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The Ontogeny of Cannabinoid Receptors in the Brain of Postnatal and Aging Rats

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BELUE, R. C., A. C. HOWLETT, T. M. WESTLAKE AND D. E. HUTCHINGS. *The ontogeny of cannabinoid receptors in the brain of postnatal and aging rats.* NEUROTOXICOL TERATOL 17(1) 25-30, 1995.—It is recogized that a number of the biological effects of Δ^9-tetrahydrocannabinol (THC) can be attributed to a cannabinoid receptor found in abundance in the brain. Due to observations that cannabinoid drugs exert some developmental toxicity, it was of interest to examine the developmental pattern of cannabinoid receptors in the brain of neonatal rats through young adulthood, and then to further examine the cannabinoid receptor during the aging process in the brain of rats 3 to 32 months of age. Using radioligand binding assays, this study demonstrated that cannabinoid receptor binding capacity increases progressively from birth to postnatal day (PND) 60. Within the striatum, a significant increase in binding occurred between PNDs 14 and 21. In the cerebellum, cannabinoid receptor binding capacity doubled at 7-day postnatal intervals until adulthood. Cannabinoid receptor binding in the cortex doubled between PNDs 7 and 14. Within the hippocampus, there were small incremental increases until the final adult level was reached at PND 21. There was no significant alteration in the affinity for CP-55940 during development. These findings might reflect an increased differentiation of neurons into cells possessing cannabinoid receptors, or an increase in the number of cannabinoid receptors on cell bodies or projections in regions undergoing developmental changes. Once the adult cannabinoid receptor levels have been reached, binding activity in the whole brain preparation neither increased nor declined during the normal aging process.

THC Cannabinoid receptor Embryolethality

THE PRENATAL administration of Δ^9-THC to rats has been found to produce dose-related toxic effects in the offspring independent of cannabinoid-induced maternal toxicity. These include increased embryolethality of female conceptuses (21) as well as delays in postnatal growth (6) and brain protein synthesis (24). Moreover, studies of perinatal and postnatal administration of cannabinoids have found effects on endocrine systems and sexually mediated behaviors (for review, see ref. 1).

It is now recognized that a number of the biological effects of Δ^9-THC can be attributed to a cannabinoid receptor found in abundance in the brain (see refs. 2,18 for review). The cannabinoid receptor is coupled to adenylate cyclase by a G protein to ultimately inhibit cyclic AMP production (17,19). Recent pharmacological evidence also indicates that the cannabinoid receptor can regulate ion channels in certain target neurons (10,23). In adult rats, the cannabinoid receptor is found to be abundant in the cortex, hippocampus, basal ganglia, and cerebellum (3). Highly sophisticated quantitative densitometry of [^{3}H]CP-55940 binding to brain slices from rats and other mammals including humans has defined the brain regions that exhibit the greatest density of cannabinoid receptors (13,14,15). Cannabinoid receptors appear on cell bodies located within the striatum and on the projections from these cells to the *globus pallidus*, *entopeduncular nucleus*, and *substantia nigra pars reticulata* (13). Within the cerebellum, axons of granule cells within the molecular layer bear cannabinoid receptors (15). Within the hippocampus, cannabinoid receptors are located with greatest density in the molecular layer of the dentate gyrus and the CA3 region (14,15). Cannabinoid receptor density is sparse in the hypothalamus (13, 14,15), and yet studies have described endocrine effects of

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Δ^9-THC that can be attributed to functions under the regulatory control of the hypothalamus (7,31).

Because of the observations that cannabinoid drugs exert some developmental toxicity and the knowledge that extensive neuronal systems in the brain contain cannabinoid receptors, it was of interest to examine the developmental pattern of cannabinoid receptors in the brain of neonatal rats through young adulthood, and then to further examine the cannabinoid receptors during the aging process in the brain of rats 3 to 32 months of age. The present studies have demonstrated using radioligand equilibrium binding assays that cannabinoid receptor number increases progressively during development in various brain regions. The increase in binding during development can be characterized by regional growth spurts at postnatal ages. In adults, once the binding maxima has been reached, it neither increases nor declines during the aging process. No apparent changes in K_d for CP-55940 were observed during development or aging.

EXPERIMENTAL METHOD

Animals were sacrificed by decapitation at postnatal days (PND) 0, 7, 14, 21, and 60. For PND 0 brains were obtained from two males and two females from each of two litters and placed directly on dry ice. On PNDs 7, 14, and 21, brains were rapidly removed and frozen in methyl butane at −30°C for 2 min and then placed directly on dry ice. Frozen brains were free-hand dissected into cortex, caudate, hippocampus, hypothalamus, and cerebellum. One litter was used on each day of sacrifice. Samples were taken from 5 pups for each brain area and approximately equal numbers of males and females were included. On PND 60 ± 2, brains were obtained from adult males and placed on dry ice and stored at −80°C until use. Adult brains were thawed and free-hand dissected for immediate membrane preparation. Crude washed membrane fractions were prepared by homogenizing the tissue in a solution containing 320 mM sucrose, 2 mM Tris-EDTA, and 5 mM $MgCl_2$ (20 ml per g wet weight). The homogenate was sedimented at 1000 × g for 5 min. The supernatant was saved and the pellet was resuspended in the same volume of sucrose solution and resedimented. The combined supernatant fractions were sedimented at 39,000 × g for 15 min and resuspended in one half volume of TME buffer (50 mM TrisCl, pH 7.4, 3 mM $MgCl_2$ and 1 mM TrisEDTA). The pellet was washed by incubation in two steps, first at 37°C for 10 min and the second at 30°C for 30 min, each followed by sedimentation and resuspension. The final pellet was resuspended in 5 ml TME per g original wet weight. The protein content was determined using the assay of Bradford (5), and was usually 5 to 10 mg/ml.

Cannabinoid receptor binding in Wistar rats for the developmental studies (Table 1 and Fig. 2) were performed using a centrifugation assay as described previously by Devane et al. (11). However, cannabinoid receptor binding for the aging studies and strain comparisons (Fig. 1 and Table 2) was determined using a filtration method to separate bound from free radioligand (16). The assay mixture (200 μl) containing 20 mM TrisCl pH 7.4, 3 mM $MgCl_2$, 1 mM Tris-EDTA, 0.1 mg/ml fatty acid-deficient bovine serum albumin, 30 μg membrane protein, and 0.1 nM [^{3}H]CP-55940. To determine nonspecific binding, 0.1 μM desacetyllevonantradol was added to displace radioligand from high affinity receptor sites. Homologous equilibrium competiton curves were produced using concentrations of CP-55940 from 0.01 nM to 100 nM for the determination of K_d values for CP-55940. Heterologous displacement of [^{3}H]CP-55940 for data in Fig. 1 used a range of 0.1 to 100

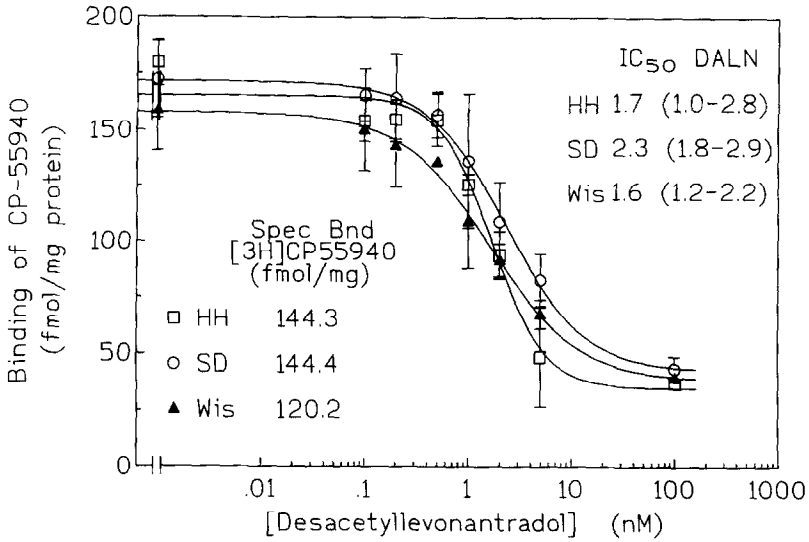

FIG. 1. Competitive displacement by desacetyllevonantradol in three strains of adult rats. Whole adult brains were obtained from Hooded Hybrid, Sprague-Dawley, and Wistar rats and membranes were prepared. Specific binding of [^{3}H]CP-55940 was determined at 0.2 nM and competition for binding is shown for the indicated concentrations of desacetyllevonantradol. Data were analyzed by combining the data from $n = 3$ experiments for nonlinear regression analysis using Graphpad Inplot, where variables of maximum, minimum, IC_{50} and slope factor were determined. Data points are means and SEM, and the error of specific binding and affinity for desacetyllevonantradol are represented as 95% confidence intervals for the curve.

nM desacetyllevonantradol. Incubations were for 60 min at 30°C. To separate bound from free radioligand using filtration, 250 μl of 50 mg/ml bovine serum albumin was added and the samples were immediately filtered over Whatman 934-AH filters and washed with five 3-ml volumes of ice cold buffer containing 20 mM TrisCl, pH 7.4, and 2 mM $MgCl_2$. The filters were placed in scintillation vials and shaken for 2 h at room temperature with 1 ml 0.1% sodium dodecyl sulfate in 20 mM NaHEPES pH 7.0. Scintillation cocktail (4 ml) was added and radioactivity was counted in a Beckman LS1800 liquid scintillation counter.

Equilibrium binding data were analyzed using the computer program Graphpad Inplot. Values were also analyzed by linear least squares regression analysis using the Hill transformation (9). K_i values were calculated from IC_{50} using the assumptions of Cheng and Prusoff (8). Statistical comparisons of the binding data were made by analysis of variance (ANOVA) followed by Tukey's posthoc test. Statistical differences were considered to be at the 0.05 level of significance.

Sprague Dawley rats 60 days of age were supplied by Harlan Laboratory. Wistar rats ages 1 to 60 days and Hooded Hybrids ages 3- to 32-months were supplied by Charles River Breeding Laboratories. The Hooded Hybrids were generously donated by NIA as a preliminary study. All rats were sacrificed several days after arrival at the local animal care facilities.

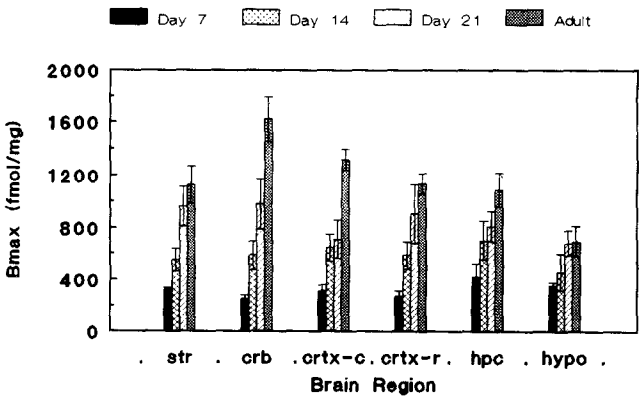

FIG. 2. Developmental differences in cannabinoid receptor binding capacity in various regions of the rat brain. Samples were dissected from Wistar rats of indicated age, and stored at −80°C until they were assayed. Membranes were prepared from individual samples and cannabinoid receptor binding activity was assessed in each sample. Binding maxima values were analyzed by ANOVA followed by Tukey's posthoc test and were found to be significant at the 0.05 level for the following comparisons: Striatum (str): D7 versus Adult; Cerebellum (crb): D7 versus Adult; D14 versus Adult; Cortex-caudal (ctx-c): D7 versus Adult; D14 versus Adult; D21 versus. Adult; Cortex-rostral (ctx-r): D7 versus D21, Adult; D14 versus Adult.

RESULTS

In previous studies, our laboratory used only Sprague-Dawley rats. However, the longevity of the Hooded Hybrid and the fact that in other laboratories different strains of rats are used, it became necessary to compare cannabinoid receptor binding between strains of young adult (60 day) male Sprague-Dawley, Wistar, and Hooded Hybrid (Fig. 1). Binding capacity for [^{3}H]CP-55940 was not significantly different between strains of rat: the fraction of [^{3}H]CP-55940 that was bound was the same, and the fraction of total binding that was specific as defined by displacement with 0.1 μM desacetyllevonantradol was the same. As shown by the competitive displacement curves, desacetyllevonantradol exhibited the same affinity for each rat strain. This was an important determination to make because desacetyllevonantradol is used as the agonist to define specific binding to the cannabinoid receptor and it is clear from these curves that maximal displacement was attained at 0.1 nM for each strain of rat. Although a pharmacological profile was not obtained for a series of agonists across strains, it might be assumed that factors that influence agonist binding affinity (e.g., receptor : G protein stoichiometry, cellular guanine nucleotide content) would exert the same influence on all cannabinoid agonists.

TABLE 1

CANNABINOID RECEPTOR BINDING IN WHOLE BRAINS FROM POSTNATAL DEVELOPING RATS

Age	B_{max} (fmol/mg)	K_d for [^{3}H]CP-55940 (nM)
Day 0	313 ( ), 306 ( )	0.5 ( ), 0.3 ( )
Day 7	640 ± 61	0.70 ± 0.18
Day 14	670 ± 24	0.43 ± 0.13
Day 21	970 ± 49	0.55 ± 0.25
Adult	1470 ± 22	0.55 ± 0.33

Data for PND 7 to adult are mean ± SEM from $n = 4$ whole brains from individual male or female Wistar rats. PND 0 rat brains were pooled brain samples from rats of either male or female gender, and the results are reported as a single number from pooled tissue. The adult data are from male rats only. ANOVA analysis had indicated that there were no differences between males and female whole brain B_{max} or K_d data; therefore, the data for the two genders were combined for each age group. No significant differences were found by ANOVA at the 0.05 level of significance for K_d values. All values of B_{max} differed from each other value at the 0.05 level of significance, with the exception of PND 7 and PND 14.

The developmental pattern of cannabinoid receptor binding in the Wistar rat is shown in Table 1. During postnatal development, a five-fold increase in cannabinoid receptor numbers appears in the brain. No difference in affinity was evident over the postnatal developmental period. The data were also analyzed for gender differences in either B_{max} or K_d values for [^{3}H]CP-55940. Gender was not a significant influence on either B_{max} or K_d.

In order to more fully appreciate the developmental pattern observed regionally in the brain, specific areas were examined from Wistar rat brains at 7-day intervals. Figure 2 shows the binding capacity in various brain regions. When the combined data from all regions were examined by ANOVA, a statistically significant increase was evident at each age increment. Analysis of the data for individual regions gave some insight regarding the timing of incremental increases in cannabinoid receptor binding. Within the striatum, a statistically significant increase in binding occurred between PND 14 and PND 21, during which time cannabinoid receptor capacity nearly doubled. Within the cerebellum, cannabinoid receptor binding capacity nearly doubled in every 7-day interval. Cannabinoid receptor binding in the cortex doubled between PND 7 and PND 14, and then increased in smaller increments to the final adult level. Within the hippocampus, there were small incremental increases until the final adult level was reached. The

TABLE 2

CANNABINOID RECEPTOR BINDING IN AGED HOODED HYBRID RATS

Rat Age (Month)	Cannabinoid Receptor Binding X ± SEM (fmol/mg)
3	97.0 ± 8.2
8	92.7 ± 10.5
12	95.3 ± 12
24	102 ± 6.3
32	100 ± 7.5

Data are presented as fmol [^{3}H]CP-55940/mg specifically bound at 0.1 nM [^{3}H]CP-55940. Data are means of n = 3 rats, and each was assayed in triplicate at 32 μg and at 45 μg membrane protein per assay, and the data were analyzed by ANOVA. Data were not different at the p = 0.05 level of signficance for the two different protein concentrations. The data obtained at the two different protein concentrations were pooled for presentation here. No significant differences were found between age groups.

hypothalamus was unique in being the only region in which cannabinoid receptor binding reached its maximal level at PND 21 rather than at adulthood.

Data were analyzed by ANOVA for possible gender differences in binding capacity at the various age groups and within various regions. When the data were examined by age as a group comprising all brain regions, it appeared that cannabinoid receptor binding was greater in males than in females for PND 14 and PND 21 age groups only. However, when these data were analyzed by region for these two age groups, no significant gender differences were found. Thus, no single individual region appeared responsible for contributing to an apparently larger cannabinoid receptor binding activity in male brains in these postnatal age groups.

The K_d values for binding of CP-55940 determined by homologous displacement equilibrium binding studies for each region at each age group ranged from 0.34 nM to 1.0 nM. Statistical analysis by ANOVA indicated no significant differences associated with gender within an age group, or between age groups for either gender. The data were further analyzed for differences between regions at different age groups but no significant differences in K_d values were noted.

The whole brain cannabinoid receptor binding capacity as a function of age in adult Hooded Hybrid rats between 3 and 32 months was examined (Table 2). Specific binding of [^{3}H]CP-55940 was determined at two different protein concentrations, within the linear range (32 μg/tube and 45 μg/tube) and found not to be different, so the data were combined for Table 2. Statistical analysis by ANOVA revealed no significant difference between cannabiniod recepter levels with age. Thus, no major increases or decreases in cannabinoid receptor binding is evident as a function of the normal aging process in these rats.

DISCUSSION

The processes of neuronal differentiation and cell migration are of major importance during the early postnatal period of brain development in the rat. This period is important for assessing consequences of human exposure to marihuana inasmuch as this period is comparible to the third trimester of human prenatal development. Here we found that during this period, significant increases in the numbers of cannabinoid receptors appeared regionally in the developing rat brain. Our studies can be compared with studies of Rodriguez de Fonseca et al. (27), which reported similar findings. In our studies, cannabinoid receptor binding continuously increased until the maximum adult level was reached at adult day 60. In Rodriguez de Fonseca's studies (27), binding also continuously increased until around PND 30, and then binding began to slightly decrease until adult binding maximum was reached. The K_d for CP-55940 in both studies were relatively similar.

Our study showed that cannabinoid receptors in the striatum nearly doubled between PND 7 and PND 14, and doubled again between PND 14 and PND 21. Striatal dopaminergic activity has been reported to be influenced by pre- and perinatal cannabinoid exposure during this period (25,26,28,29). Chronic treatment of pregnant and lactating mothers with crude marihuana extract (but not with Δ^9-THC alone) decreased the D_2 receptor binding capacity at PNDs 10 and 20, and reduced tyrosine hydroxylase activity between PNDs 20 and 40 (28,29). Using a similar protocol of maternal exposure to crude marihuana extract, Rodriguez de Fonseca and colleauges (25,26) observed that D_1 and D_2 receptor binding capacities decreased slightly between PND 15 and PND 20, and then increased dramatically between PNDs 30 and 40 (25). At PND 20, cannbinoid exposure was associated with a 20% smaller D_1 receptor binding capacity and about a 20% increase in the DOPAC/DA ratio (indicating increased dopaminergic release and metabolism) in females but a 20% increase in D_2 receptor binding capacity and a reduced tyrosine hydroxylase activity in males (25,26). Because these protocols exposed the offspring to cannabinoid drugs for extended pre- and postnatal periods, and given that Δ^9-THC has been found to accumulate following multiple dosing (22), it is unclear at which point during development the cannabinoid drugs were influencing these multiple events in the dopaminergic system. Moreover, Δ^9-THC not only inhibits food and water intake but can also inhibit oxytocin and prolactin in nursing dams (for discussion of these methodological issues, see ref. 20). It is thus imperative that studies of the developmental toxicity of the cannabinoid drugs include appropriate controls to distinguish primary drug effects from secondary effects of maternal and offspring toxicity. These problems aside, however, pharmacological studies have indicated that in adult rats, cannabinoid receptors are indeed present on neurons possessing D_1 receptors that can stimulate cyclic AMP production in the striatum and are likely to coexist with D_2 receptors that inhibit cyclic AMP production on these same neurons (4,13).

The present studies did not reveal a region-selective gender difference in the developmental pattern of cannabinoid receptors. However, Rodriguez de Fonseca et al. (27) found higher cannabiniod receptor binding in the forebrains of PND 2 females than males, and higher receptor binding in PND 5 males than females. Perhaps a more selective dissection and more discrete timing of intervals may discern variations attributable to gender effects.

Δ^9-THC has been reported to exert an influence on reproductive endocrine functions during late postnatal development. Δ^9-THC administered daily to female rats beginning as late as PND 22 was found to delay the day of vaginal opening by 2 days (12,30). Although Δ^9-THC administration in those studies had been discontinued at the day of vaginal opening, persistent endocrine dysfunction was evidenced by irregular

estrous cycles, reduction in the number of ova, and decreased serum LH and FSH levels (30). These findings suggest that cannabinoid receptors are present on neurons integral to neuroendocrine pathways that are undergoing developmental changes during this critical prepubertal period in the female rat.

Regional increases in cannabinoid receptors occurred in cerebral and cerebellar tissue over the postnatal period. The present study demonstrates that cannabinoid receptors are indeed present at birth, and thus the influence of prenatal exposure to cannabinoid compounds can directly alter the activity of cannabinoid receptor-bearing neurons at that early stage of development. As postnatal development progresses in the rat, the observation of large increases in cannabinoid receptors in multiple brain regions could be an indication of increased differentiation of neurons into cells possessing cannabinoid receptors. Alternatively, precursor cells already possessing cannabinoid receptors may undergo an induction of those receptors, or else expand the numbers of those receptors on their projections as they may migrate in the process of synapse formation.

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