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EFFECT OF CANNABINOIDS ON NEUROTRANSMITTER UPTAKE, ATPASE ACTIVITY AND MORPHOLOGY OF MOUSE BRAIN SYNAPTOSOMES

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Abstract—Δ^1-Tetrahydrocannabinol (Δ^1-THC) and cannabidiol (CBD), a psychoactive and a nonpsychoactive constituent of marijuana respectively, inhibit the uptake of ^{3}H-labelled norepinephrine (NE), dopamine (DA), γ-aminobutyric acid (GABA) and serotonin (5-HT), by mouse brain synaptosomes. CBD is more effective than Δ^1-THC in the inhibition of neurotransmitter uptake. At 5×10^{-5} M both CBD and Δ^1-THC inhibit uptake by 60–100%. The one exception to the above is the differential effect of Δ^1-THC and CBD on 5-HT uptake. At 10^{-6} M of Δ^1-THC the uptake is twice that of the control value and at 5×10^{-5} M uptake is still equal to control value. At the former concentration CBD has no effect on 5-HT uptake whereas at the latter concentration a 50 per cent inhibition is observed. Both Δ^1-THC and CBD inhibit Na^+-K^+-ATPase and Mg-ATPase activities; at 5×10^{-5} M inhibition amounts to 40 per cent. Electron microscopy reveals that synaptosomal preparations are highly damaged at 5×10^{-5} M. Thus inhibition of uptake could stem from either failure of ATPase activity, from disruption of synaptosomes, or from both.

Δ^1-Tetrahydrocannabinol (Δ^1-THC) a component of cannabis sativa extracts [1] has been shown to exert psychomimetic effects in man analogous to those evoked by hashish and marijuana [2, 3]. In an attempt to identify the level at which cannabinoids affect the central nervous system several laboratories have investigated the effect of Δ^1-THC on brain content of serotonin [4, 5], norepinephrine [4] and dopamine [6]. The *in vivo* studies produced contradictory results and failed to show a consistent correlation between the psychoactive state brought about by Δ^1-THC and total brain neurotransmitter content.

Studies of the uptake of different neurotransmitters by isolated brain synaptosomes [7–9] revealed that Δ^1-THC as well as other cannabinoids (nonpsychoactive derivatives) exert an inhibitory effect on the uptake of various neurotransmitters.

Studies in our laboratory [10–14] as well as those of other groups [15] have established that both Δ^1-THC and cannabidiol (CBD), a nonpsychoactive compound constituting 40 per cent of the cannabis extract [16], have similar effects on various membrane systems. Lysosomal lysis [11] mitochondrial swelling and rupture [17], leakage of K^+ from bull spermatozoa [18], cytotoxicity towards macro-phages [14] are only few examples of damage to biological membranes both in structure and function. Δ^1-THC and CBD are also potent disorganizers of phospholipid bilayer membranes; i.e. they reduce membrane electrical resistance [12] and the gel–liquid crystalline transition temperature [13].

In the following we report about studies concerning the effects of Δ^1-THC and CBD on the uptake of different neurotransmitters by isolated brain synaptosomes. A wide range of cannabinoid concentrations was tested in order to assess whether there exists a differential effect of Δ^1-THC and CBD. Since neurotransmitter uptake is an energy dependent process [19, 20] that also requires structural intactness of the organelle, the effect of cannabinoids on symaptosomal ATPases and on synaptosome morphology (as revealed by electron microscopy) have also been studied.

MATERIALS AND METHODS

Chemicals. Δ^1-Tetrahydrocannabinol and cannabadiol were obtained from Makor Chemicals (Jerusalem, Israel). Unlabeled neurotransmitters, Pargyline and ATP were obtained from Sigma Chemical Corp. (St. Louis, MO). [^{3}H]serotonin (2.72 Ci/m-mole), [^{3}H]γ aminobutyric acid (36.7 Ci/m-mole) were obtained from New England Nuclear Corp. (Boston, MA) and L-[^{3}H]norepinephrine (10.9 Ci/m-mole) and [^{3}H]dopamine (2.3 Ci/m-mole) were obtained from the Radiochemical Centre (Amersham, England).

Synaptosome preparations. Male (5–6 weeks old) BALB/C strain mice (20–25 gr) were killed by decapitation and the brains were removed, rinsed in cold saline and homogenized (Elvehjem homogenizer with a teflon pestle, 400 rotations per min, 10 up and down movements of the pestle) in 10 volumes of ice cold 0.32 M sucrose (pH 7.0). The homogenates were centrifuged at 1000 *g* for 10 min at 4° in a Sorvall RC 2B refrigerated centrifuge. The supernatant was saved and the pellet was resuspended in a 10 ml of the above sucrose solution and centrifuged again at 1000 *g* for 10 min. The two supernatants were pooled and centrifuged at 13,000 *g* (20 min, 4°). The resulting pellet was resuspended in 0.32 M sucrose and layered on top of a Ficoll (Pharmacia) discontinuous gradient consisting of 17%, 13% and 7% Ficoll in 0.32 M sucrose. The gradients were centrifuged at 56,000 *g* for 60 min in a Beckman centrifuge (SW 27 rotor).

The synaptosome containing interface layers between 17% and 13% Ficoll and between 13% and 7% Ficoll were collected, pooled, and diluted with 3 volumes of 0.32 M sucrose. The suspensions were centrifuged at 56,000 *g* for 15 min, the pellet washed once again with the sucrose solution and resuspended in a medium (Buffer A) consisting of 125 mM NaCl, 10 mM glucose, 10 mM $MgSO_4$, 5 mM KH_2PO_4, 1 mM $CaCl_2$, 25 mM sucrose, 1 μM Pargyline (a monoamine oxidase inhibitor) and 0.2 mg/ml of ascorbic acid, pH 7.0.

Assay of neurotransmitter uptake. Aliquots of 0.5 ml of synaptosome suspensions containing 1.25 mg protein were added to 1.5 ml of buffer A pre-gassed with O_2 for 10 min. The specified cannabinoids dissolved in absolute ethanol (at most 20 μl per incubation mixture) or an equivalent amount of ethanol for control experiments were added to buffer A prior to the addition of synaptosome preparations. The suspensions were preincubated for 10 min at 37° in a shaking bath under a 95% O_2 + 5% CO_2 atmosphere after which radioactive neurotransmitter at a final concentration of 10^{-7} M was added and the suspensions further incubated for 10 min at 37°. For assessment of uptake of GABA incubation was carried out at 25°. At the end of incubation, the tubes were transferred to an ice-water bath and their content diluted with a 4 ml of saline containing 10^{-5} M of the corresponding nonlabelled neurotransmitter. Incubation mixtures were either filtered through cellulose ester Millipore filters (0.45 μm-HA filters) or centrifuged at 27,000 *g* for 30 min. The filters were washed with 0.32 M sucrose containing 10^{-5} M of the nonlabelled neurotransmitter and then counted in toluene-triton scintillation liquid. When synaptosomes were collected by centrifugation, the pellet was washed as above and recentrifuged (27,000 *g*, 15 min). The resulting pellets were dissolved in 1% sodium dodecyl sulfate (SDS) and their radioactivity assessed as above in a Packard 3385 liquid scintillation spectrometer. Radioactivity associated with synaptosome suspensions incubated with radioactive labelled neurotransmitters at 0° was used to correct for nonspecific binding and uptake.

Assay of ATPase activity. Synaptosome preparations (0.2 mg protein/ml) were ruptured by freeze-thawing in a glycylglycine-imidazole buffer (65 mM) containing 100 mM NaCl, 5 mM KCl and 5 mM of $mgCl_2$, pH 7.4 (Buffer B). Cannabinoids or ethanol were added in aliquots of 4 μl to 0.75 ml of the ruptured synaptosomal preparation 15 min prior to the addition of ATP (final concentration 1 mM) (Sigma Chem. Co., St. Louis, MO). The suspensions were incubated with ATP for 10 min at 37° after which reaction was terminated by the addition of cold silicotungstic acid (8% in 1.2 M perchloric acid, 0.3 ml). The mixture was cooled for 60 min at 4°and then centrifuged (39000 *g* for 10 min at 4°). Inorganic phosphate in the supernatant was determined according to [21]. The differentiation between Mg-ATPase activity and Na^+-K^+ dependent ATPase activity was made by carrying out parallel assays in the presence of 1 mM ouabain. Total ATPase activity minus Mg-ATPase activity (not inhibited by ouabain) yields the value for Na^+-K^+-ATPase activity.

Electron microscopy of synaptosomes. Synaptosome preparations were incubated with either Δ^1-THC or CBD ($1 \times 10^{-5} \times 10^{-4}$ M) dissolved in ethanol or with an equivalent amount of ethanol for 10 min, at 37°. The amount of ethanol added at all cannabinoid concentrations was constant (5 μl/ml suspensions). Subsequent to centrifugation (10000 *g* for 20 min, 4°) the pellets were resuspended in a solution of 2% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.4. After 60 min at 4°, the fixed synaptosomes were pelleted again, washed in the above cacodylate buffer, and postfixed for 1 hr at 22° with 1% OsO_4 in cacodylate buffer. The pellets were dehydrated in a series of increasing alcohol concentrations and by propylene oxide. The pellets were embedded in epoxy resin according to Spurr [22]. Thin sectioning was carried out with a Sorvall, Porter-Blum MT-2-B-ultramicrotome, the sections were stained with lead citrate prior to microscopic examination. The electron microscope (Philips EM-300) was operated at 80 KV.

RESULTS

The effect of Δ^1-THC and CBD on neurotransmitter uptake. Δ^1-THC and CBD exert a dose-dependent inhibition of neurotransmitter uptake by isolated mouse brain synaptosomes. The dependence of norepinephrine (NE), dopamine (DA), γ-aminobutyric acid (GABA) and serotonin (5-HT) uptake on cannabinoid concentrations is given in Figs 1–4, respectively. The degree of inhibition of uptake of the various neurotransmitters by Δ^1-THC and CBD show certain variations, CBD exhibits in all cases a higher degree of inhibition of uptake. At 5×10^{-6} M Δ^1-THC is noninhibitory except for an observed reduction of uptake of DA of about 20 per cent. At the same concentration CBD reduced NE uptake by about 15 per cent and that of DA by 40 per cent. At a concentration of 5×10^{-5} M both Δ^1-THC and CBD exhibit remarkable inhibitory effects on neurotransmitter uptake; i.e. DA and NE uptake is inhibited by 60–65 per cent and GABA uptake is inhibited by 80–100

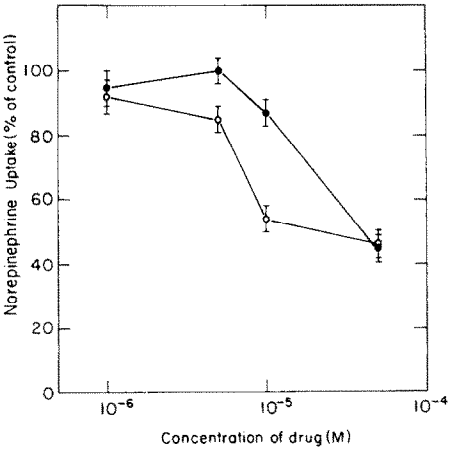

Fig. 1. Effect of Δ^1-THC (●——●) and CBD (○——○) on noreponephrine (NE) uptake by synaptosomal preparations. Values on graphs are means ± S.E.M. ($n = 8$). Experimental details are given in Methods. The uptake of [^{3}H]NE by control preparations (1 per cent ethanol) (100 per cent uptake) was 2.65 ± 0.16 pmole/min·mg protein.

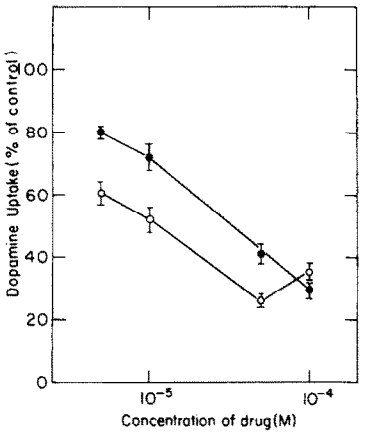

Fig. 2. Effect of Δ^1-THC (●——●) and CBD (○——○) on dopamine (DA) uptake by synaptosomal preparations. Values on graphs are means ± S.E.M. (n = 6). Experimental details are given in Methods. The uptake of [^{3}H]DA by control preparations (1% ethanol (100% uptake) was 3.10 ± 0.5, pmole/min·mg protein.

per cent. The dependence of 5-HT uptake on cannabinoid concentration differes significantly from that of the other three neurotransmitters. A remarkable differential effect of Δ^1-THC and CBD is shown in Fig. 4. CBD affects 5-HT uptake according to essentially the same pattern of its effect on the other neurotransmitters. Δ^1-THC at 5 × 10^{-5} M fails to inhibit 5-HT uptake and its inhibitory effect on uptake becomes significant only at 10^{-4} M. Since at a concentration of 5 × 10^{-6} M an increase in uptake was observed (up to 25 per cent increase) the effect of lower concentrations of Δ^1-THC were assessed. An optimum in the enhancing effect on 5-HT uptake (a 2-fold increase over control values) was observed at 10^{-6} M Δ^1-THC, a concentration at which CBD has no effect on uptake.

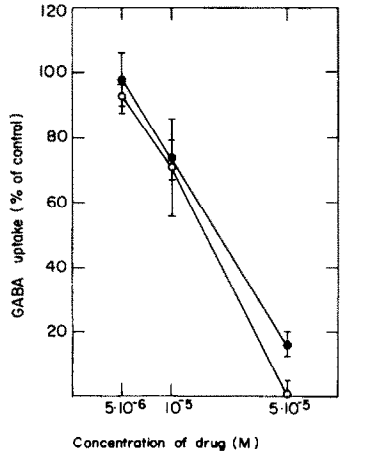

Fig. 3. Effect of Δ^1-THC (●——●) and CBD (○——○) on γ-aminobutyric acid (GABA) uptake by synaptosomal preparations. Values on graphs are means ± S.E.M. (n = 4). Experimental details are given in Methods. The uptake of [^{3}H]GABA by control preparations (1% ethanol) (100% uptake) was 10.8 ± 1.8 pmole/min·mg protein.

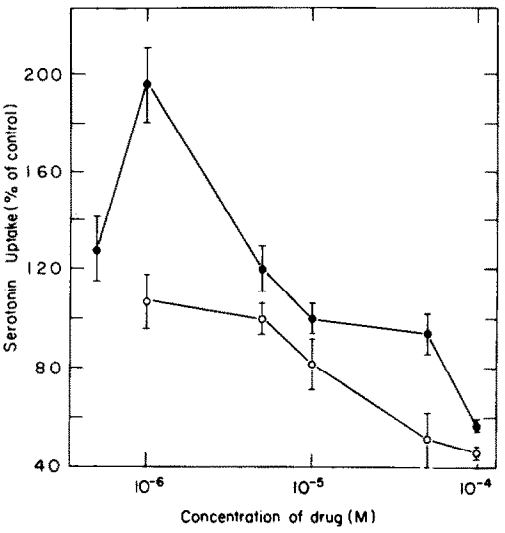

Fig. 4. Effect of Δ^1-THC (●——●) and CBD (○——○) on serotonin (5-HT) uptake by synaptosomal preparations. Values on graphs are means ± S.E. (n = 8). Experimental details are given in Methods. The uptake of [^{3}H]5-HT by control preparations (1% ethanol) (100% uptake) was 0.44 ± 0.05 pmole min·mg protein.

At 10^{-5} M neither Δ^1-THC nor CBD had a significant effect on neurotransmitter release from synaptosomes preloaded with [^{3}H]NE, [^{3}H]5-HT or [^{3}H]DA.

Effect of Δ^1-THC and CBD on ATPase activity. Neurotramsmitter uptake depends on a functional ATPase [19, 20]. Since ATPases from various sources have been shown to be sensitive to depressant drugs [23] and to Δ^1-THC [24] it was of interest to assess the activity of both Δ^1-THC and CBD on brain synaptosomal ATPases. Δ^1-THC and CBD are inhibitory to both the synaptosomal Mg-ATPase and the Na^+-K^+-ATPase (Fig. 5). Inhibitory effects are observed already at 5 × 10^{-6} M of the cannabinoids. At 5 × 10^{-5} M inhibition of both enzymes by either

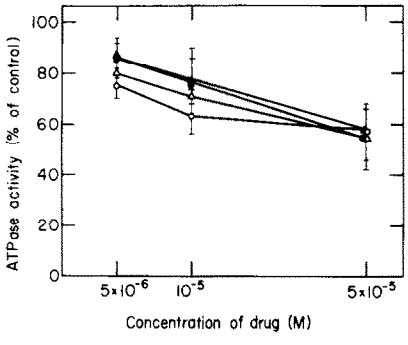

Fig. 5. The effect of Δ^1-THC (circles) and CBD (triangles) on brain synaptosoma Na^+-K^+-ATPase (empty symbols) and Mg-ATPase (full symbols). Values on graphs are means ± S.E.M. (n = 6). Details of assay procedure and calculation of activities are given in Methods. The mean value of control preparations (1% ethanol) (100% activity) was 1.6 ± 0.18 μmole Pi/10 min·mg protein for the Na^+-K^+-ATPase and 1.48 ± 0.12 μmol Pi/10 min·mg protein for the Mg-ATPase.

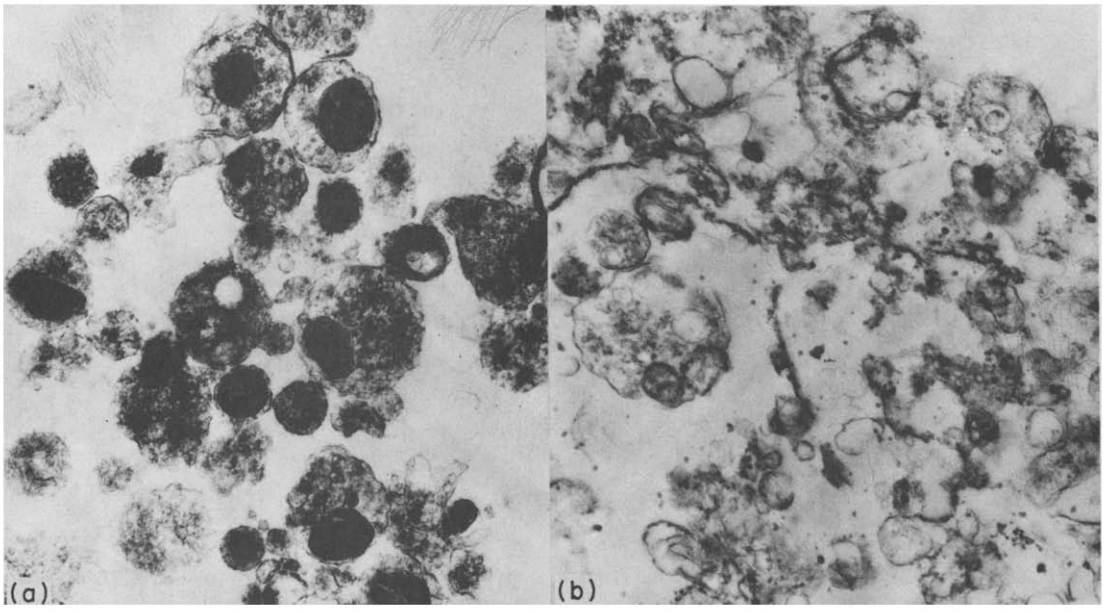

Fig. 6. The effect of Δ^1-THC on the morphology of synaptosomal preparations. (a) Control preparations exposed to 1 per cent ethanol (20 min, 37°). (b) Preparations exposed to 5×10^{-5} M Δ^1-THC (20 min, 37°). ×33,000.

of the drugs amounted to 40 per cent of the control activity. The Na^+-K^+-ATPase is more sensitive to cannabinoids than the Mg-ATPase.

Effect of cannabinoids on synaptosomal morphology. Cannabinoids have been shown to be deleterious to organelles and to cells in tissue culture. The observed inhibition of neurotransmitter uptake may stem from inhibition of the uptake mechanism or energy production or else from loss of synaptosomal integrity. An electron microscope study of the morphological appearance of synaptosomes exposed to increasing concentrations of Δ^1-THC and CBD revealed that at a cannabinoid concentration of 5×10^{-5} M synaptosomal suspensions are highly disrupted (compare Fig. 6b with Fig. 6a). At a lower concentrations, 1×10^{-5} M both Δ^1-THC and CBD had no visible effect on synaptosomal preparations although electron microscopy is hardly adequate for the detection of effects less extreme than burst and rupture.

DISCUSSION

Both Δ^1-THC and CBD exert a dose-dependent inhibition on the uptake of NE, DA, GABA and 5-HT. The fact that CBD seems to be more potent than Δ^1-THC in inhibition of uptake as well as its higher potency in other biological systems [11, 14] may in part be due to its higher water solubility [8]. Both Δ^1-THC and CBD have a very limited water solubility. At a concentration of 8×10^{-6} M of the cannabinoids in water the compounds precipitate out of solution and appear as a white suspension [12]. Banerjee *et al.* [8] have measured the solubility of Δ^1-THC and CBD in Krebs–Henseleit buffer and found that at a final concentration of 5×10^{-5} M only 2 per cent of Δ^1-THC and 4 per cent of CBD are in solution, the rest being a precipitate removed by centrifugation. Thus at concentrations $> 10^{-5}$ M the soluble drug concentrations is at saturation and the dose response must be interpreted as a partition of the drugs from the precipitate via buffer into the synaptosomal membranes. This situation means that it is difficult to compare effects of cannabinoids on different systems and that the experimental conditions, e.g. membrane/cannabinoid ratio may shift the results to the range of a high or low potency. Using a crude rat synaptosome preparation Banerjee *et al.* [8] obtained a comparable inhibition at cannabinoid concentration of 5×10^{-5} M of 5-HT and NE uptake to that observed by us. In contrast to our results, which showed a 90 per cent inhibition of GABA uptake at 5×10^{-5} M Δ^1-THC or CBD, they observed only a mild inhibition of GABA uptake (10 and 3 per cent). At a concentration of 10^{-6} M Δ^1-THC, a concentration at which we observed a 2-fold increase in 5-HT uptake, Banerjee *et al.* [8] observed a 5 per cent inhibition of uptake. The inhibition of 5-HT, NE, DA and GABA uptake by cannabinoids is noncompetitive [8].

The correlation between the inhibition of both the Mg-ATPase and the Na^+- K^+-ATPase and the inhibition of NE and DA uptake could implicate ATPase inhibition as the cause for uptake inhibition. The fact that GABA uptake is inhibited 90–100 per cent and that at 5×10^{-5} M of Δ^1-THC, serotonin uptake is in the range of uptake in control experiments, excludes this interpretation as being the major determinant in the effect. Since the various neurotransmitters are confined within different synaptosomes it could still be argued that those populations have ATPases differing in their sensitivity to cannabinoids, but this explanation is not very appealing.

Membrane-bound ATPases from various origins have been shown to be sensitive to the physical state

of the lipids in their microenvironment [25, 23]. Laurent and Roy [24] have shown that both the Na^+-K^+-ATPase and Mg-ATPase from the microsomal fraction of rat proximal ileum are inhibited by Δ^1-THC. Roufogalis [23] has shown that depressant drugs inhibit bovine brain Na^+-K^+-ATPase in a pattern suggesting that the more lipid soluble phenothiazines, penetrate into and react with, hydrophobic areas of membranes evoking a perturbation of Na activation, whereas the less lipid soluble drugs interfere with K^+ activation.

Isolated organelles (lysomes [11]; mitochondria [17]) and cells exposed to cannabinoids *in vitro* (bull sperm [18], and macrophages [14]) have been shown to undergo extensive functional and morphological changes. Damage to cellular membranes results in leak of intracellular components and affects any transport mechanism against concentration gradients. The electron micrographs of synaptosomes exposed to Δ^1-THC and CBD confirm the potency of cannabinoids in disrupting membraneous bound structures. The concentration at which cannabinoids exhibit extensive damaging potency on synaptosome suspensions, 5×10^{-5} M, correlates with the condition at which neurotransmitter uptake is inhibited by at least 60 per cent. The fact that serotonin uptake is less sensitive to Δ^1-THC than the other three neurotransmitters tested (at 5×10^{-5} M of Δ^1-THC, uptake amounts to 100 per cent of control value) could be due to the difference in packing of the various neurotransmitters in synaptic vesicles. However, the damaged synaptosomes still show a residual uptake of the other neurotransmitters tested in this study, 20–40 per cent of the control uptake value. This residual uptake may be a result of synaptic vesicles which were not as affected by the cannabinoids at higher concentrations as was the synaptic plasma membrane. When synaptosomes are exposed to hypotonic medium, uptake is inhibited to only 30 per cent of the control value.

Conditions of low concentration of drugs at which membrane structure is only mildly affected, are necessary when searching for the specific biological activity of Δ^1-THC. Cannabinoids like anaesthetic compounds may exhibit stabilizing effects on membranes. Their interaction with membranes follows a bi-phasic pattern, i.e. at low concentrations they are apt to stabilize whereas at high concentrations they lead to membrane labilization [10, 11].

Moreover, under conditions of low drug concentration it should be possible to prove differential effects of Δ^1-THC and CBD before implying relevance to the *in vivo* pharmacological effect. Cannabinoids *in vivo* are carried by serum proteins, and under these conditions they are less potent by at least one order of magnitude with regard to their cytotoxicity [14]. This fact must be taken into account when concentrations of cannabinoids used in *in vitro* studies are compared to their effects *in vivo*. Recent studies with the "unnatural" (+) isomer of Δ^1-THC have demonstrated that the difference in potency between the two optical isomers does not arise from metabolic or distributional differences and that the site of action is significantly asymmetric [26].

In mice intravenous injection of Δ^1-THC results in a dose-dependent state of immobility [27]. In our experiments, Δ^1-THC injection into the mouse tail caused an immediate cataleptic (or sedative) effect whereas CBD had no effect on the injected mouse. Crude brain synaptosomal preparations of mice injected with Δ^1-THC exhibited increased 5-HT and DA uptake while those derived from mice injected with CBD showed no significant change in uptake as compared to that observed in control mice injected with the carrier alone (Hershkowitz, in preparation). The enhanced 5-HT uptake in synaptosomal preparations subsequent to *in vitro* exposure to Δ^1-THC could thus be of relevance to the *in vivo* effects of Δ^1-THC on mouse brain.

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