GABAergic interneurons are the targets of cannabinoid actions in the human hippocampus

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Abstract—Cannabinoids have been shown to disrupt memory processes in mammals including humans. Although the CB1 neuronal cannabinoid receptor was identified several years ago, neuronal network mechanisms mediating cannabinoid effects are still controversial in animals, and even more obscure in humans. In the present study, the localization of CB1 receptors was investigated at the cellular and subcellular levels in the human hippocampus, using control post mortem and epileptic lobectomy tissue. The latter tissue was also used for [3H]GABA release experiments, testing the predictions of the anatomical data. Detectable expression of CB1 was confined to interneurons, most of which were found to be cholecystokinin-containing basket cells. CB1-positive cell bodies showed immunostaining in their perinuclear cytoplasm, but not in their somadendritic plasmamembrane. CB1-immunoreactive axon terminals densely covered the entire hippocampus, forming symmetrical synapses characteristic of GABA-ergic boutons. Human temporal lobectomy samples were used in the release experiments, as they were similar to the controls regarding cellular and subcellular distribution of CB1 receptors. We found that the CB1 receptor agonist, WIN 55,212-2, strongly reduced [3H]GABA release, and this effect was fully prevented by the specific CB1 receptor antagonist SR 141716A.

This unique expression pattern and the presynaptic modulation of GABA release suggests a conserved role for CB1 receptors in controlling inhibitory networks of the hippocampus that are responsible for the generation and maintenance of fast and slow oscillatory patterns. Therefore, a likely mechanism by which cannabinoids may impair memory and associative processes is an alteration of the fine-tuning of synchronized, rhythmic population events.

Key words: CB1 receptor, cholecystokinin, presynaptic, synchronization, memory, release.

Most behavioral effects of the active compound of marijuana and hashish are mediated by the CB1 cannabinoid receptor (CB1). Although the impacts of cannabinoid-consumption on human and animal behaviour are well known,1,3,17 the underlying processes and the precise sites of cannabinoid actions in neural networks remain to be identified. The hippocampal formation is one of the brain areas with the highest level of CB1 receptor expression.18,20 In accordance, at the behavioral level, cannabinoids typically interfere with hippocampal functions, i.e. they disrupt memory consolidation and associations both in humans and animals.3,17 Although several recent experiments attempted to determine the mechanisms of cannabinoid action and the precise cellular and subcellular localization of the CB1 receptor in the rodent and primate hippocampus, the data they provide are inconsistent.1,20,27,34,35,38,44,45

In the rodent hippocampus, a specific class of GABAergic interneurons was shown to express CB1 receptors.20,27,45 Detailed electron microscopic investigation revealed that CB1 is located presynaptically on the axons of these interneurons. Moreover, cannabinoids were shown to inhibit GABA release by activating these receptors and suppressing GABA_A-receptor mediated inhibitory postsynaptic currents in CA1 pyramidal cells. However, beside the expression in GABAergic neurons, some electrophysiological experiments also suggested that glutamatergic principal cells may carry presynaptic CB1 receptors, which may explain their interference with long-term potentiation induction.4,31,38 In contrast, CB1 mRNA was only found in these principal cells at low levels, slightly above the background, or low to moderate levels in the aged human hippocampus.25–27,29,46

In the present study, we aimed to identify the precise cellular expression pattern and the subcellular localization (final plasma membrane destination) of CB1 receptors, and to investigate cannabinoid effects on GABA release in the human hippocampus.

EXPERIMENTAL PROCEDURES

Four control post mortem human hippocampi and seven temporal lobe samples surgically removed from patients with tumor-induced epilepsy were used in this study. Control brain tissue was obtained from a 53-year-old man who died from suffocation, and from a 58-year-old woman, a 51-year-old man and a 65-year-old man who all died following a cardiac arrest. None of the control subjects had any history of neurological disorders. Brains were removed 2 h after death. The dissection was performed in the Semmelweis University Medical School, and the regulations of the Hungarian Ministry of Health were followed. Patients with intractable tumor-induced temporal lobe epilepsy underwent surgery within the framework of the Hungarian Cooperative Epilepsy Surgery Program, and part of the temporal cortex with the hippocampal formation (anterior half towards the uncus) was removed. After the post mortem or surgical removal, the tissue for anatomical experiments was immediately dissected by a blade into 2-mm thick blocks, and immersed in a fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde and 0.2% picric acid in 4% glutaraldehyde.
0.1 M phosphate buffer. The fixative was changed to a fresh solution every hour with constant agitation for 6 h, then the blocks were post-fixed in the same fixative overnight.

Immuno-staining of the slices was performed as described earlier. For immunoperoxidase and immunogold staining polyclonal rabbit antiserum against CB1 receptor N-terminal end (1:1000), CB1 receptor C-terminal tail (1:5000; specificity was confirmed by lack of immuno-staining in the CB1 knockout mice, parvalbumin, (PV, 1:2000), and cholceystokinin, (CCK, 1:10,000) were used as described earlier. For immunofluorescence staining, the rabbit antibodies against CB1 were used together with monoclonal mouse antiseria against PV (1:1000, Sigma, St Louis, MO, USA) or CCK (1:2000; CURE Gastro-enterology Center, Los Angeles, CA, USA). Cy3-conjugated anti-rabbit immunogold globulin (IgG) made in donkey (1:200) and FITC-conjugated anti-mouse IgG made in goat (1:100; both from Jackson ImmunoResearch, West Grove, PA, USA) were used as secondary antibodies. The specificity of each antiseria has been thoroughly tested by the laboratories of origin. In some immunogold-stained sections, silver-intensification was followed by incubation in 0.05 M AuCl3 for 8 min at 4°C to prevent extensive loss of silver granules during dehydration. This treatment results in gold particles with irregular shapes instead of the conventional round ones. This data is not shown. These fibers were studied with numerous axon terminals. Light microscopically, the N-termminus and C-termminus antibodies labeled the same cell population and showed matching patterns of axon distribution, but the novel C-terminal antibody visualized a much larger number of axon terminals.

CB1-immunoreactivity was restricted to interneurons (Fig. 1A, B). Neither granule cells of the dentate gyrus (Fig. 1A), nor pyramidal cells in the cornu ammonis and the subicular complex (Fig. 1) were positive for CB1. Based on the axonal staining pattern (the strongest labeling was found in the principal cell layers, suggesting that perisomatic inhibitory cells predominate among the CB1-positive types) and previous results in rodents, we investigated whether CB1 is expressed by the CCK- or PV-containing interneurons. These markers primarily visualize, although CCK not exclusively, perisomatic inhibitory cells (basket cells) in both the human and rodent hippocampus. We found that co-localize with CCK in all layers of the hippocampal formation and the subiculum (Fig. 1C, D). In contrast, the PV-containing perisomatic inhibitory cells were always negative for CB1 (Fig. 1E, F). Both evaluation methods gave similar co-localization ratios in all cases (see Tables 1 and 2 for details). As predicted by these results, CCK- and PV-immunoreactive neurons indeed represent non-overlapping populations, since none of the PV-immunoreactive neurons were positive for CCK (n = 30) or vice versa (n = 24).

RESULTS

One possible explanation for the controversy over which cell types express CB1 in the hippocampus may be the existence of different splice variants of CB1 mRNA. Therefore, together with our former antibody directed against the extra-cellular N-termminus epitope of CB1, we also used a novel antibody directed against the intracellular C-terminal tail of the receptor. This domain is identical in the two mRNA isoforms described to date. Both antibodies were verified to be specific for CB1 by using CB1 knockout mice, cell transfection experiments and western blotting. All immunostaining described below were performed with both antisera.

Expression of the CB1 neuronal cannabinoid receptor is confined to specific interneuron types in the rodent hippocampus

Immuno-staining for CB1 revealed numerous CB1-positive cell bodies scattered in all layers of the dentate gyrus, cornu ammonis and the subicular complex (Fig. 1). Immuno-staining of dendrites was not observed, but a dense meshwork of CB1-immunoreactive axons covered the entire hippocampal formation. The strongest axonal labeling was found in the stratum molecular of the dentate gyrus (Fig. 1A), and in stratum pyramidale of CA1–CA3 (Fig. 1B) and the subicular complex (data not shown). These fibers were studied with numerous axon terminals. Light microscopically, the N-term minus and C-term minus antibodies labeled the same cell population and showed matching patterns of axon distribution, but the novel C-terminal antibody visualized a much larger number of axon terminals.

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CB1 cannabinoid receptors are located presynaptically on GABAergic axon terminals in the human hippocampus

At the electron microscopic level, CB1 was located exclusively on axon terminals that form synaptic specializations, indicating that only GABAergic neurones express this receptor (Fig. 2). These boutons innervated both somata and dendrites of presumed pyramidal and granule cells (Fig. 2F, G). Immunogold staining confirmed the remarkable specificity of the antibodies, since labeling with the N-terminal antibody was located exclusively on the extracellular surface of the axon terminal membrane, whereas the C-terminal antibody labeled only the intracellular side. Due to its greater sensitivity, the C-terminal antibody revealed an extremely high density of CB1 receptors on these GABAergic boutons. Essentially the entire bouton, except the synaptic active zone, was covered by gold particles representing CB1 (Fig. 2D, E and G). These boutons contained several dense-core vesicles (Fig. 2A), possibly the sites of CCK storage. Indeed, combined immunogold–immunoperoxidase
Fig. 1. CB1-immunostaining with the C-terminus antibody in the human hippocampus. (A) Low-power light micrograph showing the distribution of CB1-immunoreactive profiles in the human dentate gyrus. Cell bodies typical of interneurons (arrows) contain CB1. Note the lack of dendritic labeling. In contrast to interneurons, granule cells (asterisks) are always negative for CB1. The vast majority of staining is confined to a dense meshwork of axons (arrowheads depict some typical fibers with chains of boutons) in all layers. (B) In the CA1 subfield, the most profuse axonal immunostaining is present in stratum pyramidale. Arrowheads point to bouton-laden axon collaterals. Compare the CB1-immunoreactive cell body (thin arrow) with the CB1-negative principal cells (some labeled by asterisks). Thick arrow depicts lipofuscin granules in a characteristic, immunonegative pyramidal cell. (D–F) Double immunofluorescence staining reveals co-localization of CCK with CB1 in a cell body (s in C and D), whereas PV does not co-distribute with CB1 (E, F). The thick white arrow depicts the lipofuscin granules in the soma of a PV-positive, but CB1-negative neuron. Small white arrows indicate other lipofuscin granules used as landmarks. sm, stratum moleculare; sg, stratum granulosum; h, hilus; DG, dentate gyrus; CA1, subfield of cornu Ammonis. Scale bars 80 μm (A), 20 μm (B: C; also applies to D–F).
Fig. 2. Presynaptic localization of CB1 on inhibitory axon terminals in the human hippocampus. Silver-intensified gold particles (thin arrows) representing CB1-immunoreactivity outline axon terminals. The antibody directed against the extracellular N-terminal portion of CB1 labels the extracellular side of the plasma membrane (A–C in serial sections, which are separated by −0.25 μm in the tissue and F), whereas the one directed against the intracellular C-terminal tail of CB1 gives rise to immunostaining on the intracellular surface of the bouton membrane (D–E in serial sections, which are separated by −0.25 μm in the tissue and G). The difference in the shape of gold particles is due to gold stabilization of the silver granules as described in Methods. Both antibodies label boutons (b) that establish symmetric, presumably GABAergic synapses (thick arrows). Boutons establishing asymmetric synapses (star in D and E) are always negative for CB1. The CB1-immunoreactive axon terminals frequently contain dense-core vesicles (arrowheads in A and H). Combined immunogold-immunoperoxidase staining reveals that the vast majority of CB1-positive boutons contain CCK, a neuropeptide present in a subset of basket cells. Note the localization of the electron dense DAB precipitate representing CCK in boutons covered by gold particles identifying CB1 receptors (F, G). As shown in H, PV, a calcium-binding protein present in another subset of basket cells, does not co-localize with CB1. Asterisk depicts a PV-immunoreactive axon terminal.

The major postsynaptic targets of CB1-positive boutons are dendrites (d in F) and somata (s in G) of pyramidal and granule cells. Scale bars = 0.2 μm.
staining for CB1 and CCK revealed the co-localization at the axon terminal level as well (Fig. 2F, G; Tables 1, 2). In contrast, PV-positive axon terminals did not carry CB1 receptors (Fig. 2H). Glutamatergic boutons forming asymmetric synapses were extensively searched for the presence of CB1 in all layers, but none of them were CB1-positive (Fig. 2D, E). Except for occasional gold particles not associated with any subcellular compartment or the plasma membrane, we could not find CB1 receptors postsynaptically or extrasynaptically, either on dendrites or somata.

**CB1 cannabinoid receptor activation reduces [3H]GABA release in the human hippocampal slice**

To understand the functional consequences of presynaptic cannabinoid receptor activation on GABAergic networks of the human hippocampus, [3H]GABA release from human hippocampal slices was studied. The slices were surgically removed from patients with tumor-induced, therapy-resistant epilepsy, and showed no sclerotic damage in the CA1 subfield (see Experimental procedures). The pattern of CB1-immuno-staining in all of these patients was similar to post mortem controls (Table 1). After 60-min incubation, the radioactivity uptake in the slices was $9.70 \pm 1.42 \times 10^6$ Bq/g ($n = 16$), and the overall resting [3H]GABA efflux was $0.173 \pm 0.019\%$ ($n = 16$). This is comparable to the results in our previous experiments performed on superfused rat hippocampal slices. Electrical field stimulation (25 V, 3 ms, 10 Hz, 360 pulses) caused a reversible increase in tritium outflow ($S_1 = 0.148 \pm 0.05\%$, $n = 7$), and a similar increase was

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<th>Table 1. CB1 containing interneurons express cholecystokinin, but not parvalbumin in the human hippocampus</th>
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<td><strong>Ratio of CB1-immunoreactive profiles</strong>&lt;br&gt;(cell bodies or axon terminals) immunoreactive also for the interneuronal markers</td>
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At the somatic level, the mirror technique and double-immunofluorescence staining was used to establish the proportion of CB1-containing cells expressing the neurochemical markers of distinct interneuron types. At the electron microscopic level, combined immunogold (for CB1) and immunoperoxidase (for CCK or PV) staining was used. $n = the total number of investigated profiles in a given experiment.

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<th>Table 2. Characteristic interneuron types of the human hippocampus visualized by their neurochemical markers differ in their CB1 receptor content.</th>
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At the somatic level, the mirror technique and double-immunofluorescence staining was used to establish the proportion of cells of distinct interneuron types expressing CB1 receptor. At the electron microscopic level, combined immunogold (for CB1) and immunoperoxidase (for CCK or PV) staining was used. $n = the total number of investigated profiles in a given experiment.
observed in response to the subsequent stimulation period ($S_2$), resulting in an $S_2/S_1$ ratio of 1.03 ± 0.02, ($n = 7$) in control experiments (Fig. 3A). We used 1 μM WIN 55,212-2, because this concentration is known to be the maximal selective concentration for cannabinoid receptors and elicited maximal effects in rat hippocampal slices as well.

Indeed, 1 μM WIN 55,212-2 reduced electrical field stimulation-induced [$\text{H}]$GABA release (Fig. 3B): the $S_2/S_1$ ratio was 0.51 ± 0.09 ($P < 0.01$ vs control, $n = 9$), which corresponds to approximately 49% inhibition. This is in good agreement with the results obtained in rat (0.53 ± 0.06, $n = 7$, 48% inhibition, see Ref. 18). However, the basal outflow of [$\text{H}]$GABA did not change in the presence of WIN 55,212-2 (Fig. 3B). The effect of WIN 55,212-2 (1 μM) was also examined under the blockade of N-methyl-D-aspartate (NMDA) and non-NMDA-type glutamate receptors, i.e. in the presence of AP-5 (10 μM) and CNQX (10 μM). The inhibitory action of WIN 55,212-2 on stimulation-induced [$\text{H}]$GABA outflow remained unaffected under these conditions (Fig. 3C). In the presence of SR 141716A (1 μM), the CB1 receptor antagonist, neither the basal, nor the stimulation-induced outflow of tritium were altered significantly (data not shown). However, SR 141716A effectively prevented the action of WIN 55,212-2 to reduce field stimulation-evoked [$\text{H}]$GABA efflux, resulting in $S_2/S_1$ ratios similar to control (Fig. 3C).


**DISCUSSION**

**Similar distribution pattern of CB1 cannabinoid receptors in the human and rodent hippocampus**

Our results suggest that the CB1 receptor is located presynaptically on GABAergic axon terminals, since CB1-immunoreactivity was confined to varicosities forming exclusively symmetrical synapses, and the CB1 receptor agonist was shown to inhibit GABA release. Alternatively, it is possible that subcortical pathways also forming symmetrical synapses may express the CB1 receptor as well.

*In situ* hybridization studies indicate low to moderate CB1 expression levels in pyramidal cells, but despite the extensive search and use of novel antisera recognizing both proposed splice variants of CB1, neither the cell bodies and dendrites, nor the axon terminals of pyramidal cells were found to express this receptor. In contrast, this complete lack of immunostaining in pyramidal cells does not exclude the possibility that our antibodies could recognize only those cells, which express high levels of CB1. No CB1 labeling was observed on postsynaptic membranes in this study with either antibodies (N- and C-terminals), in contrast with previous experiments on monkeys. In that study, the same antibody, recognizing the N-terminal domain of CB1, was used as here. However, despite the extracellular localization of the epitope, the labeling was observed intra- or extracellularly. Although an extremely high rate of internalization of the receptor may theoretically lead to this pattern, our results failed to confirm this. Furthermore, the selective expression pattern of CB1 in different interneuron types of the human hippocampus matches that found in rodents. Namely, CB1 was expressed in interneuron types characterized by the presence of the neuropeptide CCK. Taken together with the surprisingly conserved structure of the CB1 gene in mammals, which may underlie the same expression pattern and subcellular targeting, these data strongly suggest that the localization and, thereby, the function of CB1 in the hippocampus is evolutionarily conserved. Interestingly, a strikingly similar localization of CB1 was recently shown in the retina of different vertebrate species as well.
CB1 cannabinoid receptor activation leads to a reduction of GABA release both in the human and rodent hippocampus

In parallel with the similarity of anatomical results obtained in rats and humans, we found that the release of [3H]GABA measured from the superfused human hippocampal slices is inhibited by CB1 receptor activation to a similar degree as in rats. The amount of restitute and stimulation-induced outflow of [3H]GABA was in a range similar to that in rats indicating that, in spite of differences in preparation, human hippocampal slices preserve their functional integrity to take up and release [3H]GABA from neuronal stores. Thus, it served as a reliable model system to study the effects of cannabinoids on the release of GABA. WIN 55,212-2 (1 μM), reduced field stimulation-evoked release of [3H]GABA from human hippocampal slices, and its effect was prevented by the selective CB1 receptor antagonist SR 141716A. As the possible effect of WIN 55,212-2 on the uptake of [3H]GABA has already been excluded in our previous study and its effect was preserved in the presence of the NMDA and non-NMDA receptor antagonists CNQX and AP-5, these results suggest that the evoked release of [3H]GABA in the human hippocampus is inhibited by presynaptic CB1 receptors located on the nerve terminals of CCK-positive interneurons. Whether CB1 receptors negatively modulate the release of CCK from these axon terminals as well is also a very important issue, and is quite likely considering the strikingly opposite effects of CCK and cannabinoids on hippocampus-related behaviours. CCK has been shown to enhance memory formation by acting on hippocampal neurons, and have strong anxiogenic effects, whereas cannabinoids interfere with memory formation and the endogenous tone they mediate may alleviate anxiety. 13,32,37,41

One may argue that the magnitude of suppression of GABA release (~50%) is much higher than the number of CCK-containing interneurons in the hippocampus estimated to represent only 10–20% of all hippocampal interneurons in the rat. Indeed, not all CB1-containing interneurons expressed CCK (see Table 1 for details). In the tissue derived from tumor-induced epileptic tissue (in which the GABA release experiments have been done), we found that almost one-third of the CB1-positive cells were negative for CCK. In contrast, the absolute number and size of the boutons may vary widely across different interneuron types,12,16,30 which likely correlates with the amount of released GABA, and may also explain the discrepancy between the quantitative data of the anatomical and pharmacological experiments.

Implications of CB1 receptor localization for network synchrony and memory functions

Our results indicate that the hippocampus-related behavioral influences of exogenous cannabinoids and the effects of endocannabinoids present in human brain as well11 are likely due to their actions on GABAergic interneuronal networks. Although the ultrastructure of post mortem human controls did not allow precise quantification of the percentage of GABAergic synapses bearing CB1, the magnitude of reduction in GABA release (~50%) and the dense meshwork of CB1-positive axons in all hippocampal layers corresponding to the distribution and density of cannabinoid binding sites of the human hippocampus14,18,46 suggest that cannabinoids have a profound effect on hippocampal inhibitory circuits mostly on interneurons containing CCK that include basket cells and others arborizing in dendritic layers. Even though the interneuron types in the human hippocampal formation are not as well defined as in rat,12 the strong correlation of the human and rat expression patterns and the dense axonal arborization in the principal cell layers indicate that perisomatic inhibitory interneurons (basket cells) are the major targets of cannabinoids.

Interneurons are necessary for synchronization of the large principal cell populations10,47,49 underlying memory consolidation and precise association of distinct external inputs.8,40 Interestingly, another drug with similar effects on associations, namely morphine, was shown to disrupt the long-range synchronized oscillatory patterns of rodent hippocampal networks.48

CONCLUSION

We propose that cannabinoids, by acting presynaptically on GABAergic interneurons, may also disrupt synchronized oscillations in certain frequency ranges, and thereby interfere with memory consolidation and internal representations in humans.

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