

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 Δ^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 Δ^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 diversity^{1–4}. 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 molecules^{3,5}. 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 activation^{6,7}. 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 synapse^{10,11}. Two major forms of CB₁-containing GABAergic interneurons are specialized to target either the perisomatic or dendritic regions of principal cells^{1,12,13}. 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 Δ^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 pathological alterations involving CB₁ receptors. THC has been suggested to cause cognitive deficits primarily by activating

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cannabinoid receptors on GABAergic boutons^{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 imaging¹⁹. We developed a rapid and versatile approach based on stochastic optical reconstruction microscopy (STORM)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 CB₁ 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

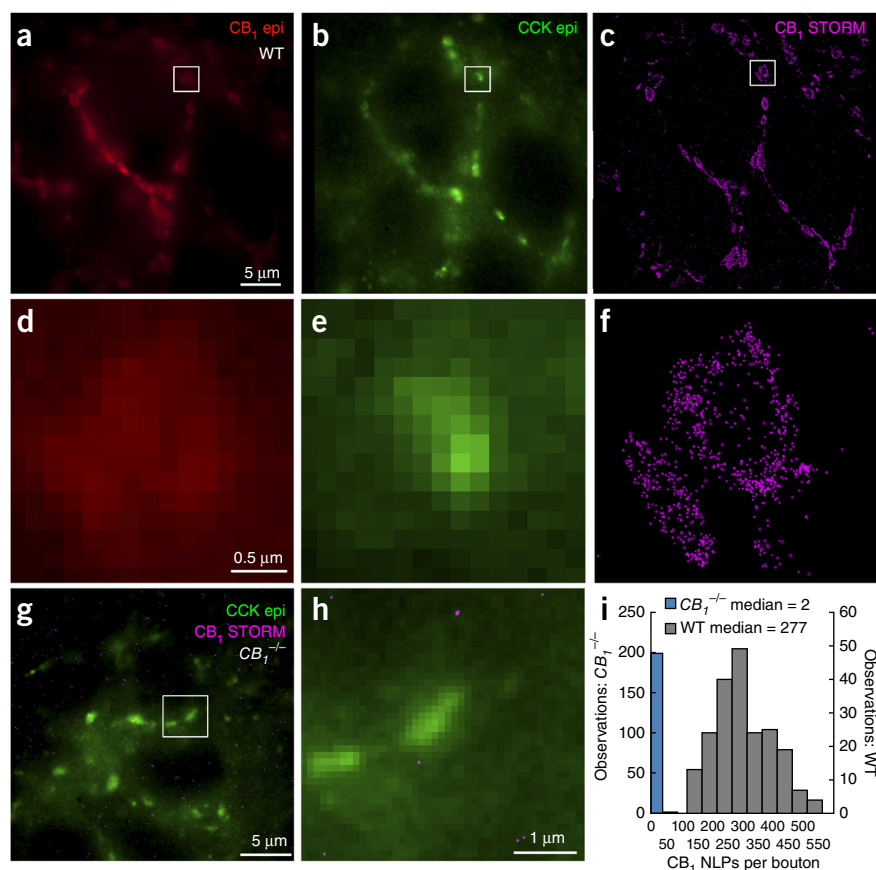
STORM super-resolution imaging of CB₁ cannabinoid receptors

We first developed an approach to combine confocal and STORM imaging and tested whether it reliably determines CB₁ receptor position in a cellular membrane environment using an EGFP-tagged CB₁ 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 CB₁ 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 CB₁ localization points (CB₁ 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 CB₁ 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 CB₁^{+/+} (also known as *Cnr1*) mice, STORM imaging uncovered high CB₁ receptor density on cholecystokinin (CCK)-containing GABAergic axon terminals forming typical basket-like arrays around the

Figure 1 3D-STORM imaging of CB₁ receptors on hippocampal GABAergic axon terminals. (a) Immunofluorescence labeling of CB₁ receptors in the CA1 stratum pyramidale of CB₁^{+/+} mice showed a basket-like arrangement of CB₁-positive axon terminals encircling a CB₁-immunonegative pyramidal cell somata. (b) These axon terminals could also be visualized by immunofluorescence staining for the neuropeptide CCK, a neurochemical marker of CB₁-expressing interneurons. (c) STORM imaging of CB₁ 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 CB₁ receptors with the axon terminals. (f) In contrast, the improved spatial resolution of 3D-STORM microscopy made it possible to reliably discern individual CB₁-positive boutons located adjacently to one another, as well as to reveal localization points representing the nanoscale position of CB₁ receptors. (g,h) To validate the specificity of our approach, we also processed and imaged hippocampal sections from littermate CB₁^{-/-} mice under the same conditions. In these sections, we found an almost complete absence of STORM localization points representing CB₁ in CCK-immunolabeled boutons. (i) Histogram of CB₁ NLPs in individual CCK-positive varicosities in the CA1 stratum pyramidale of sections derived from littermate CB₁^{+/+} ($n = 3$ animals, $n = 208$ axon terminals) or CB₁^{-/-} ($n = 2$ animals, $n = 200$ axon terminals) mice. Note that the markedly reduced CB₁ NLP value in CB₁^{-/-} 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.



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 manner³. 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 regulation⁸, 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 interneurons¹⁵. These cells were filled with biocytin during recording and then imaged and reconstructed by confocal microscopy and Neurolucida (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 ($n = 997 \pm 58$ boutons per cell, $n = 79$ cells, Online Methods). Notably, BDI unequivocally discriminated these cells as either perisomatic interneurons (BDI > 1), such as basket cells^{13,21}, which synapse on the somata and proximal dendrites of principal cells in stratum pyramidale (Fig. 2b,c), or dendritic interneurons (BDI < 0.5), such as Schaffer collateral-associated cells^{13,21}, which preferentially target the distal dendritic shafts of pyramidal neurons in stratum 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 ($n = 46 \pm 2$ 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 connections¹⁵, 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 ($88 \pm 1\%$ and $85 \pm 1\%$ 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 correlative 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

Figure 2 Cell type-specific integrated analysis of physiological properties, anatomical parameters and nanoscale molecular imaging. **(a)** Representative voltage traces in response to depolarizing and hyperpolarizing current steps recorded in whole-cell patch-clamp configuration from a multipolar neuron in CA1 stratum radiatum revealed the characteristic accommodating, regular-spiking pattern. **(b)** The neuron was filled with biocytin during recording to allow *post hoc* reconstruction. Although the dendrites (blue) arborized in all hippocampal layers, the axon cloud (red) was restricted to the stratum pyramidal. **(c)** Measurement of the relative laminar location of the recovered axonal varicosities confirmed a bouton distribution biased toward the pyramidal layer. The BDI based on the descriptive statistics of the distance distribution classified this cell as a perisomatic interneuron (BDI > 1). **(d)** Maximum intensity projection of a deconvolved confocal stack of the biocytin-filled axon terminals. **(e,f)** Subsequent STORM super-resolution imaging revealed CB₁ immunolabeling on the identified boutons. **(f)** This STORM image depicts CB₁ receptor localization points in the axon terminal shown in the boxed region in **e**. **(g)** Traces recorded from another interneuron also showed accommodating firing pattern. **(h)** This multipolar interneuron exhibited a distinct axonal morphology from the cell presented in **b**. **(i)** BDI classified this cell as a dendritic interneuron (BDI < 0.5). **(j–l)** Combined confocal and STORM imaging visualized the nanoscale distribution of CB₁ receptors in identified boutons of the same dendritic interneuron. Boutons were imaged at a similar tissue depth (~5 μ m) to equalize antibody penetration probability and the effect of light scattering. We processed and evaluated 80 interneurons in the same manner.

biocytin localization points. This geometric approach generated a polyhedron representation of the bouton volume and facilitated intuitive evaluation of the acquired images (Fig. 3i,l). 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 nanodomains^{22–24}, 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

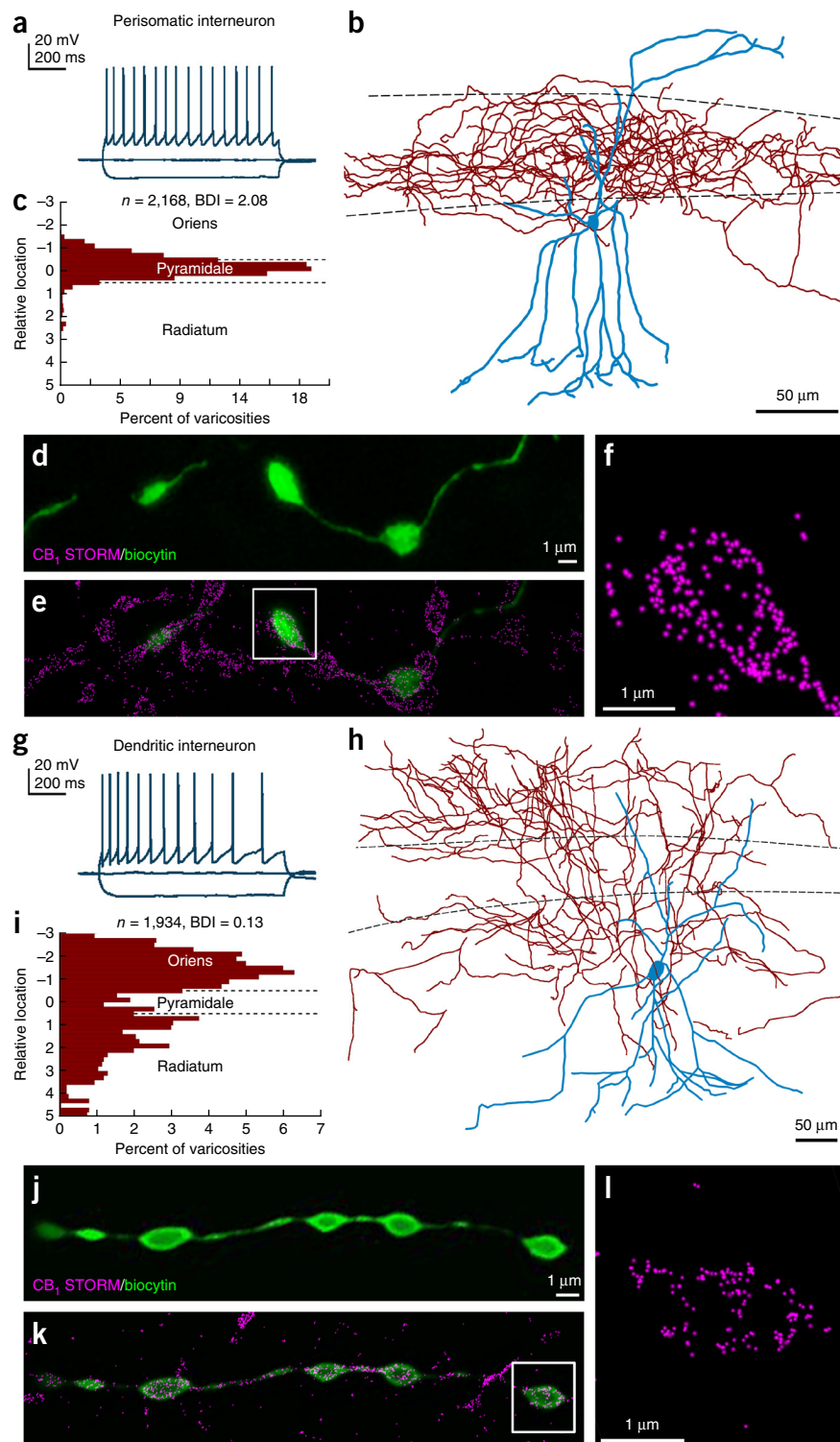
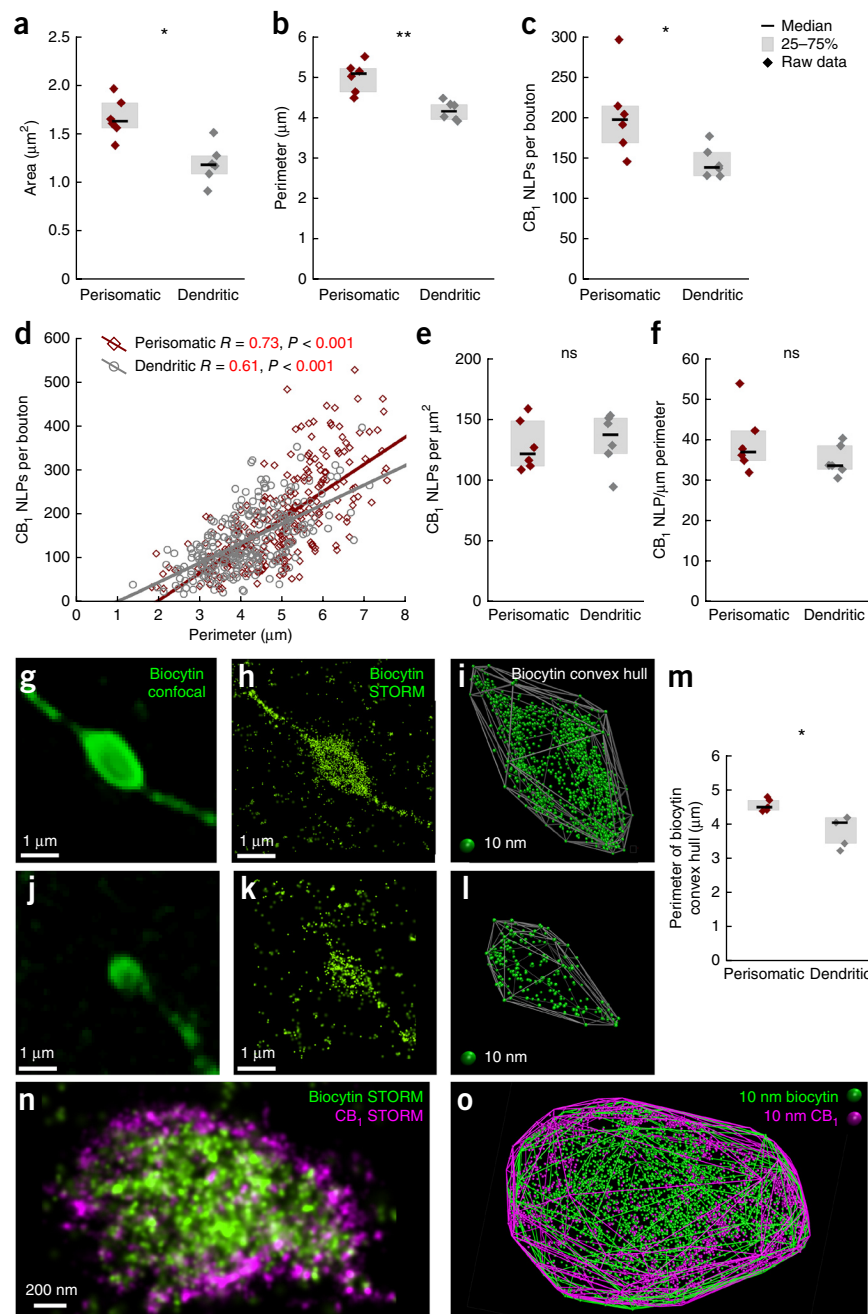


Figure 3 Perisomatic boutons are larger and contain proportionally more CB₁ receptors compared with dendritic boutons. (**a,b**) Perisomatic interneuron terminals were larger than boutons of dendritic interneurons ($n = 6$ cells per cell type, Mann-Whitney U test, $*P = 0.004$, $U = 1$ for area, $**P = 0.002$, $U = 0$ for perimeter). (**c**) Perisomatic axon terminals also carried more presynaptic CB₁ receptors ($*P = 0.015$, $U = 3$). (**d**) CB₁ NLPs showed correlation with bouton size in both interneuron types ($n = 279$ and 334 boutons, Spearman's correlation). (**e,f**) In contrast, the density of CB₁ was similar between the two cell types (Mann-Whitney U test, $P = 0.7$ and 0.3 , $U = 15$ and 11 for NLP over area and perimeter, respectively; ns, $P > 0.05$). (**g,j**) Biocytin-filled axon terminals of CB₁-positive perisomatic (**g**) and dendritic (**j**) interneurons labeled by a mixture of Alexa488- and Alexa647-conjugated streptavidin were imaged in confocal (**g,j**) and STORM (**h,k**) modes. (**i,l**) A three-dimensional convex hull was fitted on the localization points to visualize the surface of axon terminals. (**m**) Consistent with the size difference of CB₁ two-dimensional convex hulls (**b**), the perimeter of biocytin two-dimensional convex hulls of perisomatic boutons was significantly larger ($n = 5$ cells per cell type, Mann-Whitney U test, $*P = 0.012$, $U = 0$). (**n**) Dual-channel directSTORM imaging of an interneuron bouton labeled by Alexa488-conjugated streptavidin and immunostained for CB₁ using Alexa647. (**o**) Fitting three-dimensional convex hulls on the biocytin or on the CB₁ localization points resulted in markedly similar contours of the structure. Representative images are shown from experiments repeated successfully on two biocytin-filled perisomatic interneurons. Graphs show raw data and median $\pm$ interquartile range (IQR).

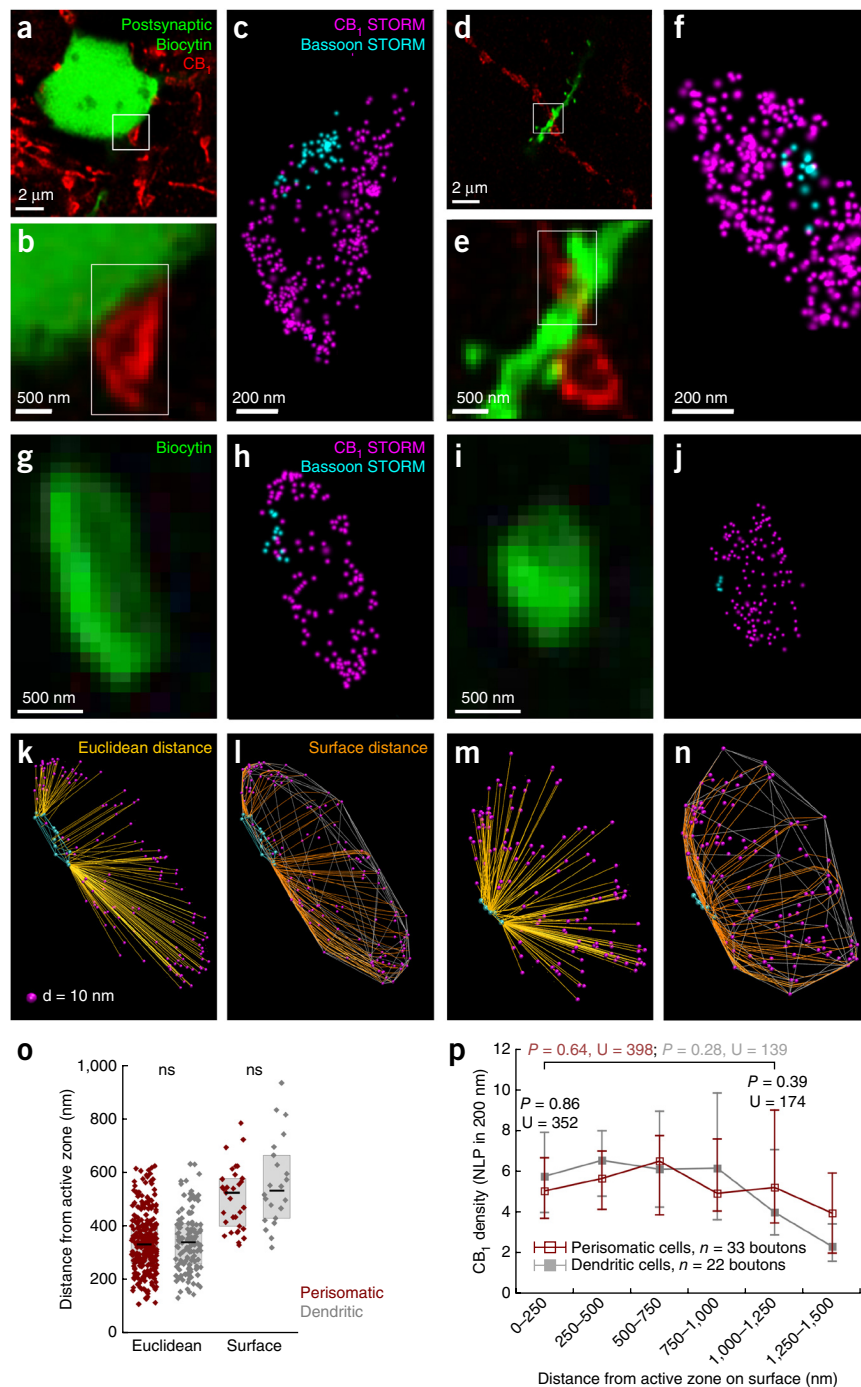


VGCC distribution in the active zone²⁸. We first asked whether 3D-STORM imaging reliably visualizes bassoon in the active zone in CB₁-containing perisomatic and dendritic axon terminals (Fig. 4a–f). CB₁-positive varicosities impinging on the somatodendritic surface of biocytin-labeled postsynaptic CA1 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^{22–24}, 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 $\beta\gamma$ subunits in a plasma membrane-delimited manner²⁹, 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 plasma-membrane in both interneuron types ($79 \pm 1\%$ and $81 \pm 1\%$, $n = 280$ and 141 perisomatic and dendritic axon terminals, respectively). To make molecular distance measurements possible on the polyhedron surface,

Figure 4 Cell type-specific multicolor STORM imaging illuminates homogeneous nanoscale distribution of CB₁ receptors on GABAergic axon terminals. (a,d) Deconvolved confocal images show a biocytin-filled CA1 pyramidal neuron and adjacent CB₁-positive boutons. (b,e) Putative afferents of the labeled neuron were identified. (c,f) Dual-channel 3D-STORM demonstrated accumulation of the active zone protein bassoon at the contact site, indicative of a perisomatic and dendritic afferent synapses. Representative images are shown from experiments repeated on ten biocytin-filled pyramidal neurons. (g,i) Confocal images of axon terminals of biocytin-filled perisomatic and dendritic interneurons, respectively. (h,j) Dual-channel 3D-STORM revealed the nanoscale bassoon and CB₁ distribution. (k,m) Localization points were visualized in three dimensions. The Euclidean distances between CB₁ and bassoon localization points could be readily calculated. (l,n) To allow intermolecular distance measurements along the structure surface, we determined the shortest paths between CB₁ and bassoon localization points on the bouton surface after fitting a three-dimensional convex hull on the CB₁ localization points. (o) Neither the Euclidean nor the surface distance was different between axon terminals of perisomatic and dendritic interneurons ($n = 311$ and 141 boutons, Mann-Whitney U test, $P = 0.69$, $U = 21420$ for Euclidean distance; $n = 33$ and 22 boutons, $P = 0.31$, $U = 305$ for surface distance; ns, $P > 0.05$). Graphs show raw data and median $\pm$ IQR. (p) The density of receptors (CB₁ NLP in 200 nm from random points of the surface, median $\pm$ IQR) was plotted against surface distance from bassoon. Perisomatic (within 250 nm) and extrasynaptic (1,000–1,250 nm) CB₁ densities were similar in boutons, and between cell types. Scale bar in panel g also applies to panel h; scale bar in panel i also applies to panel j.

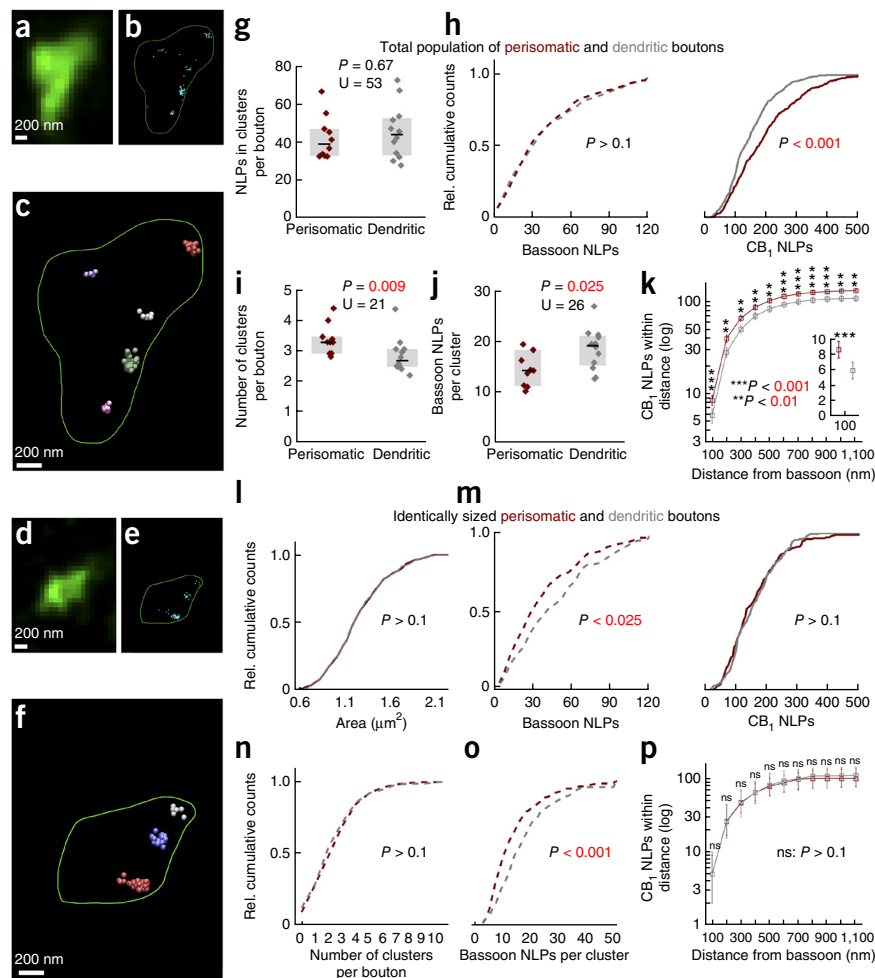


we developed an approximative mathematical algorithm (Supplementary Fig. 8d and Online Methods). In selected boutons containing a single cluster of bassoon localization points, a second convex hull was fitted onto this bassoon cluster. Because bassoon is not an integral membrane protein, points on this second hull were then projected onto the CB₁ polyhedron surface. The shortest route between each CB₁ localization point and the nearest projected bassoon point along the polyhedron surface was then calculated (Fig. 4k,n). In accordance with the Euclidean distance measurements, we found no difference in CB₁ distances from the active zone between perisomatic and dendritic axon terminals (Fig. 4o). We also determined the local density of CB₁ receptors on the polyhedron surface in relation to the active zone (validated by simulation of enriched receptor distributions; Supplementary Fig. 9), which also revealed a uniform CB₁ density distribution across the bouton surface and gave similar results for the two cell types (Fig. 4p). Taken together, these measurements introduce new tools, which are easily applicable to any single-molecule localization-based microscopy data to determine molecular distances on the surface of identified subcellular compartments. Moreover, in contrast with the clustered organization of postsynaptic ionotropic

receptors^{22–24}, 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

Figure 5 Cell type-specific and size-independent active zone architecture of GABAergic interneurons. (**a,d**) Deconvolved confocal images of boutons of perisomatic 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 perisomatic and dendritic interneurons contained similar NLPs ($n = 349$ and 374 boutons from 10 perisomatic and 12 dendritic cells, respectively, Mann-Whitney U test). (**h**) In contrast with the comparable bassoon NLPs (Kolmogorov-Smirnov test), perisomatic 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 perisomatic interneurons contained more bassoon clusters (Mann-Whitney U test), whereas NLPs in individual clusters was lower (**j**). Graphs show raw data and median $\pm$ IQR. (**k**) Dual-channel STORM imaging demonstrated that CB₁ NLPs in any given distance from bassoon labeling was higher in axon terminals of perisomatic interneurons ($n = 80$ – 80 randomly selected boutons, Kolmogorov-Smirnov test, plot shows median $\pm$ 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, perisomatic axon terminals contained less bassoon, but a similar amount of CB₁. (**n**) Although the number of bassoon clusters was similar, clusters in boutons of perisomatic interneurons had less NLPs (**o**). (**p**) 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).



combined confocal and STORM imaging uncovered dense CB₁ receptor labeling with high localization precision on all investigated axon terminals ($n = 129$; **Supplementary Fig. 10d–f**). Notably, normalized CB₁ NLP distributions were similar in hippocampal GABAergic interneurons filled under *in vitro* or *in vivo* conditions (**Supplementary Fig. 10g**). Moreover, CB₁ surface distribution was also similar under both conditions (**Supplementary Fig. 10h**). Finally, CB₁ quantity on individual boutons was in positive linear correlation ($P < 0.001$) with the size of the axon terminals (**Supplementary Fig. 10i**). These measurements highlight the feasibility and power of the cell type-specific methodology for an integrated analysis of *in vivo* physiological, anatomical and molecular parameters in intact brain circuits and validate our *in vitro* findings.

Different nanoscale structure of interneuron active zones

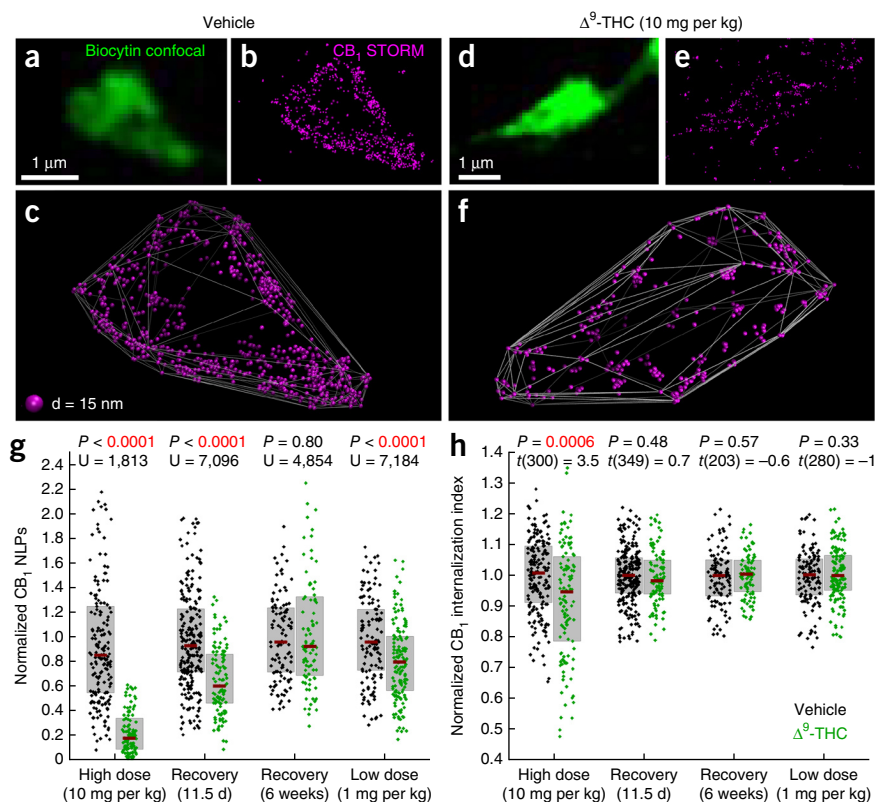
The two observations that CB₁ receptor density and nanoscale distribution pattern are similar on interneuron boutons imply that other molecular parameters account for cell type-specific cannabinoid signaling efficacy on GABA release probability. At central synapses, active zone size tightly correlates with VGCC abundance and transmitter release probability³⁰. The copy numbers of protein components in the active zone, including the quantity of bassoon and VGCC subunits, also correlate closely³¹. 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 perisomatic 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 perisomatic interneuron terminals had, on average, 49% more CB₁ receptors than those of dendritic interneurons. Given that bassoon and VGCC numbers in release machineries correlate³¹, 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,

Figure 6 Chronic THC treatment induces long-lasting loss of CB₁ from the plasma membrane of GABAergic axon terminals in a dose-dependent manner. (a,d) Deconvolved confocal images of axon terminals of perisomatic interneurons, recorded in slices prepared from mice treated with vehicle or THC (intraperitoneal injections twice a day for 6.5 d). (b,e) STORM imaging revealed a marked THC-evoked reduction of CB₁ localization points. A CB₁ antibody with improved sensitivity derived from transgenic rabbits (Online Methods) was used to enable reliable visualization of CB₁ receptors at low and high levels alike. (c,f) Visualization of CB₁ localization points in three dimensions demonstrated an increased intraterminal accumulation of CB₁ in THC-treated animals. (g) Following chronic administration of 10 mg per kg THC, CB₁ NLPs were reduced by 74% ($n = 185$ and 117 axon terminals from 3 vehicle- and 2 THC-treated animals, Mann-Whitney U test). After a drug-free period of 11.5 d, 53% of CB₁ receptors recovered, but the reduction in NLPs (35%) was still significant ($n = 283$ and 113 boutons from 3 and 3 animals). A longer recovery period of 6 weeks, however, allowed complete rescue ($n = 113$ and 92 boutons from 2 and 3 animals). Chronic treatment with a lower (1 mg per kg) dose resulted in a slight (16%) decrease in NLPs ($n = 129$ and 153 boutons from 2 and 3 animals). (h) The remaining CB₁ localization points were shifted toward the center of the axon terminal after a high dose of THC (unpaired two-sided t test). However, internalization was not detectable after recovery for 11.5 d or 6 weeks, or after low dose of THC. Graphs show raw data and median $\pm$ IQR.



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 surface²⁷) 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)³², 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

(Fig. 6a–g). 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^{35,36}. 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, release¹⁶. LTP only partially recovers after THC withdrawal and remains persistently weaker for weeks¹⁶, 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 recovered³², but substantially weaker hippocampal LTP still persists¹⁶. 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 effects³⁹. 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 rodents⁴⁰. 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 pathophysiologically 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 subfield¹. A stunning molecular diversity of the synapses connecting these various cell types supports the specific computing tasks of given brain circuits³. 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 perisynaptic position of a major endocannabinoid-synthesizing enzyme⁴⁵. 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, respectively²². 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 imaging¹⁹. 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 populations²⁷ 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 perisomatically-projecting and dendritically-projecting hippocampal GABAergic interneurons, which exhibit marked differences in the strength of cannabinoid signaling¹⁵. 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^{22–24}, 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^{22–24}. However, similar anchoring mechanisms for presynaptic CB₁ receptors have not yet been identified, despite the fact that other presynaptic GPCRs exhibit notable synaptic segregation patterns⁴⁶. Constitutive CB₁ trafficking occurs between the plasmalemmal surface of the pre-terminal axon and boutons⁴⁷; 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 dendritic CB₁-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^{27,31}. Moreover, bigger axon terminals have larger total active zone areas, accommodating more VGCCs and other release machinery components³⁰. 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 dendritic 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 dendritic GABAergic synapses on the somatodendritic surface of pyramidal neurons^{1,14}, the lower bassoon number in individual bassoon clusters in perisomatic boutons is an interesting finding. Because bassoon copy numbers correlate with the number of VGCC subunits³¹, 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 interneurons¹⁵. Because saturating levels of cannabinoids are equally effective in reducing GABAergic transmission at both terminal types¹⁵, 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 humans⁴⁸ and chronic cannabis users exhibit strong, long-lasting cognitive deficits, including impairments in hippocampus-dependent memory functions³⁷. 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 regions³⁸. In accordance with the circuit-specific effects, chronic THC administration causes a strong reduction in cannabinoid efficacy selectively on GABAergic transmission¹⁶, 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 treatment³², it was surprising that CB₁ receptors only partially recovered at this time point. However, hippocampal LTP is still substantially impaired after 2 weeks¹⁶ and human memory deficits persist even after 28 d of abstinence³⁷. 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 deficits^{17,18}, and sets the threshold for LTP at excitatory synapses^{35,36}. 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 content^{33,34,49}, 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 applications^{39,40} 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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AUTHOR CONTRIBUTIONS

B.D. and L.B. designed and performed STORM experiments and corresponding analytical tools, analyzed data, prepared the figures, and wrote the manuscript. M.L. carried out patch-clamp electrophysiological recordings, cell reconstructions, STORM imaging and data analysis. S.I.S. conducted and analyzed *in vitro* electrophysiological measurements as well as participated in STORM imaging and data analysis. E.S., B.P., S.G.W. and R.N. developed immunostaining, tissue processing and antibody-labeling procedures for STORM imaging and contributed to data acquisition and analysis. C.M.H. performed cell culture and electron microscopy experiments and data analysis. G.Y.B., J.C., I. Kacsóvics and M.W. developed and validated antibodies. C.V. performed *in vivo* electrophysiology. S.-H.L. carried out *in vitro* electrophysiological recordings. M. Matolcsi developed mathematical analysis tools. C.S., M. Melis and M.P. designed and performed the chronic drug administration experiment. I.S. designed and supervised *in vitro* and *in vivo* electrophysiological experiments and contributed to manuscript editing. I. Katona conceived and supervised the project, performed *in vitro* electrophysiological recordings, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the [online version of the paper](#).

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ONLINE METHODS

Maintenance, transfection and immunostaining of HEK293 cells. HEK293 cells were a kind gift from B. Gereben (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-D-lysine (20 µg ml⁻¹) coated glass bottomed dishes. Transfection was performed using 1 µg of cDNA encoding an N-terminally EGFP-tagged CB₁ protein⁵¹ 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 Alexa488-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 heterogeneous 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/6H mice⁵³ (kindly provided by A. Zimmer, University of Bonn) were deeply anesthetized with intraperitoneal injection of ketamine-xylazine (25 mg ml⁻¹ ketamine, 5 mg ml⁻¹ xylazine, 0.1% (m/m) 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)⁵² as described earlier⁵⁴. 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^{-/-} mice⁵³. 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 earlier⁵⁵, 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 from the mode and interquartile range of the relative positions

$$\left(\text{BDI} = \frac{0.5}{|M| \cdot (Q3 - Q1)} \right)$$

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 Neurolucida 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 earlier⁵², 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 mice⁵⁶). 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 unlabeled 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 protocol⁵⁷) 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 previously²⁷ containing 5% (m/v) glucose, 0.1 M mercaptoethylamine, 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) activity⁵⁸, as they carry and express one extra copy of the rabbit FcRn α -chain encoding gene (rabbit FCGRT) in addition to the endogenous rabbit FCGRT gene on New Zealand White rabbit genetic background, were used in this study. These rabbits are coded as NZW Tg1 rabbit FCGRT (78), wherein 78 refers to the founder line. Tg rabbits and their wild-type littermates were housed in specified pathogen-free facility. All the treatments of rabbits 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 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'-MHRAESCIKSTVKIAKVT MSVSTDTSAEAL-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 Immobilization 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 imaging⁵⁹). The setup was controlled by Nikon NIS-Elements AR software with N-STORM module. For STORM imaging, a 300-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 2 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/far-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 definition⁶⁰, 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 ImageJ⁶¹. 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 on coverslips. 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 deconvolved 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

measurement functions of ImageJ. Next, the STORM localization points belonging to the particular bouton were determined. In the specific case of CB₁-CCK double immunostaining comparison in wild-type and CB₁ knockout mice, rectangles were placed onto the CCK-immunopositive boutons. The coordinates of localizations in the selected volume were saved for further analysis. The perimeter of the bouton was determined as the perimeter of a two-dimensional convex hull fitted on the CB₁ localizations.

For the measurement of surface distances, a tri-dimensional convex hull was fitted onto the outermost CB₁ localizations, and the shortest distance between two localizations on the surface of this polyhedron was determined. First, we approximated the bouton surface by calculating the three-dimensional convex hull of the CB₁ localizations belonging to a single biocytin-filled bouton using Matlab software (Mathworks). Localization points were projected onto the surface by determining the nearest point of the convex hull. To avoid possible problems arising from projection to a wrong face, internal localizations with a distance of more than 80 nm from the surface were rejected from the analysis. A second convex hull was fitted on the bassoon localization points, and the nearest point 'Q' on the surface of this polyhedron to each CB₁ localization point 'P1' was determined. This point Q was then projected onto the bouton surface by determining the point 'P2' of the CB₁ convex hull that was nearest to Q. Given the two points P1 and P2, the shortest route between P1 and P2 on the surface of the bouton was approximated by the following algorithm. An axis was defined by connecting P1 and P2. If any half-plane S is fit on this axis then S intersects the surface of the convex hull in a path connecting P1 and P2. This path can be determined explicitly by the points of intersection of S and the edges of the convex hull. Hence the length of this route is also explicitly computable for each S. Finally, we rotated S around the axis degree by degree, and the minimum of the arising trajectories was assumed as the approximately shortest route between P1 and P2 on the surface of the convex hull.

For the measurement of CB₁ localization point densities, random points of the hull surface were first selected at a high density. Afterwards, the number of CB₁ localization points within 200 nm of each point was determined together with the surface distance of each measurement point from the nearest point of a convex hull fitted onto the bassoon localization points.

For the measurements of bassoon localization point clusters, we implemented the widely used Density-Based Spatial Clustering of Applications with Noise⁶² (DBSCAN) in Matlab. This method finds dense clusters in a set of three-dimensional points with the advantage that there is no need to define a priori number of clusters. A point P is considered as a member of a cluster, if it has at least minP number of neighbors within epsilon, whereas P is considered as noise otherwise. All 21 possible combinations of several settings of the epsilon (50–150 nm) and minP (2–4) parameters of the algorithm were run on the data, and yielded similar results. Data are shown for the epsilon = 100 nm and minP = 3 setting.

Detecting multiple blinkings of the same fluorophore can potentially lead to artificial clustering of signal in single-molecule localization-based super-resolution microscopy data⁶³. However, the following three measurements and observations indicate that the detected clusters represent bassoon distribution and cannot be explained by multiple detections of fluorophores or apparent clustering of randomly distributed signal. First of all, the vast majority of detected fluorophores blinked only once during the time of the imaging (~80%), as images of isolated fluorophores appeared as single localizations, and rarely as doublets or triplets, while the bassoon clusters typically comprised of 10–20 localization points (on average 15.46). Second, the size of the bassoon clusters were much larger than the distribution of multiple localizations of single fluorophores. Although the mean diameter of a bassoon cluster was 201 nm, the distribution of multiple localizations of isolated fluorophores was restricted to 40 nm diameter (Supplementary Fig. 2a–c). Third, when the same clustering analysis was performed on data from regions of interest taken from pyramidal cell nuclei then the analysis could not reproduce a cluster/noise ratio or cluster size distribution specific for data from regions of interest obtained from axon terminals.

To discern whether given molecular parameters are related to axon terminal size or represent cell type-specific features, we analyzed two samples of boutons with identical size distribution from both interneuron types. The samples were generated by picking an equal number of boutons from each cell type from in the interdecile range of bouton sizes of the given cell type, at random. Then, for

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.

Chronic drug treatment. 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^9 -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 6 weeks after THC withdrawal or vehicle treatment, respectively. In a separate experiment, two groups of mice received either a subthreshold dose of THC (1 ml kg⁻¹, intraperitoneal), which does not elicit classical behavioral effects of cannabinoids⁴⁰, 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 points 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 ($\alpha = 0.05$) 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

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.

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