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Quantitative changes in hippocampal structure following long-term exposure to Δ^9-tetrahydrocannabinol: possible mediation by glucocorticoid systems

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Although cannabinoids exert strong effects on brain function, there have been no extensive analyses of the long-term effects of cannabinoids on mammalian brain structure. Consequently, we conducted quantitative light and electron microscopic studies on the brains of rats treated chronically with Δ^9-tetrahydrocannabinol (THC) (5× weekly for 8 months — approximately 30% of the lifespan). In these studies, we found significant THC-induced changes in hippocampal structure: specifically, THC-treated animals exhibited decreased neuronal density and increased glial cell reactivity (i.e. an increase of cytoplasmic inclusions). In addition, we confirmed prior reports of THC-induced increases in adrenal-pituitary activity, since both adrenocorticotropic hormone (ACTH) and corticosterone were elevated substantially during an acute stress. However, the animals appeared to be only minimally affected behaviorally by the doses used (highest dose: 8 mg/kg) and no effects of THC were observed on several ultrastructural variables, including synaptic density. The observed hippocampal morphometric effects of chronic THC are similar to apparent glucocorticoid-dependent changes that previously have been found to develop in rat hippocampus during normal aging. Given that cannabinoids and steroids are similar in chemical structure in several respects, therefore, the present results seem to raise the possibility that chronic THC exposure may alter hippocampal anatomical structure by interactions with, or mimicry of, adrenal steroid activity.

INTRODUCTION

It has been recognized for some years that synaptic elements exhibit degeneration and 'turnover' in the central nervous system (CNS) of adult mammals[40]. In peripheral systems, trophic factors and neural stimulation have been shown to play an important role in neuronal maintenance as well as development. However, little is known about the factors which govern neuronal degeneration and regrowth in the mammalian CNS[9,30].

In recent years, interest also has begun to focus on a possible role for adrenal steroid hormones in regulating adult brain structure, particularly in relation to the brain aging process. That is, brain aging has often been found to be associated with declines in neuronal and/or synaptic density, and with increases in glial reactivity[2,3,11,19,45,48] (although not all structures exhibit clear-cut neuronal decline with age; cf. ref. 6). At least some aspects of this brain aging pattern appear to be glucocorticoid-dependent, since chronic adrenalectomy can retard the age-related decline in rat hippocampal neuronal density, and the increase in hippocampal astrocyte inclusions[17,18,21]. Other studies have shown that the chronic administration of high doses of corticosterone to adult/aging rats can

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reduce the density of those hippocampal neurons rich in corticosterone receptors[35], or can affect axon sprouting[7].

Δ^9-Tetrahydrocannabinol (THC), the major psychoactive constituent of marijuana[12], appears to interact with endogenous steroid systems in several ways. THC or marijuana increases the release of glucocorticoids in rats[8,23,33], possibly by interfering with negative feedback[8]. In addition, some[32,38], but not all[29], investigators have found that THC binds to estrogen receptors. Further, there is considerable similarity between the chemical structures of THC and some endogenous glucocorticoids[44]. Marijuana can also affect at least some endocrine processes in humans, although it is still unclear whether human adrenal corticoid levels are altered by cannabinoids[16].

Thus, it seems possible that chronic THC exposure might either mimic or increase endogenous adrenal steroid activity, and might thereby alter the anatomical structure of the hippocampus. Indirect support for the possibility that THC might alter brain structure derives from studies indicating that THC influences the electrical activity of the hippocampus[4,10,26] and other central systems[46], and that there may be residual behavioral effects following chronic exposure to cannabinoids[28,42].

Despite the extensive use of marijuana, however, almost nothing is known about the possible brain anatomic effects of long-term exposure to THC and related compounds. A recent abstract reported that oral administration of THC (20 mg/kg) to rats for 90 days resulted in reduced synaptic density in the hippocampus[36]. In several earlier studies (cf. refs. 13, 14), THC was administered to electrode-implanted monkeys for 6 months, but the analyses of brain structural effects relied on very small n's, and limited quantitative approaches. In addition, some of the changes reported (e.g. clumped vesicles, large intercellular spaces, etc.) could have been related to nonoptimal tissue fixation, since such changes also are seen with poor fixation. Further, the 6-month trial represented only approximately 2% of the animals' life span, which might not have been long enough to detect the gradual effects that could arise from interactions with steroid systems.

The scarcity of data on the brain structural effects of THC, therefore, suggests that quantitative analyses of the chronic effects of THC on hippocampal anatomy might provide important information both on the nature of factors that modulate adult brain structure and on the mechanisms of THC-induced behavioral change. Therefore, to test the possibility that chronic THC exposure acts in part analogously to chronic glucocorticoid exposure, we administered THC to relatively large groups of rats on a near-daily basis, for approximately 30% of their life span (8 months), and then conducted extensive quantitative light and electron microscopic analyses of a hippocampal region (CA1) known to be affected by chronic alterations of glucocorticoid levels[18,35]. In addition, we studied plasma concentrations of corticosterone and adrenocorticotropic hormone (ACTH) in chronic THC-treated animals.

MATERIALS AND METHODS

Subjects and animal maintenance

Subjects were 57 Fischer 344 male rats, 3–4 months of age at the start of the study. The animals were housed doubly in an animal facility containing a HEPA (high efficiency particulate; absolute) filter/laminar air flow system which filtered all particles larger than 0.3 μm from the air. Although the colony is not fully sterile, it is isolated from other rats in the vivarium and the air filter removes airborne bacteria. Administration of agents was carried out by an investigator wearing a clean lab coat and surgical mask and gloves, using sterile needles.

Two experiments on chronic THC treatment were conducted in the present series. In experiment 1, 3 groups of animals (n = 7 per group) were administered either THC (8 mg/kg), vehicle, or saline, 5× weekly (see below) for 4 months, and plasma samples were taken from the animals on two occasions. In experiment 2, 3 groups of animals (n = 12 per group) were administered either 4 mg/kg of THC, 8–10 mg/kg of THC, or vehicle, 5× weekly for 8 months. Behavioral and morphometric analyses were carried out on these animals. Administration was by subcutaneous injection. A 5× weekly regimen rather than a 7× weekly regimen was used in the present studies because it allowed the animals 2 days per week to recover partially from undesirable effects of the treatments (e.g. weight loss, irritability); these recovery periods appeared to contribute importantly to the

health of the animals during prolonged treatments.

THC preparation

The basic protocols used for preparation of THC, which is highly lipophilic, relied on a detergent (Pluronic F68-Wyandotte Chemicals) as the vehicle in which to suspend THC[37]. The possibility of pathological consequences of chronic treatment with the vehicle itself was also examined during these studies since, although Pluronic has been shown to be nontoxic in acute studies[37], it has not been used as a vehicle in studies lasting several months. We therefore conducted separate tests on blood chemistry and plasma endocrine factors in animals treated for 4 months (5× weekly) with the Pluronic vehicle alone, with the high dose of THC in the Pluronic vehicle, or with saline alone (experiment 1). Specific procedures for preparing THC in the Pluronic vehicle (cf. ref. 37) are given below:

(1) A small volume (1.0 ml) of the stock THC solution (100 mg/ml absolute ethanol) was added to 1 ml of Pluronic F68 Solution (150 mg/ml absolute ethanol) and 2 ml of saline in a 10-ml glass beaker. This solution was then thoroughly mixed with a small Teflon magnetic stir bar.

(2) The ethanol was then evaporated by a gentle stream of pure nitrogen gas for approximately 20 min.

(3) After removing the stir bar, the solution was then subjected to 15 s of ultrasound (W350 Sonifier, Branson) and the beaker was scraped with a glass rod to remove any remaining solution from the beaker walls. Following sonification, the solution does not stick to the glass rod. Another brief (15 s) administration of ultrasound was then given.

(4) Room temperature saline was then added slowly to the Pluronic–THC mixture, as the mixture was continuously stirred, to reach the appropriate concentrations for administration in the chronic paradigms. The residue sticking to the side of the breaker was suspended by warming the beaker in warm tap water.

Experiment 1: THC administration and blood sampling procedures

Animals in Expt. 1 were injected subcutaneously, 5× weekly with either 0.5 ml saline alone, the Pluronic F68 vehicle alone in 0.5 ml of saline, or 8 mg/kg THC in Pluronic in 0.5 ml saline, for a period of 4 months. Blood samples were collected from these animals after approximately 2.5 months and after 4 months of treatment, by tail sampling while the animals were held in a restraining cage. During sampling a small cut was made in the tail vein, a local anesthetic was applied to the cut, and blood was 'milked' into a heparinized vial. On each occasion, blood was collected at both 2 and 12 min after the animal was first handled and placed in the restraining cage. After the 4-month test, the animals were sacrificed by decapitation and a complete blood chemistry test (SMAC) was performed on the plasma.

Corticosterone assay (Expt. 1)

Corticosterone was analyzed in plasma from Expt. 1 animals with a specific double-antibody radioimmunoassay (RIA) using a gamma-labeled tracer. A volume of 25 μl plasma was extracted with methylene chloride and 100 μl of the organic layer was transferred to glass tubes, dried down and reconstituted with gelatin–phosphosaline buffer. Iodinated corticosterone and diluted antiserum (Cambridge Medical Diagnostics) were added for an overnight incubation at 4 °C. On the next day, anti-rabbit γ-globulin (ARGG) was added along with 20% polyethylene glycol (PEG). The mixture was immediately centrifuged at 4 °C, the supernatant was aspirated and discarded, and the pellets (antibody-bound hormone fraction) were counted. Results were calculated automatically by a log–logit plot of the standard responses. The readable range of the assay was 2–1000 ng/ml (cf. ref. 39 for methods). A rat plasma pool that reads approximately 100 ng/ml has an intra-assay variation of 12.5% and an inter-assay variation of 13.1%.

ACTH assay (Expt. 1)

ACTH was measured with an antiserum obtained from IgG, Nashville, TN. This is a highly sensitive and specific antibody that permits analysis of 25-μl sample volumes with no extraction or purification of the hormone. Specimen, tracer (Radioassay Systems Labs., Carson, CA) and diluted antiserum were incubated together at 4 °C for 24 h followed by addition of ARGG and a second 24-h incubation. Polyethylene glycol was then added to each tube, followed by centrifugation and counting. The assay requires a

buffer of phosphate–EDTA without saline but with Triton X-100 and Trasylol. A plasma pool in the range of 125 pg/ml has a very good intra-assay variation of 6.8%; the inter-assay variation was 14.2% (cf. ref. 39 for methods).

Experiment 2: THC administration and behavioral/morphometric procedures

After determining that the blood chemistry studies showed no indications of THC-induced renal or other major pathology, and no effects of the Pluronic vehicle alone, long-term studies of the effects of THC on brain structure were initiated. These studies included a Pluronic vehicle-alone group (n = 12), a low dose of THC (4 mg/kg) in Pluronic vehicle group (n = 12), and a high dose of THC (10 mg/kg, lowered to 8 mg/kg during the first month) in Pluronic vehicle group (n = 12). Administration of these agents was subcutaneously, in 0.5 ml saline, 5× weekly, for a period of 8 months.

Behavioral tests (Expt. 2)

On 2 occasions during the 8-month treatment regimen, animals were examined for open-field behavior, and at 2 other points, they were tested for learning in a simple 1-trial active avoidance paradigm (consisting of either spaced or massed trials). Two weeks after the cessation of the THC treatments all animals were tested for acquisition and retention of maze reversal learning.

Open field test. Animals from Expt. 2 were placed in a square plexiglass activity cage (35 × 35 × 35 cm). An observer with a counter and stopwatch quantified the number of rearings (front feet off the floor) as an index of exploratory behavior, and the number of seconds spent in walking or grooming behavior. Two 5-min activity tests were given to each animal after 2 and after 4 months of the treatment.

Active avoidance task. Animals from Expt. 2 were placed in a small square box that contained a grid floor and a safe ledge. Five s after being placed in the apparatus, a mild foot shock (0.35 mA) was applied via the grid floor. The latency of animals to respond was used as an index of motivation-arousal, as well as an index of some memory retrieval components. Animals were tested on this task on two occasions to assess levels of performance. On the first training session, (after 6 months of treatment) a single training trial was given, and 6 days following this training trial, 4 retention/training trials were given, each separated by 24 h (spaced trials). On the second avoidance session (after 8 months of treatment), 3 additional retention trials were given, each separated by 1 min (massed trials).

Maze reversal task. Animals in Expt. 2 were tested on this task after they had been removed from drug administration for 14 days, in order to assess residual, rather than acute, effects of THC on learning performance. Rats were trained to choose one arm of a T-maze, by first placing an animal in the start box, and, after 5 s, administering scrambled electric shock (0.35 mA) to the floor grids of the maze until the animal ran into the correct arm. This shock level is just above threshold for consistently motivating the animals to leave the start box. Animals were given 2 of these initial training footshock (FS) trials on the first day, and thereafter were given one FS trial per day for one week. The correct (safe) arm was then reversed (e.g. the right, instead of the left, side was now made correct), and 3 training trials (acquisition trials) were then given 1 min apart to train animals to the reversed paradigm. Acquisition data were analyzed only for the 2nd and 3rd trials since the 1st trial was always incorrect. The animals were then tested for retention of the reversal 24 h later, on 3 retention trials, 1 min apart. Five min following completion of the retention trials, the animals were again reversed to the other side, using the same training and testing protocols.

Fixation and tissue preparation for morphometric analysis (Expt. 2)

Similar procedures for intracardial perfusion and tissue preparation for electron microscopy were used for all groups. Animals from Expt. 2 were deeply anesthetized with pentobarbital, and surgery was conducted only after deep anesthesia was attained (e.g. absence of eye-blink reflex and absence of responses to deep tail pressure). The abdomen was then opened and the descending abdominal aorta clamped with a hemostat, to direct fixative solution toward the upper body. The rib cage and diaphragm were cut, and the right auricle was cut to allow drainage of the fixative solution, while the left ventricle was catheterized and perfused at a rate of 30–40 ml/min. The entire surgical procedure required approximately

35–45 s, and only 10–15 s elapsed between the time the diaphragm was cut and the solution entered the heart.

A 2% glutaraldehyde and 2% paraformaldehyde fixative solution in 0.15-M sodium cacodylate buffer containing 1.0 mM Ca and 1.0 mM Mg was used as the fixative solution. Perfusion of the fixative solution was maintained for 10–15 min, after which the brain was removed and stored in fixative overnight. Criteria for acceptable fixation quality were normal staining and appearance of cell nuclei, unswollen mitochondria, sharp membranes with little intercellular space, well-dispersed synaptic vesicles within the synaptic terminals, and normal appearing myelin. One animal from the vehicle group, two from the low THC (4 mg/kg) group, and one from the high THC (8–10 mg/kg) group were excluded because of failure to meet these criteria.

Block trimming and sample selection

The hippocampus was dissected free and transverse, 250-μm-thick slices were cut from the middle of the septotemporal axis of the structure, using a McIlwain tissue chopper. These were compared under a dissecting microscope, and the slice which conformed most closely to the cell layer patterns of the region we were attempting to study was selected for analysis. The hippocampal slice was then dehydrated, embedded in Araldite 502 and stained according to standard EM procedures. The embedded tissue was subsequently trimmed perpendicular to the medial-lateral axis of the slice, and parallel to the CA1 apical dendrites, on a line through CA1 and the dentate gyrus at a consistent point, based on the shapes of the ventral and dorsal leaves of the granule cell layer of the dentate gyrus. Landmarks of these cell layers were then used to cut semi-thin (1 μm) and thin sections (70 nm; silver-gray interference color, stained with uranyl acetate and lead citrate) from the block.

A series of electron micrographs was taken in the apical dendritic region of CA1 (stratum radiatum), from the area of densest Schaffer collateral-commissural fiber termination[43], at a point midway between the pyramidal cell somal layer (stratum pyramidale) and the fibers of the perforant path (stratum moleculare). A sequence of 20 micrographs per subject was shot in evenly spaced frames across the full width of the section at this depth, at an initial magnification of ×12,000, and photographically enlarged to ×32,500 for analysis. Another 5 micrographs (final magnification: ×14,000) were shot of the largest glial cell processes in the field.

Quantitative methods: light microscopy

For each animal, the nucleoli of pyramidal cells were counted blind (from coded slides) on 5–9 randomly selected, semi-thin sections from the stratum pyramidale. The length of this cell layer on each section was also measured with a calibrated eyepiece. Total nucleoli were then divided by total cell layer width, and data were expressed as neurons/100 μm stratum pyramidale length. Only neurons that were clearly within the pyramidal somal layer were counted.

Nucleolar diameters were also measured in all cells on 1 or 2 sections for each animal, in order to ensure that nucleolar diameter was not changed by a treatment (since large diameter particles are more likely to be counted — see below).

The length of stratum radiatum (e.g. length of apical dendrites) was also measured in all animals, in order to determine whether overall structural growth, which can reduce cell density, had occurred.

Quantitative methods: electron microscopy

Two primary methods were employed to quantify the ultrastructural variables of interest in these studies: (1) all synaptic active zones on each micrograph were counted and measured for length on an image digitizing tablet, and a numerical synaptic density was then derived for each type of synapse, using a stereological formula that corrected for the length of the active zone of that synaptic type; and (2) the relative volume occupied by each element of the neuropil was estimated on each micrograph using point counting stereological approaches. All analyses were performed blind on coded micrographs.

Synaptic active zones were defined as dark paramembranous densities which were associated with at least two synaptic vesicles. If an active zone crossed the edge of a micrograph it was counted as an 'edge synapse'. The total number of 'edge synapses' was divided by 2 before being included in the count, to prevent overestimation of synaptic density due to counting synaptic active zones twice (i.e. edge syn-

apses theoretically appear in more than one micrograph).

Each active zone length was measured using a digitizing pad and morphometric software and hardware (Bioquant), and was simultaneously coded for the type of terminal with which it was associated. Terminals were categorized as either simple axospinous, perforated, axodendritic, and multiple terminal or spine. Simple axospinous terminals were defined as those which terminated on a single dendritic spine, containing a single postsynaptic density; axodendritic terminals were defined as those which terminated directly on dendritic shafts; perforated terminals were defined as those characterized by adjacent, apparently perforated densities (which generally reflect disk-shaped synaptic contacts, containing a circular hole, or perforation, in the middle[31]). Multiple-spine synapses were defined as synapses with two spines in contact with one terminal, and multiple-terminal synapses were defined as those with two terminals contacting one spine. Mean active zone length was calculated separately for each of these categories for each animal.

The number of synapses per unit volume (N_v) can be estimated from the number of synapses per area (N_a) of micrograph, as long as stereological corrections for 3-dimensional variables are considered. That is, a single synapse that is significantly larger than a fraction of the section thickness will theoretically often be present in two or more sections, and will therefore be counted as two or more synapses. To correct for this overestimation, the average length of synapses in micrographs (d) was incorporated with section thickness (t) into the denominator of a formula for converting N_a to N_v[15,24,47].

$$N_v = \frac{N_a}{d + t}$$

It should be noted that the mean trace length of synapses on the section plane is used directly in corrections for quantifying synapses (unlike the term $(4/\pi)\cdot(d)$, which is used in formulae for spheres), because the shape of a synaptic density approximates that of a circular disk, and disks are projected differently from spherical shapes on two-dimensional section planes (cf. discussions in refs. 15, 24).

Additional corrections for polydispersion of the sizes of the particles of interest (e.g. to correct for particles of non-uniform size), or for missing or invisible caps have sometimes been applied in calculations of N_v (cf. refs. 24, 47). However, corrections for polydispersion are not thought to be necessary if the coefficient of variation (CV = (S.D./mean) ×100) is

TABLE I

SMAC tests performed on plasma from 3 groups of animals following 4 months of 5× weekly administration of saline, Pluronic vehicle alone, or the high dose of THC (8 mg/ml) in Pluronic

There were no differences between saline and vehicle, but chronic THC induced a rise in plasma glucose and cholesterol (and possibly creatinine and uric acid), and a decrease in total protein and albumin. However, no signs of major pathology were evident. LDH, lactic dehydrogenase; SGOT, serum glutamic-oxaloacetic transaminase.

	Saline	*Vehicle*	*THC*	*P value*
Na (mmol/l)	140.28 ± 1.73	137.28 ± 1.08	140.0 ± 1.17	n.s.
Cl (mmol/l)	91.00 ± 0.57	82.85 ± 7.08	91.71 ± 0.60	n.s.
Ca (mg/dl)	10.40 ± 0.09	10.27 ± 0.20	10.00 ± 0.21	n.s.
PO_4 (mg/dl)	6.17 ± 0.25	5.57 ± 0.19	5.27 ± 0.49	n.s.
BUN (mg/dl)	18.85 ± 0.88	19.42 ± 1.11	18.14 ± 1.08	n.s.
Creatinine (mg/dl)	0.62 ± 0.026	0.58 ± 0.023	0.68 ± 0.03	0.083
Uric acid (mg/dl)	1.48 ± 0.11	1.58 ± 0.15	2.02 ± 0.20	0.065
Glucose (mg/dl)	175.14 ± 7.51	177.28 ± 4.94	238.85 ± 20.04	0.003*
Cholesterol (mg/dl)	60.42 ± 1.95	59.28 ± 1.25	69.14 ± 2.77	0.007*
Iron (mg/dl)	168.71 ± 5.29	161.14 ± 5.03	173.71 ± 8.25	n.s.
Protein (g/dl)	6.50 ± 0.09	6.48 ± 0.08	5.80 ± 0.13	0.001*
Albumin (g/dl)	3.57 ± 0.04	3.57 ± 0.03	3.11 ± 0.07	0.001*
Alkaline phosphate (U/l)	246.28 ± 10.16	230.42 ± 9.01	236.00 ± 10.65	n.s.
LDH (U/l)	1561.00 ± 158.99	1645.28 ± 233.62	1682.42 ± 278.94	n.s.
SGOT (U/l)	280.85 ± 21.77	305.85 ± 39.59	252.14 ± 49.89	n.s.

less than 20%[47]. Since we separated each individual synaptic category (which substantially reduces variability in trace length), the CVs for individual categories were well below 20%. The separate synaptic categories were therefore treated as monodispersed populations in our analyses. The total N_v was determined by adding the individual category N_vs together, rather than by calculating N_v for overall N_a and mean profile. Because we have found that corrections for tissue shrinkage affect the categories relatively similarly, and by less than 1–2%, such corrections were not used in these analyses.

The estimates of relative volume for each element of the neuropil were determined using a 10 × 12 grid overlay on each micrograph. The area immediately under each intersection of the grid was examined and categorized for the element of neuropil it contained. Neuropil elements were classified as terminal axon, dendritic spine, dendritic shaft, glial process, and unknown. As each point was classified, the appropriate code was entered into a computer program developed in this laboratory and the mean and S.E.M. was calculated for each category of each micrograph, along with cumulative totals for the subject.

The volume fraction of each element in the neuropil was determined for each subject from the fraction of test points (P_p) that were classified as a given element of neuropil. This point counting approach relies on the principle that the fraction of test points P_p that cover a given element on a 2-dimensional representation is an unbiased estimator of the fractional volume (V_v) of that element[47], i.e. $P_p = V_v$.

Over 2500 test points per subject were used, yielding a theoretical error ranging from 2.5% for the more common elements to 7.0% for the rarer elements.

Mean volumes (v) per single terminal or spine elements were also calculated, by combining the above measures; that is, by dividing the volume fractions (V_v) by the appropriate N_v for each element

$$v = \frac{V_v}{N_v}$$

Statistics

Data for all synapses per animal were averaged to yield a single value on each variable for each animal. Morphometric variables were analyzed by one-way analyses of variance (ANOVA), and body weight values were analyzed by a one-way ANOVA for repeated measures. A probability level of $P < 0.05$ was used for determining statistical significance.

RESULTS

Blood chemistry studies (Expt. 1)

The data for the SMAC are shown in Table I. There were no differences between the saline and Pluronic vehicle-alone groups on any variable. However, the THC group showed significant elevations in glucose, cholesterol, and perhaps creatinine and uric acid. Other investigators have also reported that THC alters blood glucose levels[33]. THC also induced significant decreases in total protein and albumin.

The THC-treated rats also exhibited significant ($P < 0.01$) elevations in plasma ACTH and corticosterone, particularly after 12 min of restraint stress (Fig. 1). The data shown are for the 4-month test, but simi-

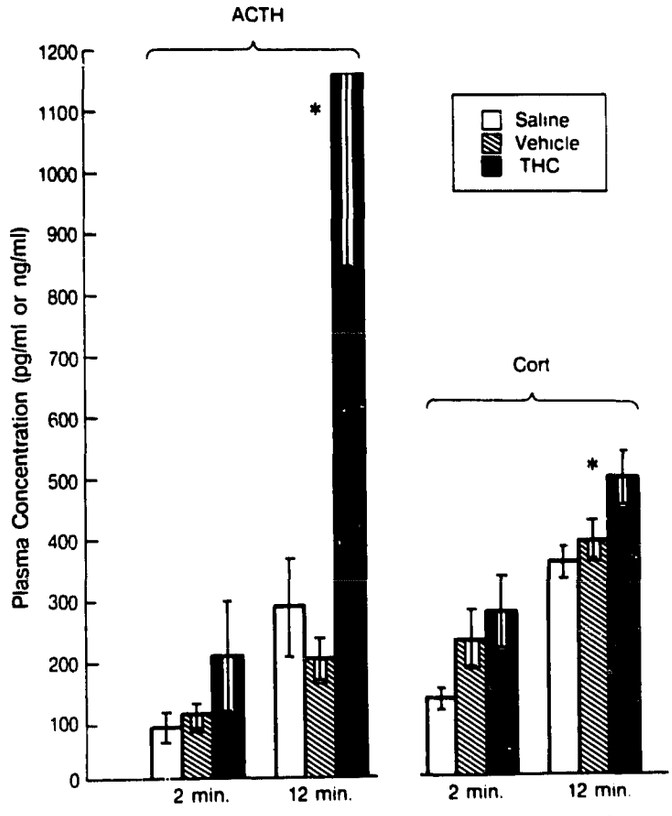

Fig. 1. Plasma concentrations of ACTH (pg/ml) and corticosterone (Cort) (ng/ml) in groups of young rats treated 5× weekly for 4 months with saline alone, Pluronic vehicle alone or THC (10 mg/kg) in Pluronic. Blood was taken from the tail vein of each animal after placement in a restraint cage, at both 2 and 12 min after first handling (means ± S.E.M.). Asterisks show time points at which the THC group was found to differ significantly from the other groups.

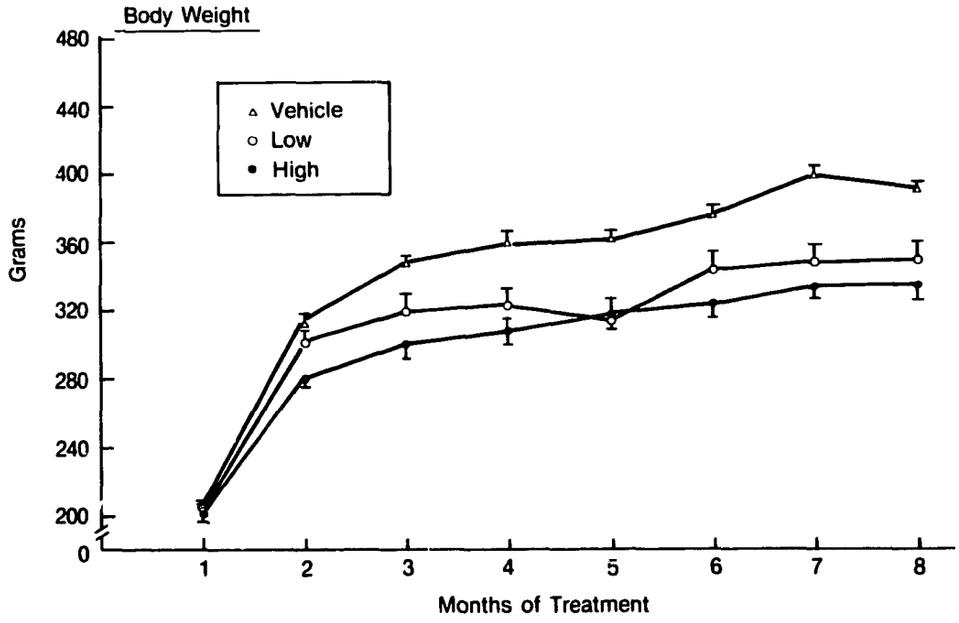

Fig. 2. Body weights of 3 groups of rats treated 5× weekly from 4–12 months of age with either Pluronic vehicle alone, with a low dose of THC (4 mg/kg) in Pluronic, or with a high dose of THC (8 mg/kg) in Pluronic. Statistically significant but moderate effects of both doses of THC on body weight were found (means ± S.E.M.).

lar and statistically significant patterns also were found on the 2-month test. Thus, the results confirm earlier reports of chronic THC-induced increases in adrenal activity, and also provide further direct evi-

TABLE II

Behavioral tests conducted on 3 groups of animals during or after an 8-month regimen of 5× weekly s.c. administration of either vehicle alone, a low dose of THC (4 mg/kg), or a high dose of THC (8 mg/kg)

Data are shown from the first 2 trials of an open-field test, from the first 2 retention trials of each of two training sessions in a simple active avoidance task, and from the acquisition and reversal phases of two reversal tests conducted 2 weeks after the end of the 8 mo-THC regimen. Only the first open field test and the first retention trial in the active avoidance task showed any effect of THC, indicating that the rats performed well under the influence of even the 'high' dose, and no obvious residual effects were present.

	Vehicle	*Low*	*High*	*P value*
A. Open field activity				
Rearing/5 min				
Test 1	18.25 ± 6.81	15.66 ± 1.38	7.25 ± 0.97	0.001*
Test 2	8.75 ± 0.98	12.33 ± 3.83	6.16 ± 0.66	n.s.
Seconds of walking/5 min				
Test 1	197.91 ± 9.72	148.66 ± 8.92	115.33 ± 12.49	0.001*
Test 2	104.16 ± 10.71	102.50 ± 11.64	140.58 ± 15.03	n.s.
B. Active avoidance (latency to respond)				
6 Months (spaced)				
Trial 1	10.75 ± 0.98	9.25 ± 0.52	18.91 ± 3.34	0.004*
Trial 2	9.16 ± 1.18	6.58 ± 0.29	8.41 ± 1.27	n.s.
8 Months (massed)				
Trial 1	8.08 ± 0.98	8.58 ± 1.37	7.58 ± 1.62	n.s.
Trial 2	6.00 ± 0.49	4.92 ± 0.89	5.42 ± 0.63	n.s.
C. T-maze reversal (latency to correct choice)				
Acquisition (mean of 2 trials)				
First reversal	12.87 ± 1.2	15.77 ± 1.8	14.27 ± 1.7	n.s.
Second reversal	13.16 ± 1.9	11.60 ± 1.6	12.00 ± 2.5	n.s.
Retention (mean of 3 trials)				
First reversal	11.21 ± 0.9	12.27 ± 1.2	9.96 ± 0.7	n.s.
Second reversal	10.81 ± 1.4	8.92 ± 0.9	8.73 ± 0.9	n.s.

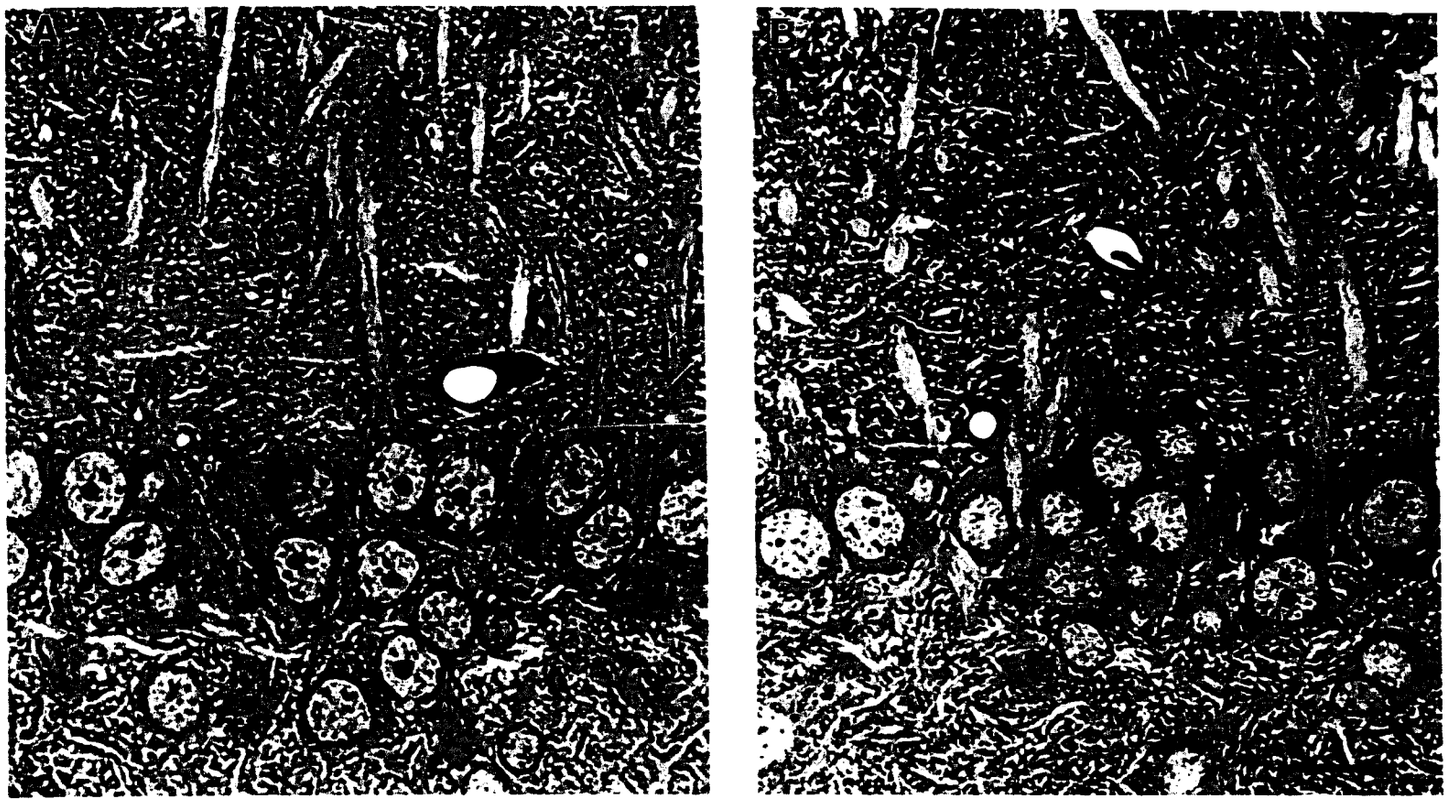

Fig. 3. Representative examples of pyramidal cell somata in stratum pyramidale of field CA1 of the hippocampus. A: semi-thin (1-μm-thick) section from a vehicle control after 8 months of treatment. B: semi-thin section from the same region of a high dose THC animal after 8 months of treatment. Qualitatively visible cell depletion could be seen in stratum pyramidale of a substantial number of the high dose animals. Bar = 20 μm.

dence that the effect is due to elevated ACTH.

Long-term morphometric and behavioral study (Expt. 2)

Both the low (4 mg/kg) and high (initially, 10 mg/kg) doses of THC caused slight initial reductions in body weight during chronic treatment (Fig. 2). High dose animals also appeared to find the treatment aversive (cf. also ref. 33). Consequently, the high dose was lowered to 8 mg/kg during the first month, and maintained at this level for the duration of the study. High dose animals still appeared irritable (at 8 mg/kg) although this behavior decreased considerably during the first several months of treatment. Food and water intake were assessed on 4 occasions during the 8-month protocol. Water intake was found to be significantly reduced in both groups of THC animals, but the reduction of food intake was not quite statistically significant. Weight loss also was moderate, and did not exhibit a clear dose dependence (Fig. 2). Therefore, the animals appeared to tolerate these doses reasonably well.

Behavior

Aspects of the behavioral data are summarized in Table II. As can be seen, the high dose of THC

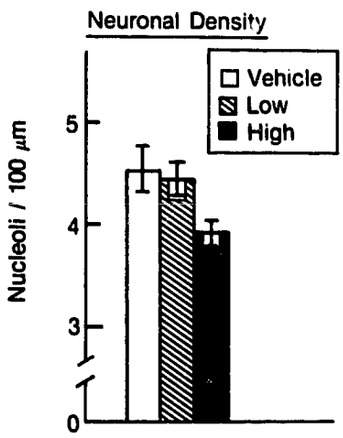

Fig. 4. Quantitative analysis of neuronal density in stratum pyramidale of the vehicle, low dose of THC (low) and high dose of THC (high) groups after 8 months of 5× weekly s.c. administration. Cell nucleoli were counted blind and corrected for length of the stratum pyramidale (means ± S.E.M.). A significant quantitative decrease was found, consistent with the visible cell depletion observed in some high dose animals (e.g. Fig. 3).

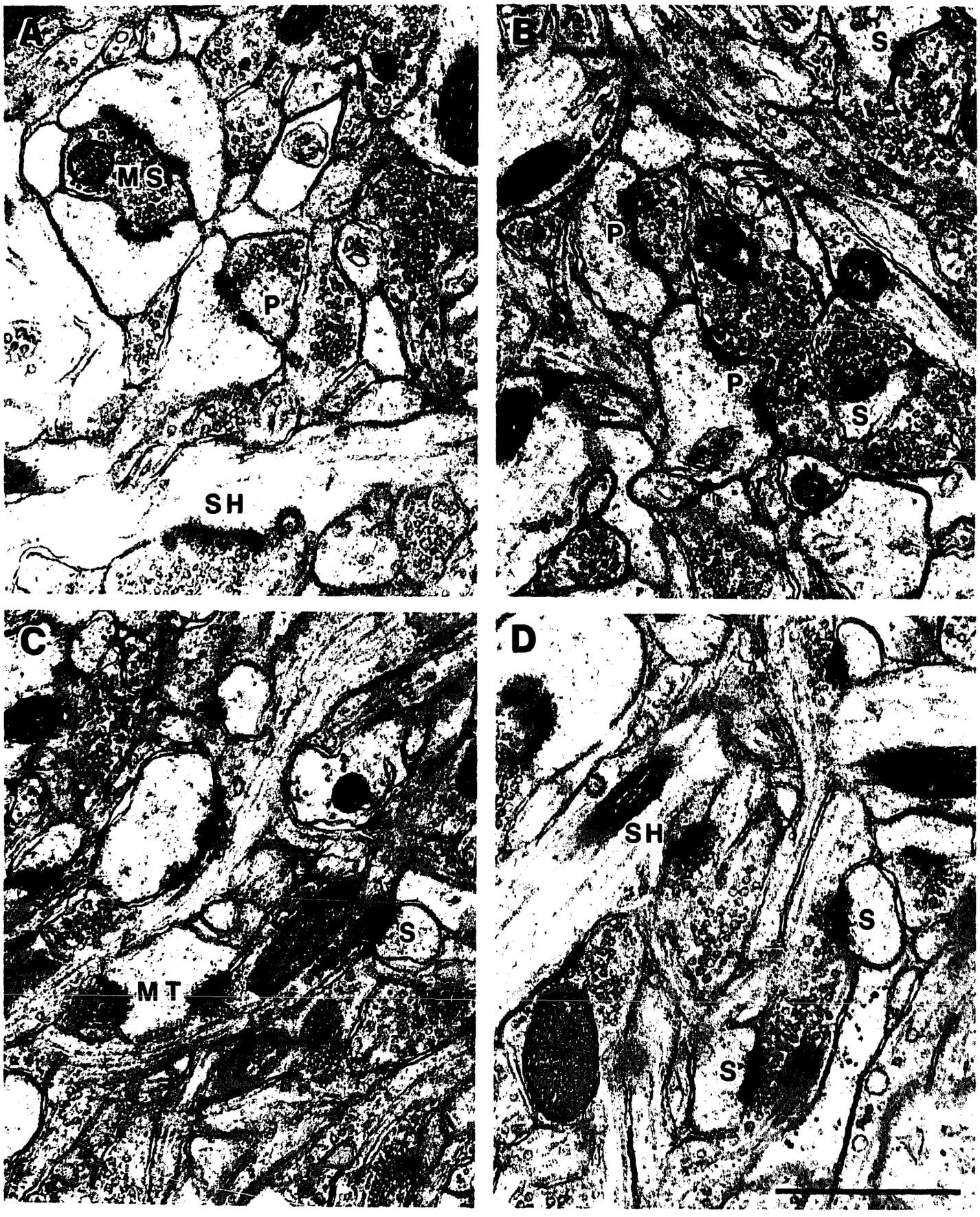

Fig. 5. Examples of the types of synapses found in stratum radiatum of field CA1. Several examples of simple axospinous contacts (1 terminal, 1 spine) (S) are seen in panels B–D. Perforated synapses (P) are seen in panels A and B. Axodendritic synaptic contacts (terminal on a dendritic shaft (SH)) are seen in panels A and D. Multiple synapses in which more than 1 spine (MS) is in contact with a single terminal, or in which more than 1 terminal (MT) contacts a single spine are seen in panels A and C. Bar = 1 μm.

exerted moderate effects on open-field activity and active avoidance training, but only on the first trial of each paradigm. After the first retention trial, the THC animals quickly matched the performance of the controls on active avoidance, and were no different from controls on the 1st trial of a subsequent active avoidance test (massed trials) at the end of 8 months. Similarly, the THC animals were no different from controls in open field activity after the first trial (although habituation affected scores of the controls after the first session).

No differences were found among groups in acquisition or performance of the T-maze reversal (Table II). Latency data only are shown, but similar patterns were seen for choice behavior.

These very moderate effects of THC on behavioral performance, even when rats were tested shortly after THC administration, suggest that 8 mg/kg s.c. was not an effectively high dose for rats, at least under chronic conditions in which metabolism and other processes may be able to adapt.

Morphometric results

After the 8-month THC treatment regimen, and the behavioral tests, animals in Expt. 2 were anesthetized and perfused intracardially with the aldehyde solution, and their brains were prepared for EM and trimmed, as described in Methods.

Fig. 3 shows examples of the hippocampal CA1 pyramidal cell somal layer (stratum pyramidale) in the semithin sections in which the light microscopic analyses were conducted. Fig. 4 shows that a significant decrease ($P < 0.05$) in pyramidal cell density was found in the high-dose THC animals.The effect was pronounced in some high-dose animals, and could be seen readily in several animals, even without quantitative analysis (e.g. Fig. 3). However, in other high-dose animals, the effect was mild and counts were within the range of the control group. Future studies in which plasma hormones and THC are measured in the same animals in which brain structure is assessed, and correlated with the brain changes, may help to clarify the bases of these individual differences in the brain response to THC administration.

Quantitative EM analyses were carried out on micrographs from thin sections from the same tissue blocks. Fig. 5 illustrates the appearance of the material, and the types of synapses that were categorized and measured. In each animal 200–300 synapses were measured on micrographs shot according to a programmed sequence, across a strictly defined region of stratum radiatum, from at least 2 thin sections.

Fig. 6 illustrates the mean lengths of the paramembranous density (active zone) for each type of synapse, in each treatment group. Fig. 7 illustrates the percent of total synapses that fell in each category and Fig. 8 shows the synaptic density (N_v) for each type of synapse (as corrected for mean active zone length), in each synaptic category. (Multiple terminal synapses were only rarely found, and were not included in the analyses.)

As can be seen in Figs. 6–8, no major changes in ultrastructure of the neuropil were found in THC-treated animals, despite the decrease in neuronal density (Fig. 4). However, a tendency for mean lengths to be larger was seen in several categories for the high-dose group (Fig. 6). In addition, a tendency toward more multiple-spine synapses was seen in THC-treated animals (Figs. 7, 8).

Although THC treatment did not substantially affect synaptic density, it did alter EM measures of astrocyte reactivity. That is, as shown in Fig. 9, dark, membranous inclusions were often present in hippo-

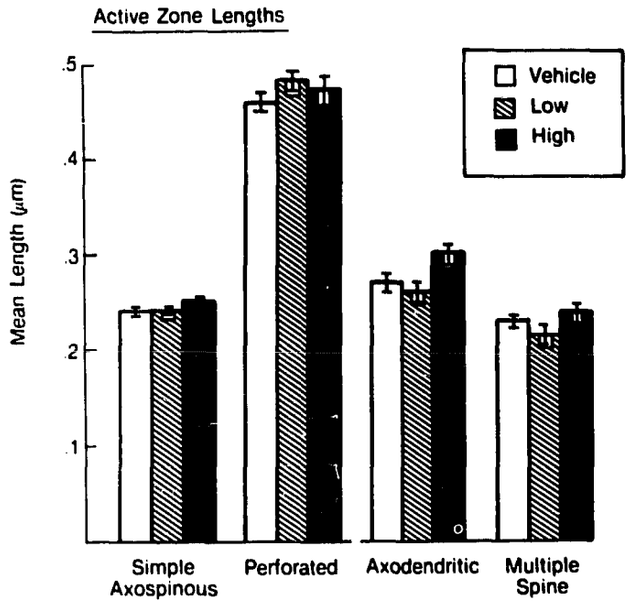

Fig. 6. Mean lengths of the active zones (paramembranous densities) of each of the major categories of synaptic contact shown in Fig. 5. No significant differences were found between the 3 groups after 8 months of treatment with vehicle alone, low THC or high THC. However, there appeared to be a consistent tendency for high-dose THC animals to exhibit longer synaptic contacts in several categories (means ± S.E.M.).

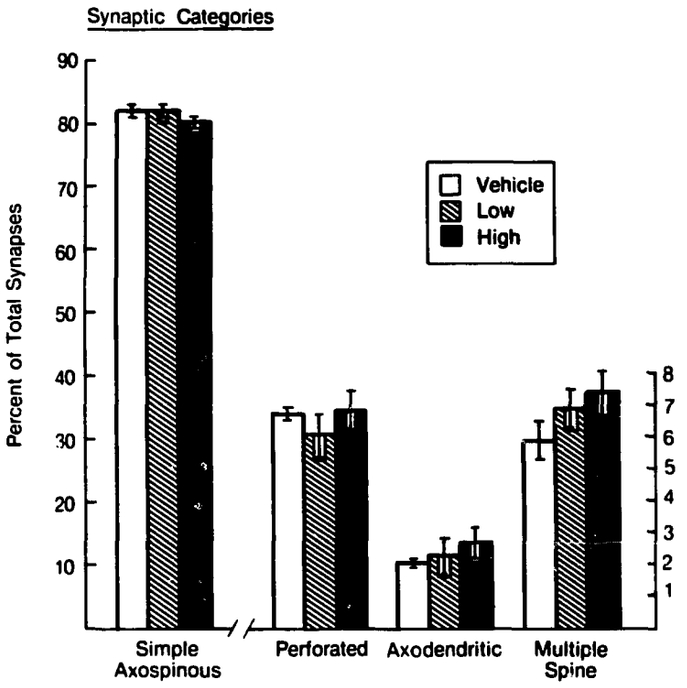

Fig. 7. Percent of total synaptic contacts accounted for by each of the several synaptic categories analyzed. No statistically significant differences were found after the 8-month treatment regimen, but in THC-treated animals, there was a nearly significant increase in the percent of synapses in the multiple spine category (means ± S.E.M.).

campal astrocyte cytoplasm in these animals. Similar inclusions, vacuoles and globules increase substantially during normal aging[1,19,20,45,48], or following experimentally induced brain lesions[5,27].

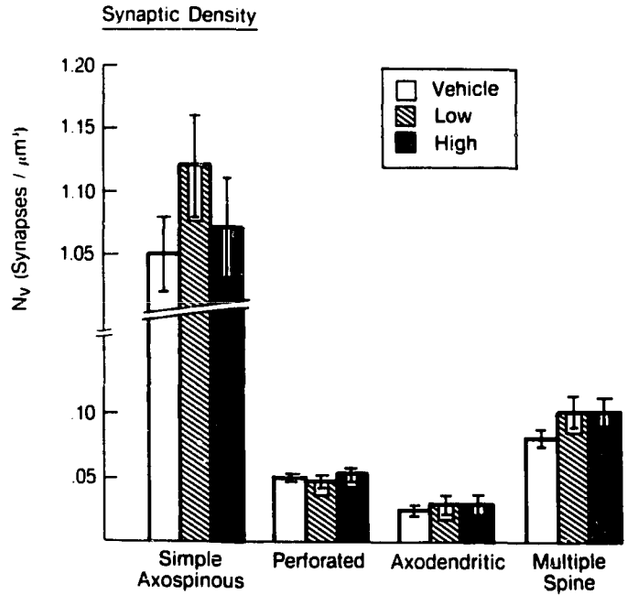

Fig. 8. Synaptic density in neuropil volume (N_v), in the same categories shown above, after correction of synaptic counts for differences in mean length of active zones. No statistically significant differences were found after the 8 month treatment regimen, but multiple spine synapses exhibited a nearly-significant increase in the THC-treated animals (means ± S.E.M.).

Fig. 10 shows that a greater fraction of the cytoplasm of astrocytes was occupied by such inclusions in THC-treated animals ($P < 0.03$), although no differences were seen between low- and high-dose THC animals.

Volume analysis

The results of the point counting volume analysis did not show significant differences among the 3 groups, although a trend toward larger mean volumes (v) per spine was seen in high-dose THC animals (data not shown).

DISCUSSION

The results indicate that chronic exposure of young-mature subjects of a mammalian species to apparently moderate doses of THC is associated with reduced neuronal density and increased glial reactivity in the hippocampus. The neuronal density effect was not seen at the lowest dose, but was observed at a dose level (8 mg/kg) which was compatible with near-normal function on an active avoidance task, and near-normal food and water intake, and which therefore did not seem to induce extreme sedation or toxicity. At the EM level, synaptic density was not found to be decreased, although a trend toward an increase in multiple spine contacts (e.g. 2 spines per terminal) was seen. Behavioral affects were also minimal.

Some of the differences between our results and those from other brain anatomical[36] or behavioral[42] studies on chronic THC administration in rats could of course arise from differences in the procedures of THC administration or of behavioral training, among other possibilities. In addition, the highest dose of THC used in the present study did not appear particularly high in effective (i.e. acute behavioral) terms.

Synaptic remodeling might also play a role in the negative findings on synaptic density in this study. That is, it has been found that the frequency of multiple synaptic contacts increases in rat hippocampus during lesion-induced reactive synaptogenesis[25,41]. Although the basis for this effect is still not clear[41], it seems possible that such contacts may reflect a compensatory formation of new contacts by spines following the loss of terminals. An adaptive mechanism of this kind could explain why neuronal density but

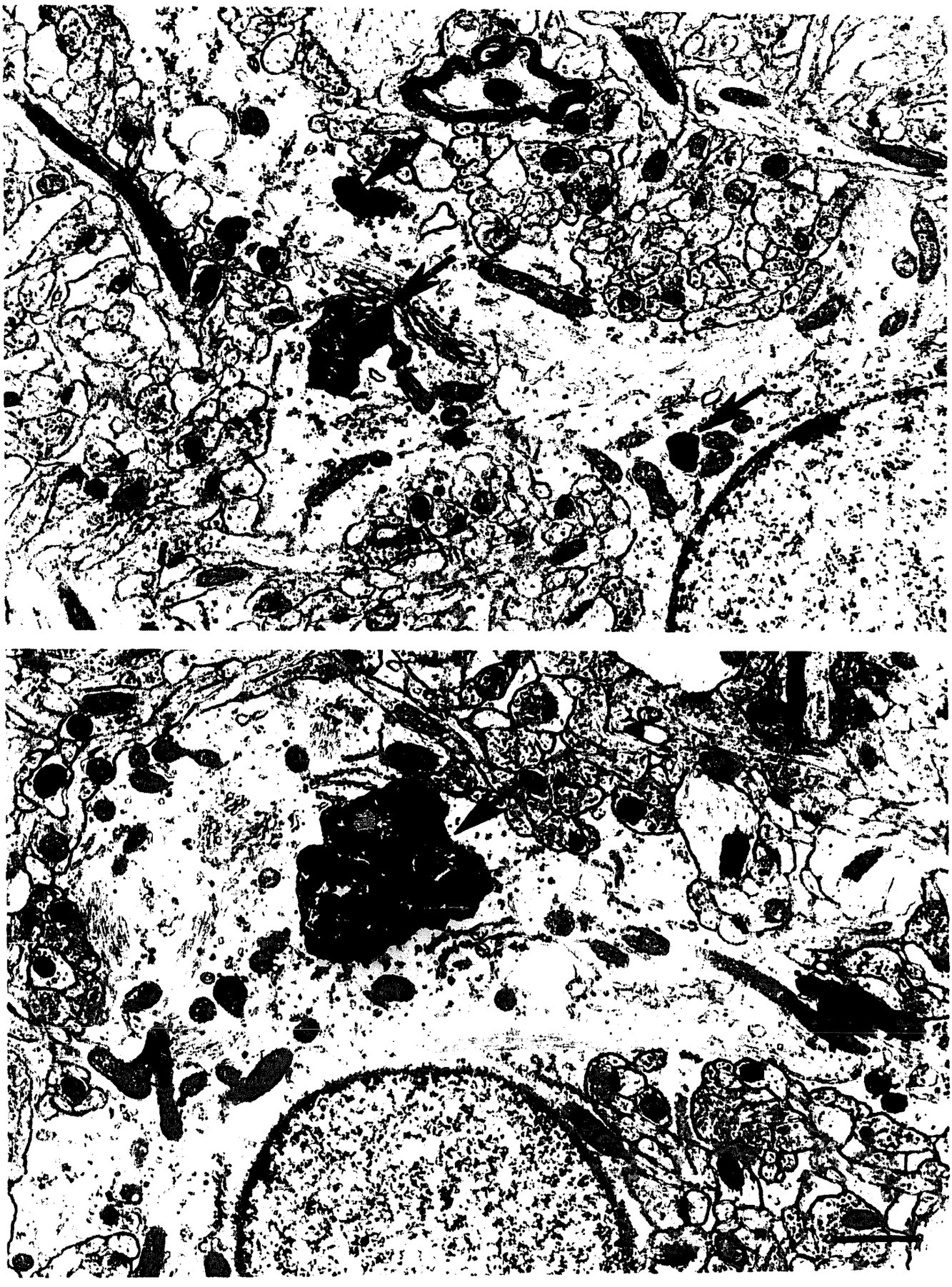

Fig. 9. Opaque, membranous inclusions (arrows) in the cytoplasm of two astrocytes (top and bottom) from stratum radiatum of high-dose THC animals. The inclusions appear similar in configuration to those which are increasingly present in hippocampal astrocytes during normal aging. Bar = 1 μm.

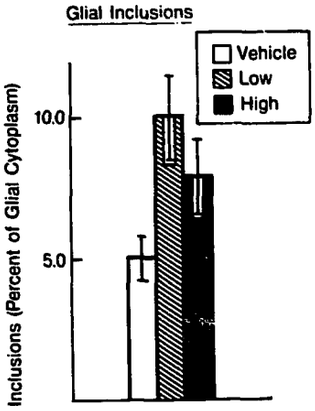

Fig. 10. Analysis of the percent of large profile astroglial cytoplasm occupied by opaque and globular inclusions following 8 months of treatment with vehicle alone, a low dose of THC, or a high dose of THC. A significant increase was observed in both the low- and high-dose THC groups, in comparison to vehicle controls (means ± S.E.M.).

not synaptic density was reduced in the high dose THC animals.

The observed glial and neuronal density changes were similar to those previously found to occur in the hippocampus during normal aging in rats[1,2,18–20,45]. Such changes can be retarded or prevented by chronic adrenalectomy[18,21], and can also be increased by chronic exposure to elevated glucocorticoids, apparently through mediation by specific corticoid brain receptors[35]. Aging rats also exhibit elevated plasma corticosterone and ACTH, particularly in response to stress[7,20,22,34].

As noted in the Introduction, these and other data have led to the hypothesis that glucocorticoids contribute to aging of the rodent hippocampus[7,17,20,35]. In this context, therefore, it seems of particular interest that these chronic treatments with THC resulted in elevations of plasma ACTH and corticosterone (Fig. 1).

One hypothesis that might account for these findings is that THC can bind to and mimic the glucocorticoid effect on steroid receptors[32,38], and thereby accelerate brain aging. An alternative possibility is that THC might interfere with negative feedback inhibition in the adrenal-pituitary axis[8,23,33,44], leading to an elevation of corticosterone. In turn, such higher endogenous corticosterone could induce aging-like brain changes.

However, if one or the other of the above hypotheses is correct, then again it is not obvious why N_v (synaptic density) was not reduced by THC in this study, or why no residual effects on maze reversal learning were found, since both of these effects are seen in aged rats[1,17,18]. A possible explanation of this apparent inconsistency could be that hippocampal cell loss in young animals has different functional consequences than does similar cell loss in aged animals. Young animals might be able to adapt to THC-induced brain changes and to compensate at least partially for neuronal loss (e.g. by learning and/or synaptic remodeling) whereas aged animals, because of a decline in adaptive capacity, may exhibit more widespread consequences of neuronal decline. A possible corollary of this view is that rats exposed chronically to THC during early life may exhibit more severe brain aging and behavioral changes in later life than same-age controls (either because of additive or synergistic effects between THC exposure and aging mechanisms), even if the animals have been withdrawn from THC for prolonged periods.

Clearly, however, defining the extent, if any, of THC interactions with adrenal steroid-dependent processes will require further research.

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