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Effects of Cannabis Sativa on Ultrastructure of the Synapse in Monkey Brain

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Electron microscopic study of brain tissues of monkeys chronically exposed to psychoactive Cannabis showed widening of the synaptic cleft, electron opaque material in the cleft and in pre- and postsynaptic regions, and some "clumping" of synaptic vesicles. In contrast, tissues of control monkeys showed no ultrastructural changes.

Key words: Cannabis sativa, delta 9-THC, electron microscopy, marihuana, monkey brain, septal region, synapse, ultrastructure

INTRODUCTION

We have previously described electroencephalographic (EEG) findings in rhesus monkeys chronically exposed to Cannabis sativa derivatives (Heath, 1976a; Heath, 1976b). Lasting changes in EEG recordings from the septal region, amygdala, and hippocampus developed after 3 months in monkeys whose dose of active marihuana was calculated to be equivalent to 7 joints per day for man (heavy-smoking), in monkeys whose dose of active marihuana was calculated to be equivalent to 1 joint per day for man (moderate-smoking), and in monkeys receiving delta 9-THC intravenously. The EEG changes persisted for 7 months following the 6 months exposure period, at which time the monkeys were sacrificed. In contrast, monkeys that were heavy smokers of inactive marihuana displayed no EEG changes. Some monkeys were left without electrodes to serve as controls for the effects of electrodes on histopathologic results. The current report describes the results of an electron microscopic study of 5 monkey brains. One was from a nonoperated heavy-smoking monkey of active marihuana. A second brain was from a monkey with electrodes, which had received intravenous delta 9-THC at a dose that induced acute EEG changes corresponding to those induced in monkeys that were heavy or moderate smokers of active marihuana. The third brain was from an electrode-implanted heavy-smoking monkey of inactive (nonpsychoactive) marihuana that displayed no EEG changes. The other 2 brains were from nonoperated control monkeys, which had not been used previously for any study.

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MATERIALS AND METHODS

Each monkey was anesthetized with sodium pentobarbitol (30 mg/kg) and subjected to sequential transcardiac perfusion of 3 solutions at 125 mm Hg; warm (37°C) heparinized (9.0%) physiologic saline until a return flow was clear of blood; warm 0.1 M phosphate-buffered 1.5% glutaraldehyde/1% paraformaldehyde (pH 7.4) for 30 minutes; and cold (4°C) 0.1 M phosphate-buffered 6% glutaraldehyde/3% paraformaldehyde (pH 7.4) for 1 hour. The brain of each monkey was then carefully removed from the skull and immersed in the last fixative until dissection (2–4 hours later).

Small pieces of the subcallosal gyrus of the septal region (Heath, 1954) (at approximate stereotaxic coordinates, A 24 and D +7) were taken in the coronal plane. The subcallosal gyrus of the septal region was selected for detailed, time-consuming ultrastructural study because it was one of 3 brain sites that showed the most consistent and lasting changes in EEG's. The current study focuses on the ultrastructure of synapses because morphologic differences could not be detected by light microscopy. In the 2 monkeys with chronically implanted electrodes, the subcallosal gyrus tissue was obtained on the side opposite the site of implantation to minimize the possible complicating effects of the implanted electrode.

During dissection, the brains were immersed in a cold fixative. Tissue samples were immediately replaced in a cold fixative for 1 hour and then postfixed in cold, 1.5% O_sO_4; dehydrated through cold, graded ethanols; and flat-embedded in Epon 812. Sections encompassing the entire depth (1.8 mm) of the sample site were cut on a Porter-Blum MT-2B ultramicrotome at 1 μ and at 700 Å in thickness, as judged by interference coloration. Semithin sections were stained with toluidin blue. Thin sections were stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and viewed with a Philips EM-300 electron microscope. Samples for quantitative study were randomly coded so that the investigators were unaware of the monkey source of the tissue under study. Photographic plates taken for measurement were standardized for magnification (39,000 X) with a carbon-grating replica (Fullam No. 1002), and enlarged with a 3.75 print factor. Ensuing prints were measured with Mitutoyo calipers under a 3 X dissecting microscope. Six hundred prints were found acceptable for measurement. The distance between outer leaflets of the confronting pre- and postsynaptic membranes were taken as the synaptic cleft width. Three measurements, at the center and near each edge, were meaned to determine the final width value for each cleft.

We chose to compare synapses of the same morphologic type from tissues of the 5 representative brains. Axodendritic synapses, described by Colonnier (1968) and by others (Mollgaard et al., 1971) as corresponding closely to Gray's asymmetrical Type I synapses (Gray, 1959), were measured. These synapses are characterized by a thickened postsynaptic cytoplasmic opacity bordering the region of synaptic contact and by a population of spherical vesicles. Only those fine structures with clear pre- and postsynaptic membranes were included, so as to ensure exact cross-sectional measurement of the cleft. Layer 5, the large pyramidal neuron layer, was the specific site chosen for study. The cytoarchitectural boundaries of this stratum, particularly when seen first in semithin reference sections, are distinct, and this layer was therefore localized within each thin section (of each of the brains) with minimal difficulty.

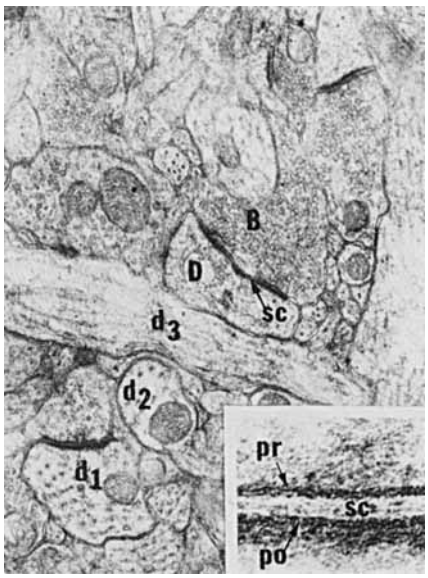

Fig. 1. Photomicrograph showing ultrastructure of tissue from a nonexposed control monkey.

RESULTS

Figure 1 illustrates the neuropil of layer 5 from a subcallosal gyrus sample (30,000 X) of the brain of a nonexposed control monkey. Dendrites are evident in various planes of section (d_1, d_2, d_3) and an electron-lucent intercellular space (100–150 Å) separates all tissue components. Axodendritic synapses can also be seen, each characterized by a presynaptic bouton (B), with a complement of spherical synaptic vesicles, a distinct synaptic cleft (sc), and a postsynaptic dendrite (D). The inset of Figure 1 shows the synaptic cleft region of such a synapse at higher magnification (80,000 X). The presynaptic membrane (pr), the cleft region (sc) (about 200 Å in width), and the postsynaptic membrane (po) with its subsynaptic opacity are evident.

In Figure 2 (30,000 X), an axodendritic synapse and astrocytic process (As) containing numerous glycogen granules (g) are seen in subcallosal layer 5 neuropil from the monkey that was exposed to large quantities of active marihuana smoke (heavy smoker). When compared to control tissue (Fig. 1), certain fine structural differences are disclosed. The synaptic cleft region and some of the extracellular space (at arrows) appear to be electron-opaque. The inset of Figure 2 shows the synaptic cleft region from a similar synapse at higher magnification (80,000 X). Electron-opaque, granular material is seen to be accumulated in the cleft and in the pre- and postsynaptic regions. The distance between opposing synaptic membranes seems appreciably widened.

Table I shows a comparison of synaptic width measurements. Tissues of monkeys exposed to active Cannabis had a greater mean synaptic width (about 75 Å wider) than tissues of unexposed control monkeys or of the heavy-smoking monkey of inactive marihuana. The analysis of variance indicates that monkeys receiving the active materials did not differ significantly from each other, but had a significantly ($p = 0.05$) larger mean

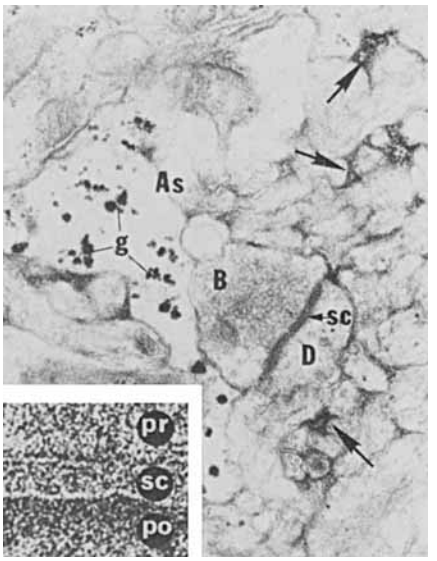

Fig. 2. Photomicrograph showing ultrastructure of tissue from monkey exposed to large quantities of psychoactive marihuana smoke (heavy smoker).

TABLE I. Comparison of Synaptic Width Measurements

Monkey brain	N	Mean synaptic cleft (Å)	Standard deviation	Standard error
Heavy-smoking active marihuana	120	275.93	32.72	2.99
Intravenous delta 9-THC	120	278.25	24.86	2.27
Unexposed control 1	120	204.18	12.43	1.13
Unexposed control 2	120	205.64	16.54	1.50
Heavy-smoking inactive marihuana	120	201.71	18.18	1.66

synaptic cleft width (277 Å) than those not exposed to psychoactive material. On the other hand, no significant differences were observed between the 2 unexposed control animals or among unexposed controls and the heavy-smoking monkey of inactive marihuana.

Figure 3 illustrates another common observation in monkeys receiving psychoactive Cannabis. The presynaptic bouton (B) appears swollen, containing aggregations of tightly packed synaptic vesicles (sv). These clumped vesicles usually occupy central regions of the bouton. Vesicles of this nature were not observed in brains of the other 3 monkeys studied.

DISCUSSION

These electron microscopic results, consistent with the EEG data previously obtained from the monkeys in this experiment, indicate that the lasting changes in brain function associated with specific changes in ultrastructure resulted from exposure to psy-

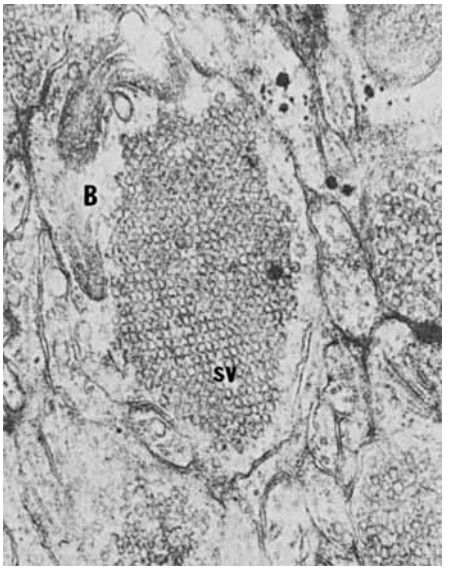

Fig. 3. Photomicrograph showing vesicle "clumping" in tissue of a monkey that received psychoactive Cannabis. 36,000 X.

choactive derivatives of Cannabis sativa, whether given intravenously or delivered as marihuana smoke. Since no EEG changes or significant ultrastructural changes were observed in the monkey smoking heavily of inactive marihuana, the influence of smoke per se on the results is questionable.

Morphologic changes observed in the samples obtained from animals exposed to active Cannabis consisted of: 1) appearance of an electron-dense (opaque) granular material in the synaptic cleft region and extracellular space, 2) a significantly widened synaptic cleft, and 3) synaptic vesicle "clumping." Each finding represents a departure from the classically defined structural characteristics of the synapse under essentially normal conditions (De Robertis, 1959; Palay, 1958; Peters et al., 1970).

Whereas tissue from animals receiving psychoactive Cannabis contained an electron-dense, granular material of unknown origin within the synaptic cleft region, as well as in the extracellular space, tissue from control animals, treated in exactly the same manner for ultrastructural study, did not. It is unlikely that this material was the result of an artifact of fixation or other preparative procedure. However, it might indicate a chemical change or a change of molecular configuration within these sites (Walberg, 1964), similar to changes described in brains of patients with certain neurologic disorders: Jakob-Creutzfeldt disease (Brion et al., 1969), thiamine (Tellez and Terry, 1968), and Vitamin E deficiency (Lampert et al., 1964). Lithium toxicity in rats also produces increased osmiophilic density and granularity of incrustation-like phenomena in synaptic vesicles (Roizin et al., 1971).

We consider widening of the synaptic cleft in monkeys exposed to psychoactive Cannabis to be a specific phenomenon. The width of the synaptic cleft is reasonably similar from one brain region to another of mammals. The preponderance of literature dealing with these species, including primates and man, indicates the most commonly observed width of the synaptic cleft averages about 200 Å, although cleft widths ranging from 100 Å to 300 Å have been reported for the "normal" brain (Palay, 1956; Karlsson,

1966; Shade and Ford, 1973; Van der Loos, 1963). Our findings show a widening of the synaptic cleft by about 27% ($p = 0.05$) in monkeys that had active material, as compared to control monkeys. Even radical treatment of brain samples, such as homogenization, centrifugation, or culture in various media, do not seem to substantially alter cleft width dimensions (Garey et al., 1972; De Robertis et al., 1967a; Ross and Bornstein, 1969). Following administration of triorthocresylphosphate, cleft widths up to 1,000 Å have been observed, but not treated statistically (Ahmed, 1971).

Clumping of synaptic vesicles similar to that reported herein has been described subsequent to axonal sectioning by numerous investigators (De Robertis, 1956; Blackstad et al., 1965; Colonnier, 1964). Aggregation of vesicles is also produced by ischemia (Williams and Grossman, 1970) and by experimental allergic encephalomyelitis (Ross and Bornstein, 1969), as well as by toxins (triorthocresylphosphate and methionine sulfoximine – De Robertis et al., 1967b) and certain neuropathies (Alzheimer's disease – Gonatas et al., 1967).

The ultrastructural changes in brains of monkeys exposed to psychoactive materials cannot be attributed to differences in section thickness or errors in magnification, as both were carefully standardized to known criteria. Further, steps were taken to minimize errors resulting from possible variations in the planes of the sections studied. Nor can the changes be attributed to effects of electrode implantation, since brain tissues of implanted monkeys were obtained from the contralateral side. Moreover, morphologic changes were equally pronounced in the brain tissues of the nonoperated, heavy-smoking monkey and of the operated monkey given delta 9-THC intravenously. Further, no significant differences were observed at the synapses of unexposed control animals when compared to the electrode-implanted monkey that smoked inactive marihuana.

Statistical analysis of such a small number of monkeys may not be completely convincing. One assumption of the analysis of variance requires that the E_{ijk} be normally distributed and independent, with mean zero and constant variance. This assumption of constant variance is violated in the current study, as indicated by the estimated error variance ranging from 93 to 776. Box (1953), and later Scheffe (1959), however, have pointed out that such a departure has little or no effect on the comparison of means, provided equal sample sizes have been taken in each group. For our analysis, equal sample sizes of animal brain tissue were obtained.

The conclusions presented here are based on comparisons of a single brain region from each of 5 monkeys, and additional studies are required. The present limited findings indicate that chronic, heavy use of psychoactive Cannabis modifies the fine structure of the synapse. It may be that these synaptic ultrastructural modifications are related to mechanisms of impulse transmission across the synapse. If so, this could account for the previously reported EEG changes in these monkeys.

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