Cell-specific STORM super-resolution imaging reveals nanoscale organization of cannabinoid signaling

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A major challenge in neuroscience is to determine the nanoscale position and quantity of signaling molecules in a cell type– and subcellular compartment–specific manner. We developed a new approach to this problem by combining cell-specific physiological and anatomical characterization with super-resolution imaging and studied the molecular and structural parameters shaping the physiological properties of synaptic endocannabinoid signaling in the mouse hippocampus. We found that axon terminals of perisomatically projecting GABAergic interneurons possessed increased CB1 receptor number, active zone complexity and receptor/effector ratio compared with dendritically projecting interneurons, consistent with higher efficiency of cannabinoid signaling at somatic versus dendritic synapses. Furthermore, chronic ∆9-tetrahydrocannabinol administration, which reduces cannabinoid efficacy on GABA release, evoked marked CB1 downregulation in a dose-dependent manner. Full receptor recovery required several weeks after the cessation of ∆9-tetrahydrocannabinol treatment. These findings indicate that cell type–specific nanoscale analysis of endogenous protein distribution is possible in brain circuits and identify previously unknown molecular properties controlling endocannabinoid signaling and cannabis-induced cognitive dysfunction.

Mechanistic understanding of biological processes requires integrated analysis of structural and functional parameters together with their underlying molecular dynamics. However, this is uniquely difficult to achieve in the brain given its cellular and molecular diversity1–4. Cortical circuits are composed of many types of excitatory principal cells and inhibitory interneurons1, which have distinct computational roles in network activity4. To sculpt their synaptic interactions, autonomous cell type– and synapse-specific processes dynamically optimize the position and density of hundreds of signaling molecules5,5. Despite considerable efforts2, cell type–specific nanoscale imaging of synaptic proteins in combination with physiological and morphological investigations has remained technically challenging in intact brain circuits. As a result, our knowledge of the quantitative molecular properties dictating various structural and functional parameters of synaptic transmission remains incomplete.

Retrograde endocannabinoid signaling mediates many forms of synaptic plasticity via CB1 cannabinoid receptor activation6,7. Although CB1 is one of the most widespread presynaptic regulators of neurotransmitter release in the brain8, the principles characterizing its cell type–specific subcellular distribution and density have remained elusive. The locus classicus for the demonstration of presynaptic CB1 receptors9 and their involvement in retrograde signaling is the hippocampal GABAergic synapse10,11. Two major forms of CB1-containing GABAergic interneurons are specialized to target either the perisomatic or dendritic regions of principal cells1,12,13. Notably, perisomatic and dendritic inhibition have markedly different physiological functions14. Consistent with this functional division of labor, endocannabinoid-mediated synaptic plasticity is remarkably stronger at synapses derived from perisomatically projecting CB1-positive interneurons compared to synapses belonging to dendritically projecting CB1-expressing cells15. Moreover, low doses of cannabinoids inhibit GABA release from perisomatic interneurons, but not from dendritic cells15. Although these findings imply that some of the molecular properties underlying CB1-dependent synaptic regulation must be different at specific circuit locations, our understanding of the molecular parameters determining the strength of retrograde synaptic transmission is still very limited.

Cannabinoid signaling is also altered in a cell type–specific manner under pathophysiological conditions. For example, exposure to ∆9-tetrahydrocannabinol (THC), the psychoactive cannabis constituent, strongly diminishes cannabinoid signaling efficacy on GABA release, but not on glutamate release16, emphasizing the cell type–specific nature of pathophysiological alterations involving CB1 receptors. THC has been suggested to cause cognitive deficits primarily by activating

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cannabinoid receptors on GABAergic boutons\textsuperscript{17,18}, but the molecular mechanisms underlying THC-induced functional tolerance and perturbed cannabinoid signaling remain poorly understood.

These findings highlight the need for a cell type–specific method for high-yield, high-resolution analysis of protein distribution in brain circuits. The recent advent of super-resolution microscopy has introduced potential alternatives for molecular imaging\textsuperscript{19}. We developed a rapid and versatile approach based on stochastic optical reconstruction microscopy (STORM)\textsuperscript{20} for cell type–specific super-resolution imaging. As a proof of principle, we applied our new methodology to the study of the molecular and structural heterogeneity and pathophysiological plasticity of synaptic endocannabinoid signaling. Our findings describe the cell type–specific molecular properties of cannabinoid-sensitive GABAergic axon terminals at a nanoscale resolution and elucidate functional tolerance-related changes in CB\textsubscript{1} distribution after chronic THC exposure, providing insights into the domain-specific efficiency of endocannabinoid signaling and the mechanisms underlying cognitive effects of cannabis use.

RESULTS

STORM super-resolution imaging of CB\textsubscript{1} cannabinoid receptors

We first developed an approach to combine confocal and STORM imaging and tested whether it reliably determines CB\textsubscript{1} receptor position in a cellular membrane environment using an EGFP-tagged CB\textsubscript{1} construct (Supplementary Fig. 1a and Online Methods). Acquiring correlated confocal and three-dimensional STORM (3D-STORM) images revealed that both the intrinsic EGFP fluorescence signal and the STORM signal representing immunostaining for CB\textsubscript{1} were concentrated in the plasma membrane of HEK cells (Supplementary Fig. 1b,c). However, although the outline of the membrane was not resolved in the confocal image as a result of light diffraction, sub-diffraction limit STORM imaging visualized sharp plasma membrane contours (Supplementary Fig. 1e,f). Notably, the number of CB\textsubscript{1} localization points (CB\textsubscript{1} NLPs) exhibited a very tight, positive correlation with the corresponding EGFP fluorescence intensity (Supplementary Fig. 1d), emphasizing the quantitative potential of 3D-STORM imaging to compare receptor distribution between distinct anatomical profiles or to measure molecular changes in the same profile.

To enable high-throughput, quantitative, cell type–specific nanoscale analysis of endogenous protein distribution in brain circuits, we then designed widely applicable tissue processing and immunolabeling protocols for 3D-STORM microscopy (Online Methods). We examined presynaptic CB\textsubscript{1} receptor distribution in 7,051 individual GABAergic axon terminals at an imaging speed of 2 min and a measured fluorophore localization precision of 6 nm in the lateral and 41 nm in the axial dimensions, as determined from the same images obtained at ~5 µm depth (Supplementary Fig. 2). Thus, this rapid and efficient workflow revealed protein distribution with high precision, but allowed a sample size that is beyond the reach of immunogold electron microscopy. In hippocampal sections derived from CB\textsubscript{1}\textsuperscript{−/−} (also known as Cnr1) mice, STORM imaging uncovered high CB\textsubscript{1} receptor density on cholecystokinin (CCK)-containing GABAergic axon terminals forming typical basket-like arrays around the

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\caption{3D-STORM imaging of CB\textsubscript{1} receptors on hippocampal GABAergic axon terminals. (a) Immunofluorescence labeling of CB\textsubscript{1} receptors in the CA1 stratum pyramidale of CB\textsubscript{1}\textsuperscript{+/+} mice showed a basket-like arrangement of CB\textsubscript{1}-positive axon terminals encircling a CB\textsubscript{1}-immunonegative pyramidal cell somata. (b) These axon terminals could also be visualized by immunofluorescence staining for the neuropeptide CCK, a neurochemical marker of CB\textsubscript{1}-expressing interneurons. (c) STORM imaging of CB\textsubscript{1} receptors revealed identical basket-like orientation of the same perisomatic GABAergic axon terminals. (d,e) Magnifying the epifluorescence images failed to delineate the morphological structure of the respective profiles located in the boxed region or the precise nanoscale position of CB\textsubscript{1} receptors with the axon terminals. (f) In contrast, the improved spatial resolution of 3D-STORM microscopy made it possible to reliably discern individual CB\textsubscript{1}-positive boutons located adjacently to one another, as well as to reveal localization points representing the nanoscale position of CB\textsubscript{1} receptors. (g,h) To validate the specificity of our approach, we also processed and imaged hippocampal sections from littermate CB\textsubscript{1}\textsuperscript{−/−} mice under the same conditions. In these sections, we found an almost complete absence of STORM localization points representing CB\textsubscript{1} in CCK-immunolabeled boutons. (i) Histogram of CB\textsubscript{1} NLPs in individual CCK-positive varicosities in the CA1 stratum pyramidale of sections derived from littermate CB\textsubscript{1}\textsuperscript{+/+} (n = 3 animals, n = 208 axon terminals) or CB\textsubscript{1}\textsuperscript{−/−} (n = 2 animals, n = 200 axon terminals) mice. Note that the markedly reduced CB\textsubscript{1} NLP value in CB\textsubscript{1}\textsuperscript{−/−} samples validates the specificity of the antibody, the staining process and the STORM imaging protocol. Scale bar in panel a also applies to panels b and c; scale bar in panel d also applies to panels e and f.}
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CB1-immunonegative somata of CA1 pyramidal neurons (Fig. 1a–c). Notably, adjacent CB1- and CCK-positive boutons were often difficult to resolve in diffraction-limited immunofluorescence images (Fig. 1d,e), whereas STORM imaging reliably resolved the same structures (Fig. 1f). In contrast, the absence of localization points in CCK-positive boutons derived from littermate CB1−/− mice validated antibody specificity and the staining and imaging protocols (Fig. 1g–i).

Taken together, these results demonstrate that nanoscale molecular investigations in brain circuits are feasible with high-yield, strong sensitivity and good specificity by using 3D-STORM imaging.

**Cell- and synapse-specific nanoscale molecular imaging**

The precise subcellular localization and density of synaptic proteins vary in relation to physiological and pathophysiological processes in a cell type–specific manner. STORM imaging provides a list of three-dimensional coordinates of target protein localizations, but does not readily position these coordinates in the cell type–specific context required for functional interpretation in light of the immense cellular complexity of brain circuits. Thus, we developed an approach combining patch-clamp electrophysiology, confocal imaging of biocytin-filled neurons and STORM super-resolution imaging of target proteins overlaid on the identified subcellular structure (Online Methods). As a major advantage, this combination of methods allows the collection of physiological and morphological information from the same neuron, providing quantitative data on multiple target protein distributions in its identified axon terminals in 1 week (Fig. 2).

Despite its prominent role in neurotransmitter release regulation, the cell type–specific principles establishing CB1 number and location on a given axon terminal type remain unknown. To address this issue, we first performed whole-cell, patch-clamp recordings from multipolar neurons located in CA1 stratum radiatum in acute slice preparations. Most neurons exhibited an accommodating regular-spiking firing pattern (Fig. 2a,g), a physiological signature of CB1- and CCK-expressing interneurons. These cells were filled with biocytin during recording and then imaged and reconstructed by confocal microscopy and Neuronlucida (Fig. 2b,h). To classify these interneurons quantitatively, we developed a bouton distribution index (BDI) based on descriptive statistics of the extent of layer-specific varicosity concentration (n = 997 ± 58 boutons per cell, n = 79 cells, Online Methods). Notably, BDI unequivocally discriminated these cells as either perisomatic interneurons (BDI > 1), such as basket cells, which synapse on the soma and proximal dendrites of principal cells in stratum pyramidale (Fig. 2b,c), or dendritic interneurons (BDI < 0.5), such as Schaffer collateral–associated cells, which preferentially target the distal dendritic shafts of pyramidal neurons in strata radiatum and oriens (Fig. 2h,i). These perisomatic and dendritic interneurons also differed in certain physiological characteristics, such as spike width, afterhyperpolarization amplitude and accommodation ratio (Supplementary Fig. 3 and Supplementary Table 1).

Subsequent CB1-immunostaining for STORM imaging was performed on 20-µm-thick free-floating sections prepared from the acute slice. Identified axon terminals of perisomatic or dendritic interneurons were imaged by confocal microscopy (n = 46 ± 2 boutons per cell; Fig. 2d,j). CB1 localization points in a given varicosity were then visualized by 3D-STORM microscopy overlaid on the corresponding confocal image (Fig. 2e,f,k,l). We conducted several control experiments, which ruled out possible distorting effects of differences in tissue handling (Supplementary Fig. 4a), in antibodies (Supplementary Fig. 4b) and in whole-cell manipulations (Supplementary Fig. 4c,d).

Collectively, these results illustrate that cell type–specific STORM imaging can readily be performed in brain circuits and showcase the combination of physiological and anatomical analyses with nanoscale molecular imaging in CB1-positive perisomatic and dendritic interneurons.

**CB1 abundance scales with bouton size in GABAergic cells**

Because cannabinoid signaling is more effective at perisomatic GABAergic synapses compared with dendritic connections, we next aimed to determine which molecular and structural parameters may contribute to this cell type–specific physiological difference. Axon terminals of perisomatic and dendritic interneurons had similar shapes (Supplementary Fig. 5), but the former were larger (Fig. 3a,b). Similarly, perisomatic terminals contained 39% more CB1 receptors than dendritic boutons (Fig. 3c). This observation obtained at the cell-type level raises the possibility that these two parameters are closely related even at the individual bouton level. Indeed, both the perimeter and the area were significantly correlated with CB1 NLPs in both cell types (P < 0.001; Fig. 3d), implying that bouton size is a major factor that predicts CB1 receptor number on a given axon terminal.

The cell type–specific difference in CB1 abundance may influence cannabinoid signaling efficacy, but it is unlikely to be the sole explanation for the higher efficacy at perisomatic synapses, as neither overall CB1 density (Fig. 3e,f) nor local CB1 density in 200-nm-sized surface nanodomains exhibited cell type–specific differences (Supplementary Fig. 6a). Additional analyses demonstrated that CB1 labeling density on interneuron terminals fell in the dynamic range of the measurement (Supplementary Fig. 6a–c), and the imaging conditions resulted in reliable CB1 distribution maps (Supplementary Fig. 6d–f). Moreover, independent experiments using CB1-immunogold labeling and electron microscopy revealed that randomly selected CB1-positive perisomatic terminals were larger and had more CB1 receptors than dendritic boutons, but CB1 density was not different (Supplementary Fig. 7). The distribution of normalized data obtained by 3D-STORM and electron microscopy did not differ (n = 94 and 200 boutons, respectively; Kolmogorov-Smirnov test, P > 0.1), corroborating that, when sampling the same bouton population, the two approaches generate comparable information.

On the other hand, CB1 NLP in STORM imaging was 2.5–5-fold greater than CB1-immunogold particle number (taking section thickness into account), offering an additional advantage for quantitative investigation of subtle molecular changes in protein quantity.

The electron microscopic analysis also showed that the vast majority of CB1 receptors were integrated into the plasma membrane (88 ± 1% and 85 ± 1% of gold particles in perisomatic and dendritic boutons, respectively; Supplementary Fig. 7a,b). Thus, the perimeter value determined from a two-dimensional convex hull fitted on CB1 localization data is expected to give good estimation of bouton size (Fig. 3b). However, CB1 receptor densely covers the entire bouton surface, whereas several other presynaptic proteins are only sparsely distributed. Thus, their visualization may not optimally support correlation morphological and molecular investigations with nanoscale precision. To circumvent this obstacle, we exploited the fact that biocytin fills out the cytoplasmic space and performed 3D-STORM imaging of streptavidin-labeled biocytin staining in interneuron boutons (Fig. 3g–i). Notably, two-dimensional convex hull fitting onto biocytin localization points allowed bouton size measurements and confirmed that axon terminals derived from perisomatically projecting interneurons were larger than those originating from dendritic interneurons (Fig. 3m). Finally, the three-dimensional coordinates of biocytin localization points were illustrated using Visual Molecular Dynamics, and a three-dimensional convex hull was fitted onto the
biocytin localization points. This geometric approach generated a polyhedron representation of the bouton volume and facilitated intuitive evaluation of the acquired images (Fig. 3i). When dual-channel directSTORM imaging was performed on biocytin-filled interneuron terminals by combining Alexa 647–CB1 immunostaining with Alexa 488–streptavidin staining (Fig. 3n), the three-dimensional convex hulls fitted onto the localization points from either channel also visualized similar bouton surfaces (Fig. 3o). Notably, this configuration also enables cell type–specific nanoscale distribution analysis of less densely expressed receptors.

Uniform CB1 receptor distribution on GABAergic boutons

Postsynaptic neurotransmitter receptors are concentrated in highly organized nanodomains22–24, but it is not known whether such topological restrictions also characterize presynaptic neuromodulator receptor distributions. Interneuron type–specific coupling distances between voltage-gated calcium channels (VGCCs) and the calcium sensor for vesicle release critically determine release properties25. A cell type–specific CB1 nanodomain organization resulting in distinct coupling distances to their effectors, the N-type VGCCs26, would also offer a potential explanation for the physiological differences in cannabinoid signaling. We therefore took advantage of another aspect of STORM microscopy, its ability to perform nanodomain-specific distribution analysis of multiple target proteins in the same sample27.

Bassoon is a presynaptic cytomatrix protein and a constituent of the release machinery with an essential function in organizing...
VGCC distribution in the active zone. We first asked whether 3D-Storm imaging reliably visualizes bassoon in the active zone in CB1-containing perisomatic and dendritic axon terminals (Fig. 4a–f). CB1-positive varicosities impinging on the somatodendritic surface of biocytin-labeled postsynaptic CA1 pyramidal neurons were identified by confocal microscopy (Fig. 4a,b,d,e). Double 3D-Storm imaging of CB1-positive terminals consistently revealed intra-terminal bassoon accumulation facing the postsynaptic pyramidal neuron (Fig. 4c,f), visualizing the position of the synaptic active zone.

To decipher the nanoscale distribution of CB1 receptors in relation to the active zone in a cell type-specific manner, we next acquired bassoon and CB1 localization coordinates in identified axon terminals of individually labeled perisomatic or dendritic GABAergic interneurons (Fig. 4g–j and Supplementary Fig. 8a). In contrast with the nanodomain-restricted accumulation of postsynaptic ionotropic receptors, presynaptic CB1 receptors showed a uniform distribution at the nanoscale level (Fig. 4k–p). The mean Euclidean distance from the bassoon localization points was not different between measured and randomized CB1 distributions (n = 452–452 boutons, Mann-Whitney U test, P = 0.55), and there was no significant difference between interneuron types (P > 0.05, Fig. 4o).

However, G protein-coupled receptors, such as CB1, inhibit VGCCs via βγ subunits in a plasma membrane–delimited manner, indicating that molecular distance measurements on the membrane surface may lead to more functionally relevant information. To this end, the plasma membrane contour was approximated by fitting a three-dimensional convex hull onto the CB1 localization points. We first measured the distance of localization points from the polyhedron surface and estimated the ratio between membrane-associated localization points and those that may represent intracellular (for example, internalized) receptors (Supplementary Fig. 8b,c and Online Methods). Consistent with our electron microscopy data, the majority of CB1 was associated with the convex hull—approximated plasmalemma in both interneuron types (79 ± 1% and 81 ± 1%, n = 280 and 141 perisomatic and dendritic axon terminals, respectively). To make molecular distance measurements possible on the polyhedron surface,
we developed an approximative mathematical algorithm (Supplementary Fig. 8d and Online Methods). In selected boutons containing a single cluster of bassoon localization points, a second convex hull was fitted onto this bassoon cluster. Because bassoon is not an integral membrane protein, points on the bouton surface after fitting a three-dimensional convex hull on the CB1 localization points could be readily calculated. To allow intermolecular distance measurements along the structure surface, we determined the shortest paths between CB1 and bassoon localization points on the surface of identified subcellular compartments. Moreover, in contrast with the clustered organization of postsynaptic ionotropic receptors22–24, these measurements unveiled a uniform distribution of presynaptic CB1 receptors on both types of GABAergic axon terminals. Thus, the differential cannabinoid sensitivity of perisomatic and dendritic interneurons is unlikely to be explained by distinct nanoscale targeting of CB1 receptors.

Next, we investigated whether these data obtained in acute hippocampal slice preparations represent the natural in vivo state (Supplementary Fig. 10). In awake mice, we recorded the firing properties of an individual GABAergic interneuron in juxtacellular configuration in relation to local field potentials during rhythmic brain activity (Supplementary Fig. 10a). Concurrent biocytin labeling enabled its post hoc anatomical characterization and classification as a dendritic interneuron (Supplementary Fig. 10b,c). Subsequent
Cell type–specific and size-independent active zone architecture of GABAergic interneurons. **(a,d)** Deconvolved confocal images of boutons of persomitic and dendritic interneurons. **(b,e)** 3D-STORM images of bassoon immunostaining in identified axon terminals. **(c,f)** Density-based clustering (DBSCAN) revealed individual bassoon clusters (highlighted in different colors). **(g)** Boutons of persomitic and dendritic interneurons contained similar NLPs ($n = 349$ and $374$ boutons from $10$ persomitic and $12$ dendritic cells, respectively, Mann-Whitney $U$ test). **(h)** In contrast with the comparable bassoon NLPs (Kolmogorov-Smirnov test), persomitic boutons contained more CB1 localization points ($n = 279$ and $334$ boutons from $6$ and $6$ cells, Kolmogorov-Smirnov test), as determined by separate single-staining experiments. **(i)** Axon terminals of persomitic interneurons contained more bassoon clusters (Mann-Whitney $U$ test), whereas NLPs in individual clusters was lower ($j$). Graphs show raw data and median ± IQR. **(k)** Dual-channel STORM imaging demonstrated that CB1 NLPs in any given distance from bassoon labeling was higher in axon terminals of persomitic interneurons ($n = 80–80$ randomly selected boutons, Kolmogorov-Smirnov test, plot shows median ± IQR). **(l)** To analyze molecular properties independently of bouton size, subsamples of boutons comprising an identical size distribution were selected at random ($n = 200–200$ boutons, Kolmogorov-Smirnov test). **(m)** In these subsamples, persomitic axon terminals contained less bassoon, but a similar amount of CB1. **(n)** Although the number of bassoon clusters was similar, clusters in boutons of persomitic interneurons had less NLPs. **(o)** CB1 NLPs in any given distance from bassoon clusters were similar in identically sized subsamples of boutons of the two interneuron types ($n = 80–80$ boutons, Kolmogorov-Smirnov test).

Different nanoscale structure of interneuron active zones

The two observations that CB1 receptor density and nanoscale distribution pattern are similar on interneuron boutons imply that other molecular parameters account for cell type–specific cannabinoid signaling efficacy on GABA release probability. At central synapses, active zone size tightly correlates with VGCC abundance and transmitter release probability. The copy numbers of protein components in the active zone, including the quantity of bassoon and VGCC subunits, also correlate closely. Thus, we hypothesized that cannabinoid effects on neurotransmitter release may be influenced by the quantitative relationship between CB1 receptors and their nearby molecular effectors. To address this issue, we next gained insights into the organizational properties of the presynaptic release machinery of the two cannabinoid-sensitive GABAergic bouton types (Fig. 5).

In contrast with the homogeneous CB1 distribution on bouton surface, cell type–specific 3D-STORM imaging of single bassoon labeling revealed clustered bassoon organization in both interneuron terminal types (Fig. 5a,b,d,e). To quantitatively evaluate clustering, we performed density-based analysis of the localization points (Fig. 5c,f and Online Methods). Considering the larger size and higher CB1 numbers of persomitic terminals, bassoon NLPs in these clusters were similar in the two interneuron bouton types (Fig. 5g,h). When CB1 numbers were normalized to bassoon numbers, the resulting cell type–specific CB1/bassoon molecular ratio showed that persomitic interneuron terminals had, on average, 49% more CB1 receptors than those of dendritic interneurons. Given that bassoon and VGCC numbers in release machineries correlate, this proportional difference in receptor/effector ratio is likely to contribute to cell type–specific cannabinoid sensitivity.

Some CB1 receptors on bouton surface may not be able to directly regulate the release machinery resulting from their distant location from the active zone. Thus, we further exploited the improved localization precision of STORM imaging to focus our analysis on CB1 receptors located in the vicinity of bassoon clusters. Notably,
perisomatic axon terminals contained more bassoon clusters than boutons derived from dendritic interneurons (Fig. 5i), but individual bassoon clusters had 26% less bassoon localization points (Fig. 5j). Furthermore, regardless of the distance from bassoon clusters, substantially more CB1 localization points were observed in perisomatic boutons than in their dendritic counterparts. For example, 46% more CB1 localization points were found in the first 100-nm bin (which includes the ~80-nm distance of bassoon from the bouton surface) in perisomatic terminals (Fig. 5k). These observations are consistent with the more fragmented bassoon cluster architecture found in perisomatic boutons and the uniform CB1 nanoscale distribution. Together with the reduced bassoon number in individual clusters, these findings indicate that a higher receptor/effector ratio in the release machinery-containing nanodomain may facilitate more efficient coupling between CB1 and its nearby molecular effectors in perisomatic axon terminals.

The differences in the molecular parameters observed at the cell-type level raise the question of which parameters are instrumental at the individual bouton level. Considering that perisomatic terminals are larger and CB1 quantity correlates with bouton size in both interneuron types, it is plausible that some of the cell type–specific differences are emergent consequences of distinct axon terminal sizes. We therefore selected perisomatic and dendritic bouton samples with identical size distributions at random (Fig. 5l). Notably, CB1 NLPs were identical between axon terminals of perisomatically and dendritically targeting interneurons, but dendritic boutons contained more bassoon LPs than perisomatic terminals of the same size (Fig. 5m). As a consequence, the CB1/bassoon ratio was 27% higher in perisomatic boutons. In addition, although the number of bassoon clusters did not differ between cell types (Fig. 5n), bassoon NLPs in individual clusters were 33% higher in dendritic boutons (Fig. 5o). In accordance with having the same total number and a homogeneous distribution of CB1 in the identically sized boutons, CB1 NLPs were identical in any given distance from bassoon clusters (Fig. 5p). These findings indicate that similar numbers of adjacent CB1 receptors need to control more release machineries in dendritic boutons when comparing boutons of the same size. In conclusion, the cell type–specific differences in receptor/effector ratio and nanoscale distance derive from distinct bassoon quantity in individual bassoon clusters and emerge from the larger number of uniformly distributed CB1 receptors on bigger perisomatic axon terminals.

**Chronic THC treatment evokes robust CB1-downregulation**

To test whether CB1 receptor number and nanoscale position are two important parameters influencing the effect of cannabinoid signaling on GABA release, we performed chronic THC treatment. Such treatment causes a strong reduction in cannabinoid efficacy on GABAergic transmission16, but the underlying molecular mechanisms of functional tolerance remain unknown. We therefore investigated the effects of repeated THC exposure on CB1 number and distribution in identified axon terminals of perisomatic GABAergic interneurons. Chronic THC treatment was performed according to the established procedure (10 mg per kg of body weight, intraperitoneal, twice a day for 6.5 d)12, which evokes behavioral tolerance to classical cannabinoid effects and results in THC blood levels in rodents comparable to those observed after marijuana smoking in humans (~100 ng ml⁻¹)16,33,34. 3D-STORM imaging uncovered robust THC-induced CB1 downregulation (74%) in perisomatic boutons.
Furthermore, the intracellular CB₁ ratio was substantially increased by 58% in THC-treated animals (Fig. 6h). Thus, chronic THC treatment has two, likely inter-related effects: a marked internalization and a notable disappearance of CB₁ receptors from the boutons. Moreover, these results indicate that the reduction of CB₁ number on axon terminals underlies decreased cannabinoid effects on GABAergic currents after chronic THC administration.

Endocannabinoid signaling at GABAergic synapses sets the threshold for synaptic potentiation at nearby glutamatergic synapses[15,16]. Accordingly, chronic THC treatment prevents long-term potentiation (LTP) of excitatory synapses, which has been postulated to be caused by functional tolerance to cannabinoid inhibition of GABA, but not glutamate, release[16]. LTP only partially recovers after THC withdrawal and remains persistently weaker for weeks[16], which parallels slow, incomplete recovery in hippocampal cannabinoid binding density and in hippocampus-dependent memory deficits in clinical studies during THC abstinence[37,38]. Whether prolonged LTP and memory defects are associated with chronically reduced CB₁ numbers on inhibitory boutons has not yet been investigated. To monitor the time course of receptor recovery, we measured CB₁ NLPs and internalization 11.5 d after THC withdrawal, when classical cannabinoid behavioral effects have recovered[33], but substantially weaker hippocampal LTP still persists[16]. Notably, CB₁ NLP was 35% lower in chronic THC-treated mice compared with vehicle-treated animals (Fig. 6g), but there was no longer any detectable difference in internalization (Fig. 6h). Notably, presynaptic CB₁ numbers fully recovered by 6 weeks after drug treatment cessation (Fig. 6g).

Medical cannabis preparations are designed to achieve lower THC blood levels (~10 ng ml⁻¹) to avoid adverse psychoactive effects[39]. We tested the effects of a lower THC dose (1 mg per kg, intraperitoneal, twice a day for 6.5 d), which results in similar drug levels in the blood and does not elicit overt behavioral effects in rodents[40]. 3D-STORM imaging revealed that 1 mg per kg THC decreased CB₁ numbers only slightly (16%; Fig. 6g) and no internalization was observed (Fig. 6h). Thus, THC administration has dose-dependent effects on CB₁ quantity in GABAergic axon terminals, and the slow time course of receptor recovery may contribute to long-lasting neurophysiological changes in synaptic transmission. Finally, these experiments illustrate the power of 3D-STORM imaging to study physiologically and pathophysiologicaly relevant molecular changes at the nanoscale in identified subcellular compartments of targeted cell types.

**DISCUSSION**

Our methodology and the corresponding analysis tools were instrumental in characterizing the abundance and nanoscale distribution of CB₁ cannabinoid receptors on GABAergic axon terminals and uncovering cell type–specific molecular differences in the active zones of interneuron bouton types, which support distinct cannabinoid regulation of somatic and dendritic GABAergic inhibition. In addition, these tools helped to reveal the extent and time course of molecular changes induced by different doses of chronic THC treatment, which contribute to the functional tolerance in cannabinoid signaling efficiency at GABAergic synapses.

**Methodological implications**

Each brain circuit consists of numerous cell types; for example, more than 20 types of GABAergic interneurons have been specified thus far in the hippocampal CA1 subfield[4]. A stunning molecular diversity of the synapses connecting these various cell types supports the specific computing tasks of given brain circuits[4]. Synaptic protein abundance and spatial localization are precisely regulated by physiological or pathophysiological mechanisms in a cell type– and synapse-specific manner. For example, hippocampal CB₁ receptor levels are elevated at GABAergic synapses, but are decreased at glutamatergic synapses in mouse and human epileptic samples[41,42]. In Fragile X syndrome, endocannabinoid-dependent synaptic plasticity is enhanced at GABAergic synapses[43,44], but is absent at glutamatergic synapses as a result of a shift in the persynaptic position of a major endocannabinoid-synthesizing enzyme[45]. These findings stressed the importance of cell type- and synapse-specific approaches to make molecular analysis at the nanoscale level feasible.

Two methods are generally used to perform molecular investigations in complex brain circuits: electron and confocal microscopy. Labor-intensive electron microscopic studies use the required spatial resolution to disclose molecular changes in a mixed synapse population. However, the cellular identity of the excitatory and inhibitory synapses usually cannot be revealed by electron microscopy[41,42,45]. As substantial benefits, we noted that tissue processing for STORM imaging was much faster, less laborious, permitted larger sample size, led to higher labeling density than pre-embedding immunogold electron microscopy and could still achieve a similar localization precision of the endogenous target protein. Confocal microscopy is optimal for simultaneous detection of multiple target proteins and cellular markers, but it does not have the necessary spatial resolution to obtain important molecular information at the nanoscale level. We found that the localization precision achieved by STORM microscopy, which is about an order of magnitude greater than with confocal microscopy, is advantageous for various reasons. First of all, STORM imaging supported more precise bouton-specific sampling of CB₁ receptor localization data, which is important because the accumulation of adjacent CB₁-positive axons forms dense meshworks and the source of collected photons often cannot be reliably determined, especially axially, by confocal microscopy. STORM imaging also revealed morphological parameters, such as bouton size, more accurately than confocal microscopy and electron microscopy because it lacked the confounding factors of light diffraction and anisotropic shrinkage, respectively[22]. Furthermore, the improved localization precision of STORM enabled the separation of functionally different surface-associated versus intracellular receptor pools, as well as the measurement of their relative changes following chronic THC treatment. Finally, STORM imaging made it possible for the first time, to the best of our knowledge, to uncover the homogeneous CB₁ distribution pattern on the bouton surface and the clustered organization of the release machinery component bassoon together with several quantitative parameters regarding their relative amounts and spatial relationships in a cell type–specific manner. The implications of these findings suggest that the receptor/effector ratio and distance at the nanoscale level are important parameters that affect the strength of synaptic endocannabinoid signaling. It is important to note here that other super-resolution microscopy approaches, such as stimulated emission depletion microscopy, also permit correlated confocal and super-resolution imaging[19]. However, single molecule–localization microscopy provides better localization precision, especially in the z axis. This was required for the accurate molecular distribution data reported here and offers better experimental flexibility for multicolor imaging, making it the method of choice for most neuroscience applications aiming for nanoscale localization of endogenous proteins.

On the basis of the advantages that we directly demonstrated, we propose that STORM imaging combines the major strengths of electron and confocal microscopy and represents a powerful approach for cell type–specific molecular investigations in brain circuits. In summary, we introduced a series of innovations to this end, including
the development of specific slice processing and immunostaining protocols to perform high-throughput STORM imaging of physiologically and morphologically characterized interneurons and to obtain excellent signal-to-noise STORM imaging data; the combination of confocal and STORM microscopy to acquire correlated morphological and molecular data from the very same identified subcellular compartment of a target neuron; and the generation of new algorithms, data processing and analysis tools to make quantitative investigation of molecular densities and distances together with morphological parameters feasible at the nanoscale level in these subcellular compartments. Our results provide cellular context to the previous ability to study proteins in random synapse populations by using biocytin labeling and correlated confocal and STORM microscopy. The morphological details revealed here, together with the multicolor feature of STORM imaging, elucidated the precise position of CB1 receptors in relation to the active zone and its reorganization after chronic THC treatment in a cell type–specific manner. Notably, our approach can be applied to other pre- or postsynaptically located molecular targets in any cell type and subcellular compartment in the future.

**Biological implications**

An important objective of our study was to ascertain molecular organizational properties that may influence cell type–specific cannabinoid efficacy on neurotransmitter release. As an example, we studied perisynaptically-projecting and dendritically-projecting hippocampal GABAergic interneurons, which exhibit marked differences in the strength of cannabinoid signaling. The cell type–specific approach identified unexpected organizational similarities and differences in the molecular architecture of cannabinoid-sensitive axon terminals belonging to these interneurons. In light of the accumulation of postsynaptic ionotropic receptors in precisely organized nanodomains, the homogeneous distribution of presynaptic CB1 receptors shown by the polyhedron surface analysis is rather surprising. Dynamic ionotropic receptors are trapped in nanoclusters by postsynaptic anchoring proteins. However, similar anchoring mechanisms for presynaptic CB1 receptors have not yet been identified, despite the fact that other presynaptic GPCRs exhibit notable synaptic segregation patterns. Constitutive CB1 trafficking occurs between the plasma membrane surface of the pre-terminal axon and boutons; thus, the homogeneous nanoscale distribution may be the consequence of unrestricted extrasynaptic receptor movement in the absence of specific anchoring mechanisms. Because homogeneous CB1 distribution proved to be a common feature of perisomatic and dendritically expressing bouton types, the subcellular distribution pattern of CB1 receptors alone does not explain the quantitative physiological differences in cannabinoid signaling.

The correlation of confocal and STORM data also revealed that larger boutons carry more presynaptic CB1 receptors. It is widely accepted that the molecular topology of the active zone is precisely orchestrated. Moreover, bigger axon terminals have larger total active zone areas, accommodating more VGCCs and other release machinery components. Our results add to this view of the highly organized presynapse by demonstrating that the number of presynaptic regulatory receptors also scales with the size of the axon terminal in both examined interneuron types. This also underlines that CB1 numbers on a given axon terminal type may be more related to the size of the bouton than to the identity of the cell type itself. Indeed, we found that identically sized perisomatic and dendritically expressing boutons contained equal amounts of CB1 receptors. Conversely, the number of the active zone protein bassoon seemed to be affected by other factors as well, as significantly more bassoon were observed in dendritic terminals than in perisomatic terminals with a similar size. It will be interesting to see whether the somatodendritic location of a given synapse predicts bassoon number and the size of the corresponding active zone.

Considering the generally different position and function of perisomatic and dendritically expressing GABAergic synapses on the somatodendritic surface of pyramidal neurons, the lower bassoon number in individual bassoon clusters in perisomatic boutons is an interesting finding. Because bassoon copy numbers correlate with the number of N-type VGCC subunits, this observation raises the possibility that less N-type VGCCs (for which a sensitive and specific antibody is not yet available) are located at individual active zones in perisomatically projecting interneurons. On the other hand, given that we found that perisomatic boutons had more CB1 receptors, which are uniformly distributed, a given VGCC- and bassoon-containing release machinery may be regulated by more adjacent CB1 receptors. Consistent with this prediction, the CB1/bassoon ratio was 46% larger within 100 nm of the bassoon cluster edges in boutons belonging to perisomatically projecting interneurons than in axon terminals originating from dendritically projecting cells. This is also consistent with the pharmacological findings that cannabinoid administration at low concentrations only inhibits GABA release from perisomatic interneurons. Because saturating levels of cannabinoids are equally effective in reducing GABAergic transmission at both terminal types, it is conceivable that the synaptic concentration of endocannabinoid ligands and the signaling efficacy of presynaptic CB1 receptors are the two principal factors that, together, determine the strength of endocannabinoid signaling at a given synapse. However, one major implication of the quantitatively different nanoscale molecular organization of CB1-containing interneuron terminals that we uncovered is that the nanoscale receptor/effector ratio and coupling distance, and not the total number of CB1 receptors on a given axon terminal alone, determines how cannabinoid signaling controls neurotransmitter release.

CB1 receptors mediate the acute psychoactive effects of cannabis preparations in humans and chronic cannabis users exhibit strong, long-lasting cognitive deficits, including impairments in hippocampus-dependent memory functions. Our understanding of how acute and chronic cannabis consumption perturbs endocannabinoid signaling has been limited, as it was previously not technically possible to determine the underlying molecular changes in a particular compartment of a particular cell type. This is of special importance considering that chronic cannabis abuse triggers non-uniform loss of CB1-specific radioligand binding sites in human brain regions. In accordance with the circuit-specific effects, chronic THC administration causes a strong reduction in cannabinoid efficacy selectively on GABAergic transmission, but the primary molecular mechanisms accounting for this reduction could not be directly investigated in identified GABAergic axon terminals until now. Our combined confocal and STORM approach revealed an astonishing loss of presynaptic CB1 receptors at the level of individual GABAergic boutons. The increased accumulation of intracellular (presumably internalized) receptors, together with the robust loss of receptors (probably as a result of protein degradation), indicate that the decreased availability of plasma membrane receptors reduces cannabinoid efficacy on GABA release following chronic THC administration.

In light of the fact that classical cannabinoid effects are regained in mice 11.5 d after the last THC treatment, it was surprising that CB1 receptors only partially recovered at this time point. However, hippocampal LTP is still substantially impaired after 2 weeks and human memory deficits persist even after 28 d of abstinence. These long-lasting neurophysiological and neurocognitive impairments may...
be influenced by the partial receptor retrieval, especially as CB1 activation on GABAergic terminals underlies THC-induced long-term memory deficits17,18, and sets the threshold for LTP at excitatory synapses15,36. Certainly, direct interpretations of findings obtained in mice and humans are difficult as a result of potentially different pharmacokinetics. Nevertheless, the dose (10 mg per kg) applied here results in similar blood THC levels as observed in humans smoking marijuana with low THC content33,34,49, and hippocampal CB1 radioligand binding density does not show reversal in chronic daily marijuana users even after 4 weeks of abstinence38. Regarding this latter observation, it is important to note that CB1 numbers returned to normal levels 6 weeks after cessation of THC administration. In addition, a low THC dose that is in the range used in medical applications39,40 caused only minor loss of CB1 receptors on GABAergic axon terminals. Considering the rapidly expanding considerations for the potential for THC-based medical treatments50, and the increasing legal recreational use of cannabis preparations with rising THC concentrations49, these findings are important for a better understanding of the molecular adaptive responses associated with chronic THC exposure.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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1. Klausberger, T. & Somogyi, P. Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. Science 321, 53–57 (2008).
2. Lichtman, J.W. & Denk, W. The big and the small: challenges of imaging the brain’s circuits. Science 334, 618–623 (2011).
3. O’Tourke, N.A., Weiler, N.C., Micheva, K.D. & Smith, S.J. Deep molecular diversity of mammalian synapses: what it matters and how to measure it. Nat. Rev. Neurosci. 13, 365–379 (2012).
4. Hangya, B., Pi, H.J., Kwistiani, D., Ranade, S.P. & Kepecs, A. From circuit motifs to computations: mapping the behavioral repertoire of cortical interneurons.Curr. Opin. Neurobiol. 26, 117–124 (2014).
5. Choquet, D. & Triller, A. The dynamic synapse. Neuron 80, 691–703 (2013).
33. Heishman, S.J., Huestis, M.A., Henningfield, J.E. & Cone, E.J. Acute and residual effects of marijuana: profiles of plasma THC levels, physiological, subjective, and performance measures. Pharmacol. Biochem. Behav. 37, 561–565 (1990).
34. Varvel, S.A. et al. Delta9-tetrahydrocannabinol accounts for the antinociceptive, hypothermic and cataleptic effects of marijuana in mice. J. Pharmacol. Exp. Ther. 314, 329–337 (2005).
35. Carlson, G., Wang, Y. & Alger, B.E. Endocannabinoids facilitate the induction of LTP in the hippocampus. Nat. Neurosci. 5, 723–724 (2002).
36. Chevaleyre, V. & Castillo, P.E. Endocannabinoid-mediated metaplasticity in the hippocampus. Neuron 43, 871–881 (2004).
37. Bolla, K.I., Brown, K., Eldreth, D., Tate, K. & Cadet, J.L. Dose-related neurocognitive effects of marijuana use. Neurology 59, 1337–1343 (2002).
38. Hirvonen, J. et al. Reversible and regionally selective downregulation of brain cannabinoid CB1 receptors in chronic daily cannabis smokers. Mol. Psychiatry 17, 642–649 (2012).
39. Stott, C.G., White, L., Wright, S., Wilbraham, D. & Guy, G.W. A phase I study to assess the single and multiple dose pharmacokinetics of THC/CBD oromucosal spray. Eur. J. Clin. Pharmacol. 69, 1135–1147 (2013).
40. DeLong, G.T., Wolf, C.E., Poklis, A. & Lichtman, A.H. Pharmacological evaluation of the natural constituent of Cannabis sativa, cannabichromene and its modulation by ∆(9)-tetrahydrocannabinol. Drug Alcolhol Depend. 112, 126–133 (2010).
41. Chen, K. et al. Long-term plasticity of endocannabinoid signaling induced by developmental febrile seizures. Neuron 39, 599–611 (2003).
42. Ludányi, A. et al. Downregulation of the CB1 cannabinoid receptor and related molecular elements of the endocannabinoid system in epileptic human hippocampus. J. Neurosci. 28, 2976–2990 (2008).
43. Maccarrone, M. et al. Abnormal mGlu 5 receptor/endocannabinoid coupling in mice lacking FMRP and BC1 RNA. Neuropsychopharmacology 35, 1500–1509 (2010).
44. Zhang, L. & Alger, B.E. Enhanced endocannabinoid signaling elevates neuronal excitability in fragile X syndrome. J. Neurosci. 30, 5724–5729 (2010).
45. Jung, K.M. et al. Uncoupling of the endocannabinoid signalling complex in a mouse model of fragile X syndrome. Nat. Commun. 3, 1080 (2012).
46. Shigemoto, R. et al. Target cell–specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. Nature 381, 523–525 (1996).
47. Mikasova, L., Groc, L., Choquet, D. & Manzoni, O.J. Altered surface trafficking of presynaptic cannabinoid type 1 receptor in and out synaptic terminals parallels receptor desensitization. Proc. Natl. Acad. Sci. USA 105, 18596–18601 (2008).
48. Huestis, M.A. et al. Blockade of effects of smoked marijuana by the CB1-selective cannabinoid receptor antagonist SR141716. Arch. Gen. Psychiatry 58, 322–328 (2001).
49. Volkow, N.D., Baler, R.D., Compton, W.M. & Weiss, S.R. Adverse health effects of marijuana use. N. Engl. J. Med. 370, 2219–2227 (2014).
50. Pertwee, R.G. Targeting the endocannabinoid system with cannabinoid receptor agonists: pharmacological strategies and therapeutic possibilities. Phil. Trans. R. Soc. Lond. B 367, 3353–3363 (2012).
ONLINE METHODS

Maintenance, transfection and immunostaining of HEK293 cells. HEK293 cells were a kind gift from B. Gerend (Institute of Experimental Medicine). Cells were maintained under routine conditions in cell culture–treated T25 flasks in high glucose-containing (25 mM d-glucose) Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated neonatal calf serum at 37 °C and 5% CO2. Cultures were regularly tested for the presence of mycoplasma contamination via DAPI staining. Before transfection, the cells were split onto poly-L-lysine (20 µg/ml) coated glass bottomed dishes. Transfection was performed using 1 µg of cDNA encoding an N-terminally EGFP-tagged CB1 protein and 1 µg Lipofectamine 2000 (Invitrogen) per dish, according to manufacturer’s instructions. Dishes were maintained at 37 °C and 5% CO2 for at least 18 h to allow for efficient cDNA incorporation and protein expression. After incubation, cells were fixed in 4% (m/v) paraformaldehyde (PFA) for 15 min, washed three times in 0.1 M phosphate buffer (PB), permeabilized in 0.1% (v/v) Triton X-100 in 0.05 M tris-buffered saline (TBS) for 10 min and immunostained with a primary antibody to CB1 receptor (guinea pig antibody to CB1, described in ref. 52, 1 µg ml−1 in TBS) for 1 h. The cells were then washed in TBS three times and stained with a secondary antibody custom-made for STORM imaging (Cy3- and Alexa647-conjugated donkey antibody to guinea pig, 1:50 in TBS) for 1 h. Cells were then washed twice each in TBS and PB. Immediately before STORM imaging, PB was removed and freshly prepared imaging medium (see below) was added to the dishes. Randomly selected HEK cells with homogeneous expression levels were imaged and included in the analysis. Imaging conditions were identical to those used for tissue samples (detailed below).

Perfusion and preparation of tissue sections. All animal experiments were approved by the Hungarian Committee of the Scientific Ethics of Animal Research (license number: XIV-1-001/2332-4/2012), and were carried out according to the Hungarian Act of Animal Care and Experimentation (1998, XXVIII, Section 243/1998), which are in accordance with the European Communities Council Directive of 24 November 1986 (86-609-EEC; Section 243/1998). All efforts were made to minimize pain and suffering and to reduce the number of animals used. Adult male littermate CB1+/+ and CB1−/− C57BL/6 mice (kindly provided by A. Zimmer, University of Bonn) were deeply anesthetized with intraperitoneal ketamine-xylazine (25 mg ml−1 ketamine, 5 mg ml−1 xylazine, 0.1% (m/p) pipolphen (wt/wt) in H2O, 1 ml per 100 g), then perfused transcardially with 0.9% (m/v) saline for 2 min, followed by 4% PFA in 0.1 M phosphate buffer (PB, pH 7.4) for 20 min. After perfusion, the brains were removed from the skull, cut into blocks and incubated in 4% PFA for 2 h. We cut either 20-µm- or 50-µm-thick coronal sections for immunofluorescence labeling or for immunogold staining, respectively, with a Leica VT-1000s vibratome.

Immunogold staining. Pre-embedding immunostaining was performed using guinea pig antibody to CB1 (1 µg ml−1) as described earlier. Electron micrographs were taken with a Hitachi (Yokohama, Japan) H-7100 electron microscope. For the quantification of CB1-immunogold labeling, random cross-sections of immunogold-containing axon terminals forming symmetrical synapses were selected in either stratum pyramidale or stratum radiatum. After collecting images of 100–100 axon terminals in each layer and each animal (n = 3), the images were analyzed using ImageJ. The area of the axon terminals was determined as the area of a freehand shape drawn along the plasma membrane. Preterminal axon segments were recognized based on their thin diameter and lack of synaptic vesicles, and were excluded from the analysis. Finally, the number of total and membrane-bound (center of particle within 50 nm of the plasma membrane) silver-intensified gold particles were counted on each terminal.

Acute slice preparation and recording. Coronal hippocampal slices (300 µm) were prepared from 25–30-d-old male C57BL/6H CB1+/+ mice or CB1−/− mice. Slices were incubated in high sucrose-containing artificial cerebrospinal fluid (ACSF; 85 mM NaCl, 75 mM sucrose, 2.5 mM KCL, 25 mM glucose, 1.25 mM NaH2PO4, 4 mM MgCl2, 0.5 mM CaCl2, and 24 mM NaHCO3) for 60 min, and then transferred to a submerged recording chamber constantly perfused with oxygenated ACSF (containing 126 mM NaCl, 2.5 mM KCl, 26 mM NaHCO3, 2 mM CaCl2, 2 mM MgCl2, 1.25 mM NaH2PO4, and 10 mM glucose, equilibrated with 95% O2 and 5% CO2). All electrophysiological recordings were performed at 33 °C in the slice chamber of a fixed stage microscope (Nikon Eclipse FN1) and infrared differential interference contrast (DIC) optics was used to visualize the slices.

Whole-cell recordings were obtained using patch pipettes (3–4 MΩ) filled with internal solution containing 126 mM potassium gluconate, 4 mM KCl, 10 mM HEPES, 4 mM ATP-Mg, 0.3 mM GTP-Na, 10 mM phosphocreatine, and 0.2% biocytin, pH 7.2, 270–290 mOsm. Pyramidal cells and interneurons were located in the strata pyramidale and radiatum of the CA1 subfield in the hippocampus, respectively. Recordings were performed using MultiClamp700B amplifiers (Molecular Devices). Signals were filtered at 3 kHz using a Bessel filter and digitized at 10 kHz with a Digidata 1440A analog-digital interface (Molecular Devices). Series resistances were carefully monitored, and the recordings were discarded if the series resistance changed >20% or reached 20 MΩ. The recorded traces were analyzed using the Clampfit 10.2 software (Molecular Devices). After ~30 min of recording, the slices were fixed immediately in 4% PFA in 0.1 M PB for 40–48 h at 4 °C. All recorded regular-spiking cells were developed after fixation, but those interneurons whose axon arbor was not visualized by biocytin labeling, or those which were found to be CB1 immunonegative, were excluded from the study.

In vivo recording. In vivo recordings were performed as described earlier, and were approved by the Institutional Animal Care and Use Committee of the University of California at Irvine. Briefly, a chronic head bar was implanted onto the skull of adult male C57BL/6 mice. After acclimation to the spherical treadmill apparatus, craniotomy surgery was performed, and borosilicate glass microelectrodes were lowered into the dorsal hippocampus. One electrode was used to record field potentials and a second one was filled with Neurobiotin-containing solution (1.5–2% in 0.5% NaCl) and used to perform juxtacellular recording and labeling of the recorded neuron.

Development and analysis of in vitro and in vivo recorded cells. After immersion fixation (in case of in vitro experiments) or after perfusion and slicing (in case of in vivo experiments), hippocampal sections were washed extensively in PB and 0.05 M Tris-buffered saline (TBS, pH = 7.4), treated with 0.5% Triton X-100 in TBS twice for 30 min, and incubated in Alexa488-Streptavidin (1:1,000, 016-540-084, Jackson Immunoresearch). Sections were then washed in TBS and PB, mounted on glass slides in Vectashield with DAPI (Vector Laboratories), coverslipped, and sealed with nail polish. Low-magnification stacks of filled neurons were collected from 300-µm-thick sections (in vitro experiments) or from 20-µm-thick sections (in vivo experiments) on a Nikon A1R confocal scan head coupled to a Nikon Ti-E inverted microscope using a CFI Plan Apo VC 20× objective (0.75 NA). After imaging, the sections were returned to PB and processed further.

The quantification of laminar bouton distribution in biocytin- or neurobiotin-filled cells was performed on maximal intensity z projections in ImageJ. The two borders of the pyramidal layer were drawn, the minimal distance of each axonal varicosity was measured from the two lines marking the layer borders, and a relative position was calculated, where 0 is the center and 1 is the thickness of the pyramidal layer. The bouton distribution index (BDI) was calculated from the ratio of the number of boutons and the relative position:

\[
BDI = \frac{0.5}{M} \left( \frac{Q_3 - Q_1}{I} \right)
\]

to reflect the extent of accumulation of boutons in the pyramidal layer. Cells with BDI > 1 were included as perisomatic, and cells with BDI < 0.5 were included as dendritic cells in the study. Finally, some representative examples of the biocytin-filled interneurons were reconstructed from confocal image stack using Neuronlucida 10 software.

Immunostaining for STORM imaging. Immersion-fixed 300-µm acute brain slices or 60-µm sections from perfused brains were embedded in 2% agarose in distilled water, and 20-µm-thick coronal sections were cut with a Leica VT-1000s Vibratome in PB. After slicing, the sections were immunostained in a free-floating manner. Following washing in TBS, and blocking with 1% (m/v) HSA (albumin from human serum, Sigma) in TBS, the sections were incubated with affinity-purified primary antibodies in TBS overnight at room temperature (20–25 °C).
on an orbital shaker. Primary antibodies used in this study included guinea pig antibody to CB₁ (1:1,000, described earlier), validated in CB₁-knockout mice (in the present study), rabbit antibody to CB₁ (1 µg ml⁻¹, described and validated in CB₁⁻⁻ mice in the present study, ImmunoGenes Kft), mouse antibody to CCK (1:3,000, 9303, CURE Gastroenteric Biology Center), mouse antibody to bassoon (1:2,000, ab82958, Abcam; specificity validated in bassoon-knockout mice). Sections were then washed in TBS and incubated with the appropriate commercial Alexa488-conjugated anti-mouse (1:400, 715-545-150) or Alexa647-conjugated anti-guinea pig (1:400, 706-605-148, Jackson ImmunoResearch) or STORM secondary antibodies (2 µg/ml, prepared using unaffiliated affinity-purified whole IgG antibody to guinea pig (703-005-155), antibody to rabbit (711-005-152) or antibody to mouse (715-005-150, Jackson ImmunoResearch), according to a previously established protocol) for 4 h. Sections used for single biocytin STORM imaging were developed with Alexa647-Streptavidin (1:1,000, 016-600-084, 4 h, Jackson ImmunoResearch) instead of the primary and secondary antibody incubations. After washing in TBS and PB, sections were mounted and dried on acetone-cleaned #1.5 borosilicate coverslips, and stored dry at 4 °C until imaging. Immediately before imaging, samples were covered with imaging medium freshly prepared as described previously containing 5% (v/v) glucose, 0.1 M mercaptoethanolamine, 1 mg ml⁻¹ glucose oxidase and catalase (2.5 µl ml⁻¹ of aqueous solution from Sigma, approximately 1,500 U ml⁻¹ final concentration) in Dulbecco’s PBS (Sigma). Finally, coverslips were sealed with nail polish, and transferred into the microscope setup after 10 min. STORM imaging was performed for up to 3 h after covering the specimens.

Preparation of the rabbit antibody to CB₁. Transgenic rabbits (Tg) that have enhanced neonatal Fc receptor (FcRn) activity, as they carry and express one extra copy of the rabbit FcRn gene on New Zealand White rabbit genetic background, were used in this study. These rabbits are coded as NZW Tg1 rabbit because they carry one extra copy of the rabbit FcRn gene on New Zealand White rabbit genetic background. In this research followed the guidelines of the Institutional Animal Care and Ethics Committee at ImmunoGenes Kft that operated in accordance with permissions 22.1/601/000/2009 and XIV-I-001/2086-4/2012 issued by the Food and Ethics Committee at ImmunoGenes Kft that operated in accordance with permissions 22.1/601/000/2009 and XIV-I-001/2086-4/2012 issued by the Food Chain Safety and Animal Health Directorate of the Government Office of Pest County, Hungary.

Tg and wild-type rabbits (3 months old female siblings) were intramuscularly immunized with a keyhole limpet hemocyanin (KLH)-conjugated polypeptide (CB₁-N′-MHRAECSIKSTVXKAVRT MSVSTDTSEAL-C′) corresponding to amino acid residues 443–473 of the mouse CB₁ receptor. Animals were immunized with 200 µg CB₁ peptide-KLH conjugate (CB₁-KLH) in complete Freund’s adjuvant and challenged multiple times with 100 µg of the conjugate in incomplete Freund’s adjuvant. Sera containing antibody to CB₁ were affinity purified with a SulfoLink Immunobilization Kit for peptides (Thermo Scientific), according to manufacturer’s instructions. Antigen-specific and total immunoglobulin levels were determined by ELISA measurements. The purified antibody from one of the FcRn Tg rabbits, which showed the highest sensitivity was selected and used in the experiments. The specificity of this antibody was validated in CB₁⁻⁻ animals.

Combined confocal/STORM image acquisition. STORM images and the correlated high-power confocal stacks were acquired via a CFI Apo TIRF 100× objective (1.49 NA) on a Nikon Ti-E inverted microscope equipped with a Nikon N-STORM system, a Nikon C2 confocal scan head and an Andor iXon Ultra 897 EMCCD (with a cylindrical lens for astigmatic 3D-STORM imaging). The setup was controlled by Nikon NIS-Elements AR software with N-STORM module. For STORM imaging, a 300–600 mW laser was used (VFL-P-300–647, MPB Communications), fiber-coupled to the laser board of the microscope setup. To obtain images, the field of view was selected based on the live EMCCD image under 488-nm illumination. A confocal stack (512 × 512 × 15 pixels, 78 × 78 × 150 nm resolution) was then collected using 488-nm excitation. The field of view of the confocal scan area and the EMCCD were set to be identical. Next, the tissue was bleached by scanning in the z axis while using maximal illumination from the 647 laser line. Finally, the STORM image was acquired using a STORM filter cube (Nikon) and the EMCCD. For single channel STORM imaging, 1,000 cycles of one activator (low-power excitation at 561 nm or 405 nm for CB₁ and bassoon, respectively) and three reporter frames (high-power 647-nm excitation), 30 ms each, were captured resulting in an imaging time of 3 min per image. For dual channel STORM imaging, 1,000 cycles of 8 × 30–ms frames were captured, each cycle consisted of: one activator (405 nm), three reporter (647 nm), one activator (561 nm) and three reporter (647 nm) frames resulting in an imaging time of 4 min per image. The applied activator laser power was determined to be low enough to avoid extensively overlapping localizations, while the reporter laser power was always set to maximum. For dual-channel direct STORM imaging, a green/gray-red dual filter cube (Nikon) was used, and each of the 10,000 imaging cycle consisted of 4–4 frames illuminated by either the 488 or the 647 laser lines at maximal power, while using continuous low-power 405-nm illumination for activation. To minimize out-of-focus background, oblique illumination was applied using the TIRF illuminator of the microscope. Localization points were collected within 300–300 nm axial distances from the center plane of the bouton at a similar tissue depth (~5 µm) for all boutons to equalize antibody penetration probabilities and the effect of light scattering.

According to the established definition, we use the term localization precision to refer to the distribution of the position of the same fluorophore calculated from multiple separate images, and report the s.d. of this distribution as localization precision values. Localization precision in STORM images obtained from the HEK cells and from hippocampal sections were determined from isolated clusters (n = 50–50) of localization points representing multiple detections of the same fluorophore.

Combined confocal/STORM image processing. All confocal image stacks of filled boutons were deconvolved with 40 iterations of the Classic Maximum Likelihood Estimation algorithm in Huygens software (SVI). To register with correlated STORM images acquired on EMCCD, deconvolved confocal images were transformed using affine transformation by the TurboReg plugin in ImageJ. The parameters of the required transformation were determined from reference image pairs of 100-nm fluorescent beads captured by both cameras. STORM images were processed to acquire coordinates of localization points using the N-STORM module in NIS-Elements AR software. Identical settings were used for every image. To exclude camera noise, the peak detection threshold was set to 2,500 gray levels (1,200 photons). Calibration of 3D-STORM was performed according to the manufacturer’s instructions using a piezo stage controller (Nano-Drive, Mad City Labs) and 100-nm fluorescent beads immobilized oncoverslips. In case of dual channel STORM images, crosstalk subtraction was applied, using the same threshold settings for every image. The batch processing of images was solved using scripts written in Python and AutoHotkey to control NIS-Elements.

Correlation of confocal and STORM images was performed using custom-written macros in ImageJ. The boutons to be included in the analysis were selected from the deconvoluted confocal image of the biocytin signal (maximum intensity z projection of three image planes centered at the plane corresponding to the focal plane of the STORM image), resampled to 1-nm per pixel resolution and localization points from the STORM data were overlaid on the image. When necessary, the confocal image was translated manually to fit the STORM data acquired on the EMCCD sensor. In the case of CB₁ staining, the confocal image was fitted to the STORM image, as the CB₁ receptor-immunostaining is dense enough to outline the membrane of the bouton. In the case of single bassoon immunostaining, the confocal image was fitted to the epifluorescence image of the bouton recorded on the EMCCD.

Visualization of STORM data were obtained by using the N-STORM module in NIS-Elements AR for two-dimensional images and by using the Visual Molecular Dynamics (VMD) software for three-dimensional renderings. The three-dimensional localization coordinates were converted to PDB (Protein Data Bank) file format. Each STORM channel was assigned to a different chain identifier or atomic type in order to visualize by VMD 1.8.7. The convex hull edges, as well as shortest-distance trajectories, were visualized as chemical bonds (see below).

Quantitative analysis of combined confocal/STORM images of axon terminals. To avoid any potential bias in assigning a given localization point to a given bouton, a freehand shape was first drawn around the center plane of the biocytin-filled bouton on the confocal image. Bouton size was measured as the area of the shape. Shape descriptors were measured using the built-in...
All procedures were performed in accordance with the Italian Ministry of Health guidelines (D.L. 116/92; D.L. 111/94-B) and EEC Council Directives (219/1990 and 220/1990). All efforts were made to minimize pain and suffering and to reduce the number of animals used. Male C57BL/6J mice (Harlan), 22–31 d of age at the beginning of the treatments, were housed (ten per cage) in a controlled environment at constant temperature (21 ± 1 °C) and humidity (60%) on a 12-h light-dark cycle (lights on at 7.00 a.m.), with free access to food and water. Animals were grouped in a randomized manner and treated either with Δ^2-tetrahydrocannabinol (THC) or its vehicle intraperitoneally at a dose of 10 mg per kg (intraperitoneal injection volume of 10 ml kg^-1) twice a day for 6.5 d. THC (THC-Pharm GmbH) was dissolved in a solution containing 1% (v/v) ethanol, 2% (v/v) Tween 80 and saline. This chronic treatment regimen was shown to induce behavioral tolerance in mice^32,64. From the six experimental groups, in the first and second groups, 24 h after the last THC or vehicle injection, respectively, mice were anesthetized with isoflurane and acute slices were cut to perform electrophysiological recordings as described above. In the third and fourth groups, the same process was carried out 11.5 d after cessation of THC or vehicle administration, respectively. In the fifth and sixth groups, the same process was performed 5 weeks after THC withdrawal or vehicle treatment, respectively. In a separate experiment, two groups of mice received either a subthreshold dose of THC (1 mg kg^-1, intraperitoneal), which does not elicit classical behavioral effects of cannabinoids^40, or vehicle twice a day for 6.5 d and electrophysiological recordings were done 24 h after finishing the treatment protocol. All recordings, developments, stainings, imaging and analyses of samples were conducted by experimenters blinded to treatment.

Internalization measurements. Due to the reduced number of localization points after chronic THC administration, using the CB1 convex hull as the approximation of the bouton surface could lead to an underestimated measure of internalization. To overcome this problem, an internalization index was calculated. First, the distance (d) of each CB1 localization point was measured from the three-dimensional center of mass of all localization points within the given axon terminal. The radius of the terminal was estimated from the area measured on the biocytin confocal image of the bouton, assuming a circular shape (r). Finally, the d/r ratio of every localization point was averaged for each bouton. This measure is sensitive to a shift of the localizations toward the center of the bouton. To estimate the extent of internalization, we first measured the ratio of membrane over total receptors in untreated animals using immunogold electron microscopy (88% for perisomatic interneurons). Subsequently, we determined a d/r threshold for individual localization points to get the same ratio from the STORM data of control perisomatic axon terminals. Finally, we assessed internalization in the data from boutons derived from THC-treated mice using the established d/r threshold.

Statistical analysis and figure preparation. Statistical analysis was performed and graphs were generated using STATISTICA 11 software (StatSoft). Based on bouton size, outlier boutons were excluded if two-sided Grubb’s test (α = 0.05) indicated the presence of outliers within the given cell. Each data set was tested for normality using Kolmogorov-Smirnov test, and for differences between animals or cells using Kruskal-Wallis test, before the appropriate statistical method was determined. Boutons from different animals were pooled if there was no significant difference between animals or cells of any group (Kruskal-Wallis test, P > 0.05). Otherwise, the mean values of individual animals or cells were used. Data always met the assumptions of the applied statistical probes. An estimate for the variance in data obtained with our approach was not available, thus no statistical methods were used to establish the number of samples analyzed. Post hoc power analysis indicated that with our measured variance in NLP, mean values from 6 animals, or 70 pooled boutons per group are required to detect a 25% standardized effect size between groups with 0.8 power and 0.05 alpha using two-tailed t test.

Figures were prepared using Photoshop CS5 (Adobe Systems, San Jose, CA). All images were modified in exactly the same way for all groups or each bouton, a corresponding second bouton, which exhibited the least difference in size and which was not yet included in the sample was identified among boutons of the other cell type.
genotypes during preparation of the figures to ensure equal comparison of animals from different treatment groups or genotypes, and comparison of cell types.

A Supplementary Methods Checklist is available.

51. Tappe-Theodor, A. et al. A molecular basis of analgesic tolerance to cannabinoids. J. Neurosci. 27, 4165–4177 (2007).
52. Fukudome, Y. et al. Two distinct classes of muscarinic action on hippocampal inhibitory synapses: M2-mediated direct suppression and M1/M3-mediated indirect suppression through endocannabinoid signalling. Eur. J. Neurosci. 19, 2682–2692 (2004).
53. Zimmer, A., Zimmer, A.M., Hohmann, A.G., Herkenham, M. & Bonner, T.I. Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. Proc. Natl. Acad. Sci. USA 96, 5780–5785 (1999).
54. Nyilas, R. et al. Enzymatic machinery for endocannabinoid biosynthesis associated with calcium stores in glutamatergic axon terminals. J. Neurosci. 28, 1058–1063 (2008).
55. Varga, C., Golshani, P. & Soltesz, I. Frequency-invariant temporal ordering of interneuronal discharges during hippocampal oscillations in awake mice. Proc. Natl. Acad. Sci. USA 109, E2726–E2734 (2012).
56. Dick, O. et al. The presynaptic active zone protein bassoon is essential for photoreceptor ribbon synapse formation in the retina. Neuron 37, 775–786 (2003).
57. Bates, M., Huang, B., Dempsey, G.T. & Zhuang, X. Multicolor super-resolution imaging with photo-switchable fluorescent probes. Science 317, 1749–1753 (2007).
58. Baranyi, M., Cervenak, J., Bender, B. & Kacskovics, I. Transgenic rabbits that overexpress the neonatal Fc receptor (FcRn) generate higher quantities and improved qualities of anti-thymocyte globulin (ATG). PLoS ONE 8, e76839 (2013).
59. Huang, B., Wang, W., Bates, M. & Zhuang, X. Three-dimensional super-resolution imaging by stochastic optical reconstruction microscopy. Science 319, 810–813 (2008).
60. Deschout, H. et al. Precisely and accurately localizing single emitters in fluorescence microscopy. Nat. Methods 11, 253–266 (2014).
61. Thévenaz, P., Ruttimann, U.E. & Unser, M. A pyramid approach to subpixel registration based on intensity. IEEE Trans. Image Process. 7, 27–41 (1998).
62. Ester, M., Kriegel, H.-P., Sander, J. & Xu, X. A density-based algorithm for discovering clusters in large spatial databases with noise. in Proc. 2nd Int. Conf. Knowl. Discov. Data Min. (eds. Simoudis, E., Han, J. & Fayyad, U.) 226–231 (1996).
63. Veatch, S.L. et al. Correlation functions quantify super-resolution images and estimate apparent clustering due to over-counting. PLoS ONE 7, e31457 (2012).
64. McKinney, D.L. et al. Dose-related differences in the regional pattern of cannabinoid receptor adaptation and in vivo tolerance development to delta9-tetrahydrocannabinol. J. Pharmacol. Exp. Ther. 324, 664–673 (2008).