.

Results

In the present study we measured the AEA contents of the pituitary and hypothalamus in pre-puberal animals, at puberty and in the aged (no more cycling) females. The AEA content in the pituitary gland fluctuated during in the investigated period with the lowest levels at 15 PN and PE1 (Fig. 1), although the differences were not significant. By contrast, AEA content in the hypothalamus (Fig. 2) increased gradually during the pre-puberal period with the highest value on the day of PE1. On the day of PE2 the AEA content was significantly lower ($p<0.01$). No differences were detected between adult and all other groups with the exception of PE1.

Discussion

In the physiology of puberty, two main aspects should be considered: (i) the mechanisms involved in the timing of the onset of puberty, and (ii) the factors affecting the tonic and basal levels of gonadotropin secretion and surges (see for review in 20). In the pre-puberal development the changes of neuroendocrine nuclei of the hypothalamus play a more important role in the onset of puberty than the pituitary (21). The LHRH neuronal system already has the capacity in the infantile hypothalamus to generate episodic secretion, which later is an

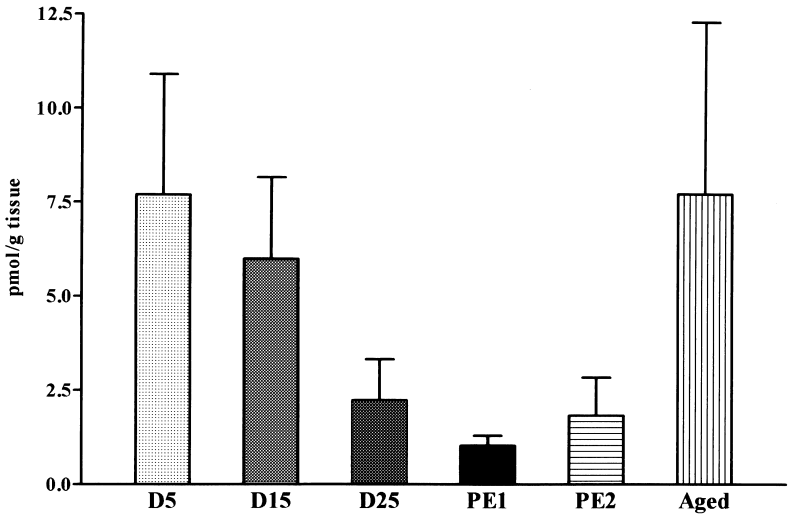

Fig. 1. Concentrations of anandamide in the pituitary gland. The values are means ± SEM of three determinations per group. D: postnatal day (5, 15, 25, respectively), PE1: 1st day of proestrus, PE2: 2nd day of proestrus, Aged: 250 days old. Details in the text.

important intrinsic property of LHRH neurones to generate the secretion of pituitary gonadotropins. The pituitary responsiveness to LHRH does not increase until the day of PE1 (20). This is in contrast with the important activation of LHRH gene expression observed in the pre-puberal period of LHRH neurones in the medial preoptic area, and with the morphologi-

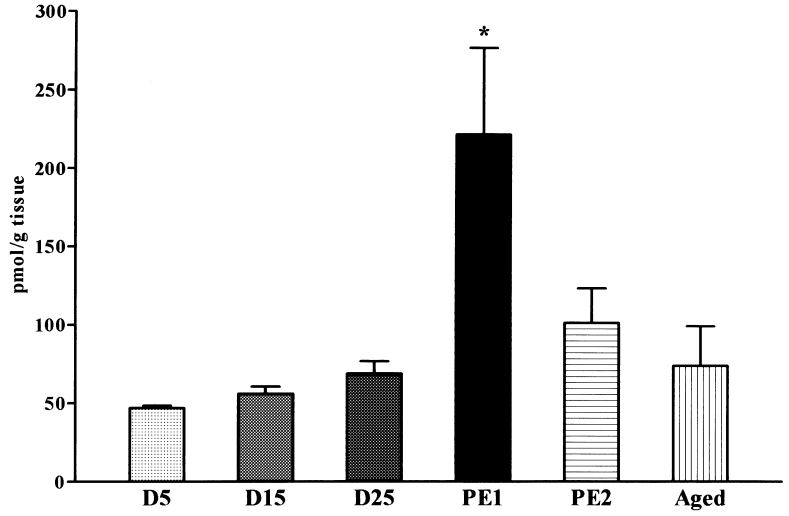

Fig. 2. Concentrations of anandamide in the hypothalamus. The values are means ± SEM of three determinations per group. D: postnatal day (5, 15, 25, respectively), PE1: 1st day of proestrus, PE2: 2nd day of proestrus, Aged: 250 days old. *: $p<0.01$ vs. All others. Details in the text.

cal maturation in this phase (22). The ovaries are under neuronal influence of the hypothalamus, too (23). The hypothalamus may, therefore, be directly responsible for both pituitary and ovarian maturation at the time of puberty.

In recent studies we have described fluctuations in the AEA content in the anterior pituitary and the hypothalamus during the estrous cycle in rats, and found the highest amounts in the estrus in pituitaries and on the day of first diestrus in hypothalami (24). The CB_1 cannabinoid receptor immunoreactivity was also shown in both pituitary (10) and hypothalamus (9). These data suggested that AEA levels in the hypothalamic-pituitary axis are under the control of sex hormones and that the endocannabinoid system intervenes in neuroendocrine regulation, particularly at the level of sexual maturation. Indeed, it has been demonstrated that cannabinoids (both exogenous and endogenous) have inhibitory effects on gonadotropin secretion. Cannabinoids caused a delay in the onset of puberty as well as alterations in reproductive functions (10, 17). Furthermore, both synthetic and endogenous cannabinoids, when exogenously administered, inhibit LH release from the pituitary. Nevertheless, data indicate that transgenic mice lacking the CB_1 receptor (CB_1 knockout mice) have significantly lower levels of LH (25), thus suggesting that endocannabinoids, unlike exogenous cannabinoids, may tonically *enhance*, rather than inhibit, LH release, possibly by acting at hypothalamic pre-synaptic neurons rather than on anterior pituitary post-synaptic cells. This also may suggest that the prepuberal hormonal changes at least partially depend upon the activation of hypothalamic CB_1 receptors. In fact, experimental data have shown that cannabinoid receptor-containing neurons in the hypothalamus, including the medial preoptic area, are all intrinsic to this brain region (26) and that pituitary CB_1 receptors might be considered as post-synaptic binding sites (27, 28) while hypothalamic receptors would be pre-synaptic. The more than two-fold increase of AEA content on the day of 1st PE, shown here for the first time, indicates that the endogenous cannabinoid system may play an important role in the onset of puberty. We hypothesise that AEA has a stimulatory effect on the initiation of anterior pituitary gonadotropin secretion at hypothalamic but not directly at pituitary level.

In conclusion, the data presented here suggest that AEA and, possibly, CB_1 receptors play a role in determining the onset of puberty in the female rat. However, this hypothesis awaits a more direct demonstration by the use of yet to be developed inhibitors of AEA synthesis, as well as of CB_1 knockout mice.

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References

1. Mechoulam R, Gaoni YJ. A total synthesis of dl-Δ^1-tetrahydrocannabinol, the active constituent of hashish. J. Amer. Chem. Soc. 1965;87:3273–5.
2. Dewey WL. Cannabinoid pharmacology. Pharmacol. Rev. 1986;38: 151–78.

3. Devane WA, Dysarz III FA, Johnson MR, Mevin LS, Howlett AC. Determination and characterization of a cannabinoid receptor in rat brain. Mol. Pharmacol. 1988; 34: 605–13.
4. Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 1990;346: 561–4.
5. Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, deCosta BR, Rice KC. Cannabinoid receptor localization in brain. Proc. Natl. Acad. Sci. USA 1990;87: 1932–6.
6. Felder CC, Veluz JS, Williams H.L, Briley EM, Matsuda LA. Mol. Pharmacol. Cannabinoid agonists stimulate both receptor- and non-receptor-mediated signal transduction pathways in cells transfected with and expressing cannabinoid receptor clones. 1992;42:838–45.
7. Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson CA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 1992;58:1946–9.
8. Wenger, T, Tóth BE, Juanéda C, Leonardelli J, Tramu G. The effects of cannabinoids on the regulation of reproduction. Life Sci. 1999;65: 695–701.
9. Moldrich G, Wenger T: Localization of the CB_1 cannabinoid receptor in the rat brain. Peptides 2000;21: 1735–42.
10. Wenger T, Croix D, Tramu G, Leonardelli J. Neuroendocrine effects of delta-9-tetrahydrocannabinol on pregnancy and puberty. In: Murphy CC, Bartke A, eds Marihuana/Cannabinoids: Neurobiology and Neurophysiology. Boca Raton, Fl: CRC Press Inc., 1992. pp. 539–560.
11. Wenger T, Fernandez-Ruiz JJ, Ramos JA. J. Immunocytochemical demonstration of CB_1cannabinoid receptors in the anterior lobe of the pituitary gland. Neuroendocrinology 1999;11: 873–8.
12. González S, Manzanares J, Berrendero F, Wenger T, Corchero J, Bisogno T, Romero J, Fuentes JA, DiMarzo V, Ramos JA, Fernández-Ruiz JJ. Identification of endocannabinoids and cannabinoid CB_1 receptor mRNA in the pituitary gland. Neuroendocrinology 1999;70: 137–45.
13. DiMarzo V, Petrocellis L. The endogenous cannabinoid signalling system: chemistry, biochemistry and physiology. Internet J. Sci-Biol. Chem. 1997;1: 1–33.
14. Felder C, Glass M. Cannabinoid receptors and their endogenous agonist. Annu. Rev. Pharmacol. Toxicol. 1998;38: 179–200.
15. Fernandez-Ruiz, JJ, Berenderro F, Hernandez ML, Ramos JA. The endogenous cannabinoid system and brain development. Trends Neurosci. 2000; 23: 14–20.
16. Ramirez VD, Endocrinology of Puberty. In: Greep RO, Astwood EB, Eds. Handbook of Physiology, Vol. II. Sec. 7. Washington: American Physiological Society 1973. pp.1–28.
17. Wenger T, Croix D, Tramu G. The effect of chronic prepubertal administration of marihuana (delta-9-tetrahydrocannabinol) on the onset of puberty and the postpubertal reproductive functions in female rats. Biol. Repr. 1988;39: 540–5.
18. Felder CC, Nielsen A, Briley EM, Palkovits M, Priller J, Axelrod J, Nguyen DN, Richardson JM, Riggin RM, Koppel GA, Paul SM, Becker GW. Isolation and measurement of the endogenous cannabinoid receptor agonist, anandamide, in brain and peripheral tissues of human and rat. FEBS Letters 1996; 393: 231–5.
19. Fontana A, DiMarzo V, Cadas H, Piomelli D. Analysis of anandamide, an endogenous cannabinoid substance, and of other natural N-acylethanolamines. Prostagl. Leukotr. Essent. Fatty Acid 1995;53: 301–8.
20. Ojeda SR, Urbanski HF. Puberty in the rat. In: Physiology of Reproduction. Knobil E, Neill JD. eds. New York : Raven Press, 1994. pp 363–409.
21. Jakubowski M, Blum M, Roberts JL. Postnatal development of gonadotropin-releasing hormone and cyclophilin gene expression in the female and male rat brain. Endocrinology 1991;128: 2702–8.
22. Wray S, Hoffman G. Neuroendocrinology Postnatal morphological changes in rat LHRH neurons correlated with sexual maturation. 1986;43: 93–7.
23. Gerendai I., Toth IE, Boldogkoi Z, Medveczky I., Halasz B. Neuronal labeling in the rat brain and spinal cord from the ovary using viral transneuronal tracing technique. Neuroendocrinology 1998;68: 244–256.
24. Gonzáles S, Bisogno T, Wenger T, Manzares J, Milone A., Berrendero F, DiMarzo V, Ramos JA, Fernández-Ruiz JJ.: Sex steroid influence on cannabinoid CB_1 receptor mRNA and endocannabinoid levels in the anterior pituitary gland. Biochem. Biophys. Res. Com. 2000;270: 260–6.

25. Wenger T, Ledent C, Csernus V, Gerendai I. The central cannabinoid receptor inactivation suppresses endocrine reproductive functions. Biochem. Biophys. Res. Com. 2001; 284: 363–9.
26. Romero J, Wenger T, de Miguel R, Ramos JA., Fernández-Ruiz JJ. Cannabinoid receptor binding did not vary in several hypothalamic nuclei after hypothalamic deafferentation. Life Sci. 1998;63: 351–6.
27. DiMarzo V, Breivogel CS, Tao Q, Briden DT, Razdan RK, Martin BR. Levels, metabolism, and pharmacological activity of anandamide in CB(1) cannabinoid receptor knockout mice: evidence for non-CB(1), non-CB(2) receptor-mediated actions of anandamide in mouse brain. J. Neurochem. 2000;75: 2432–44.
28. Szallasi A, DiMarzo V. New perspectives on enigmatic vanilloid receptors. Trends Neurosci. 2000;10: 491–7.