Title

High-throughput synapse profiling reveals cell-type-specific spatial configurations in the fly brain

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Abstract

Characterization of intracellular synapse heterogeneity aides to understand the intricate computational logic of neuronal circuits. Despite recent advances in connectomics, the spatial patterns of synapses and their inter-individual variability remain largely unknown. Using directed split-GFP reconstitution, we achieved visualization of endogenous Bruchpilot (Brp) proteins, the presynaptic active zone (AZ) scaffold, in a cell-type-specific manner. By developing a high-throughput quantification pipeline, we profiled AZ structures in identified neurons of the mushroom body circuit, where intracellular synaptic pattern is crucial due to its compartmentalized connectivity. Quantitative characterization of the pattern of Brp clusters across multiple individuals revealed cell-type-dependent synapse heterogeneity and stereotypy. Furthermore, we discovered previously unidentified sub-compartmental synapse configuration and its regulation by cAMP signaling. These results of synapse profiling uncovered multi-layered organizations of AZs, ranging from neighboring synapses to consistent patterns across individuals.

Teaser

High-throughput profiling of single active zones revealed previously unidentified multi-layered synaptic organizations.

Introduction

The brain is a complex network of neurons, and understanding their wiring strategies provides crucial insights into their roles in neuronal computation. Presynaptic sites of *Drosophila* are decorated with the electron-dense projection composed of the active-zone (AZ) scaffold protein Brp (*1–7*). These Brp projections have enabled the whole-brain connectomes, comprehensive mapping of synaptic connections at the electron microscopic level (*8–12*). While connectome-based approaches catalyzed the exploration of cell type distinctions, circuit motifs, and neurotransmitters, their application to comparisons across different conditions is still limited. Since the synaptic structure and connectivity reflect substantial variability of development and experiences (*11*, *13–18*), cross-individual comparisons are necessary. Fluorescence labeling offers high throughputs and contents

when comparing different samples. Nevertheless, characterizing endogenous synapses in specific cells using fluorescence-labeling remains challenging in the central nervous system (CNS) due to the dense synaptic connections from different neurons. This has driven growing interest in designing cell-type-specific fluorescence-tagging strategies for endogenous synaptic protein (*19*).

The circuit of *Drosophila* mushroom bodies (MBs) plays a central role in olfactory associative learning (*20*). The major intrinsic MB neuron, known as Kenyon cells (KCs) respond to odor stimulation and synapse onto the spatially segregated dendrites of MB output neurons (MBONs) that divide the MB into compartments. Distinct types of dopamine neurons (DANs) synapse onto and thereby modulate KC-MBON synapses in specific compartments (*21*). Presynaptic calcium levels of these DANs undergo local GABAergic modulation and inform the memory specificity, postulating the distinct synapse structures at individual release sites (*22*) . Two types of giant interneurons, the anterior paired lateral neuron (APL) and the dorsal paired medial (DPM) neuron extensively ramify across the MB lobes and provide recurrent modulations to KC synapses (*23–30*). Considering their comprehensive projections throughout the MB lobes, the modulation of local circuits requires intracellular synaptic tuning. These studies together underscore the importance to spatially distinguish individual synapses, while the high active zone density in the MB lobes precluded conventional approaches of fluorescent microscopy from profiling synaptic structures of specific neurons (*9*).

Here, we present a spatial analysis of individual AZs within the MB circuit at single-cell resolution, achieved by cell-type-specific visualization of endogenous Brp using the CRISPR/Cas9-mediated split-GFP tagging system. We developed a high-throughput quantification pipeline to systematically profile Brp clusters of individual AZs in different MB-innervating neurons. Characterizing multiple structural parameters revealed a significant synaptic heterogeneity within single neurons and AZ distribution stereotypy across individuals. These cell-type-specific synapse profiles suggest that AZs are organized at multiple scales, ranging from neighboring synapses to across individuals.

Results

Establishing an experimental system for cell-type-specific profiling of active zone structures

To visualize endogenous Brp in designated cell types, we employed a CRISPR/Cas9-mediated split-GFP tagging system. Using CRISPR/Cas9, we inserted the GFP11 fragment (the eleventh β-strand of the super-folder GFP) just prior to the stop codon of *brp* gene. The GFP1-10 fragment is expressed cell-type-specifically by the GAL4/UAS system. The self-assembly of GFP therefore occurs only in cells expressing GAL4 (Fig. 1A). We directed the GFP reconstitution and expressed plasma membrane marker in the single serotonergic DPM neuron using VT64246-GAL4 (Fig. 1B). Confocal microscopy revealed Brp::reconstituted GFP (Brp::rGFP) signals only to the MB lobes, matching the location of DPM axon terminals (*30*). These signals appear as puncta within plasma membrane swellings resembling presynaptic boutons (Fig. 1B), suggesting that Brp::rGFP clusters labeled individual AZs in DPM neuron.

To systematically profile Brp::rGFP clusters, we developed an image processing pipeline (Fig. 2A). In brief, image deconvolution was applied to reduce out-of-focus light and enhance signal sharpness. Individual Brp::rGFP clusters were then segmented using the 3D suite plugin in Fiji (*31*), generating the 3D region-of-interests (3D ROIs) that

encircle each cluster. Brp::rGFP signal intensity, volume and location of individual clusters were measured using the 3D ROIs. To optimize the segmentation, we systematically varied the parameters and calculated the F-score to measure the detection accuracy against manually defined ground truth (see Materials and Methods). We found that the F-score is near 1.0 in segmenting Brp::rGFP clusters within DPM and APL neurons and achieved > 0.90 in KCs with certain combinations of segmentation parameters (Fig. 2B).

To confirm that Brp clusters represent AZs, we quantified the number of Brp::rGFP clusters in DPM and APL neurons specifically and compared it to the connectome data. We found that cluster counts closely matched the number of AZs annotated by the hemibrain connectome (Fig. 2C) (*9*). This result validated our method that accurately isolated Brp::rGFP clusters corresponding to single AZs.

AZ profiles within KCs are compartmentally heterogeneous

Brp accumulation at individual AZs is known to be heterogeneous even within a single motor neuron (*5*, *6*, *32*, *33*). To study such AZ heterogeneity in CNS neurons, we quantified the signal intensity of individual Brp::rGFP clusters in MB lobes. Using KC subtype-specific drivers to direct split-GFP reconstitution in γ KC (MB009B-GAL4), α'/β' KCs (MB370B-GAL4) and α/β KCs (MB008B-GAL4). we found that Brp::rGFP intensities are highly diverse in all KC subtypes (Fig. 3A).

To visualize spatial patterns of Brp::rGFP intensity in each KC subtype, we reconstructed the 3D distribution of Brp::rGFP clusters and color-coded them by signal intensities (Fig. 3B). We found that clusters showed noticeable intensity differences between compartments in all subtypes. Adjacent compartments such as γ1 and γ2, β'2 and β'1, α2 and α3, showed drastic differences (Fig. 3B). These compartmental patterns were stable across individuals (*34*). This result suggests that AZs in different compartments have distinct structures. Since individual KCs of each subtype synapse onto all compartments (*8–10*, *35*), this Brp compartmental heterogeneity is likely formed intracellularly.

Intracellular heterogeneity of Brp concentration at individual AZs and cell-type dependent diversity

Super-resolution microscopy has shown that the Brp molecular density at individual AZs is dynamically adjusted in motor neurons (*36*, *37*). We hypothesized that the amount of Brp does not necessarily correlate with AZ cluster size. To verify this, we calculated the Brp concentration by quantifying both the volume (number of voxels within a 3D ROI) and the Brp::rGFP intensity of each cluster. We found clusters with similar sizes exhibit different signal intensities in DPM neuron, suggesting the heterogeneity of Brp concentration at individual AZs (Fig. 4A). Correlation analysis between the Brp::rGFP intensity and cluster volume revealed a substantial variability among cell types. Specifically, the correlation is high in KCs with a significant difference among KC subtypes. In contrast, the correlation is low for DPM and APL neurons (Fig. 4B-E). These results suggest a cell-type dependent variability of the Brp concentration of individual clusters.

Stereotypy of intracellular distribution of AZs

To study the spatial distribution of the Brp clusters within single neurons, we focused on DANs. MB504B-GAL4 labels four types of single PPL1 DANs projecting to distinct

compartments (*38*). Using this driver, we analyzed the Brp::rGFP distribution in each compartment and found it uneven in both α2α'2 and α3 (Fig. 5A-B). To better characterize the cluster distribution, we calculated the "AZ density" defined as the number of Brp::rGFP clusters surrounding a particular cluster within a specified radius (see Fig. 2A and Materials and Methods for detail). By color-coding Brp::rGFP clusters according to their AZ density, we found that AZs in PPL1-α3 were more localized to the core of α3. This pattern is found to be consistent across individuals (Fig. 5C-D), suggesting a stereotyped AZ distribution within PPL1-α3 neurons.

We extended the analysis to two other single pairs of neurons innervating the MBs, the DPM and APL neurons. Visualizing AZ density revealed a stereotyped and compartment specific distribution in DPM neurons (Fig. 6A). Specifically, the AZ density was constantly high in the α'/β' lobes across all individuals, consistent with the known functional importance of DPM branches in the α'/β' lobes (*28*). In contrast, this compartmental pattern is less pronounced in APL. Notably, the ratio of AZ density between α3 and α'3 is strikingly different among individuals (Fig. 6B). Taken together, these data suggest that the individual variability in AZ distribution depends on the cell type.

Local regulation of AZ structures

We have found the compartmentalized Brp distributions in KCs. Intra-compartmental variability of synaptic plasticity upon associative learnings was also reported, prompting us to analyze sub-compartmental organization of AZs (*39*, *40*). However, the Brp::rGFP intensities of neighboring clusters were highly variable (Fig. 3B). To analyze the sub-compartmental AZ patterns, we reduced such high frequency variability by calculating the "local intensity" for each cluster (the average Brp::rGFP intensity of all surrounding clusters within a specified radius *r*, see Materials and Methods for detail). By analyzing the local intensity in γ KCs, we further identified AZ "hot spots", sub-compartment-sized small patches with high local intensities, on top of the compartmental differences (Fig. 7A). Since Brp tend to localize to AZ-dense boutons in motor neurons (*5*), we hypothesized that these hot spots correspond to regions with higher AZ densities. Indeed, we found that hot spots appear in high AZ densities regions (Fig. 7A). The correlation between the local intensity and the AZ density is also high in KCs (Fig. 7C). In contrast, for DPM and APL neurons, patches of high local intensities are not associated with high AZ density regions (Fig. 7B-C).

We further examined potential local patterns among AZs in proximity by calculating the correlation between nearest neighbor clusters in terms of their Brp::rGFP intensities. This analysis revealed strong correlations in KCs but not in DPM (Fig. 7D). These results suggest previously unnoticed sub-compartmental synaptic structures organized across individual KCs. Such local structures may play an important role in the function of KCs, such as in associative learning. Given that Rutabaga (Rut), a calcium/calmodulin-dependent adenylyl cyclase, is required in KCs for associative learning (*41*), we downregulated *rut* expression in KCs using RNA interference (*42*) (RNAi). Following *rut* knock-down, the location of high local intensity patches was largely disrupted (Fig. 8A). Furthermore, both the local intensity-AZ density correlation and the nearest neighbor correlation were significantly reduced in KCs with reduced *rut* expression (Fig. 8B-C). Altogether, we propose that associative learning induces sub-compartmental memory traces in small groups of KC terminals by local regulation of cAMP signaling.

Discussion

By leveraging the CRISPR/Cas9 genome editing and split-GFP technique, we systematically profiled presynaptic structures of MB neurons in a cell-type-specific manner. This approach enabled high-throughput analysis of multiple brain samples and the detailed characterizations revealing previously inaccessible AZ structural features of CNS neurons.

Single-cell level analysis of AZ profiles revealed the cell-type-specific stereotypy in spatial regulation (Fig. 5-6). We found that the AZ density in DPM neurons is consistently high in the α'/β' lobes across individuals (Fig. 6). This AZ stereotypy of DPM explains the functional significance of the branches in the α'/β' lobes. Overexpression of a Down syndrome cell adhesion molecule (DScam) variant in DPM neurons was shown to disrupt their innervation except in the α'/β' lobe, while the memory formation remained unaffected (*28*). Similarly, our analysis identified the intracellular stereotypy in AZ distribution of the PPL1- α3 DAN within the α3 compartment (Fig. 5). This sub-compartmental organization of DANs and layered projections of α/β KCs may underlie the differential dopaminergic modulation of KC subtypes in associative learning (*43*). While such stereotypy is less pronounced in APL, we found strong correlations between Brp::rGFP intensities of neighboring AZs in contrast to DPM (Fig. 7D). This suggests that AZs in APL neurons are organized more locally, allowing them to modulate microcircuits composed of KCs, DANs and MBONs (*44*). Collectively, the spatial scale of AZ organizations depends on the cell type, reflecting the function of individual MB neurons.

We observed that Brp::rGFP intensity correlates strongly with the AZ size in KC subtypes, in contrast to DPM and APL neurons (Fig. 4). Previous studies using localization microscopy showed that the molecular density of synaptic proteins at AZs can be dynamically adjusted in motor neurons, potentially modulating the synaptic vesicle release (*36*, *37*, *45*). The variable Brp concentrations (i.e. the ratio of the Brp::rGFP intensity to the AZ size) in DPM and APL neurons may represent AZs with distinct Brp densities and therefore different functional modes (Fig. 4) (*36*). Notably, a recent study successfully predicted the neurotransmitter type of neurons using the ultrastructure of dense projections as one of the parameters (*46*), suggesting neurotransmitter-specific AZ structures. Interestingly, both the DPM and APL neurons are reported to release multiple neurotransmitters (*25*, *29*, *47–50*). Distinct Brp concentrations at AZs might thus underlie particular release modes or neurotransmitter types.

In KCs, spatial analysis revealed AZ “hot spots”, regions with high Brp localization and AZ densities. Additionally, we found strong correlations between Brp::rGFP intensities at neighboring AZs within KCs (Fig. 7). These results suggest previously unidentified, highly localized sub-compartmental AZ structures, which are likely organized across individual KCs. Presynaptic terminals of a single KC are found to be highly heterogeneous in terms of their plasticity upon dopamine modulation and associative learning (*39*, *40*). A recent study found the sub-compartmentally heterogeneous activities of dopamine terminals explain memory specificity (*22*). Such locally distinct dopamine input may be a source of the AZ hot spot formation in KC terminals. This model is consistent with the localization of two opposing dopamine receptors Dop1R1 and Dop2R to AZs (*51*) and the requirement of Rut, a calcium/calmodulin dependent adenylyl cyclase that is essential for associative memory formation (Fig. 8) (*41*). Since cAMP signaling plays a crucial role in the AZ structural

plasticity especially during associative memory formation (*34*, *45*, *52*), remodeling of sub-compartmental AZ structures may represent a structural memory trace (*17*, *18*).

Materials and Methods

Animals

Flies were maintained on standard cornmeal food at 25 °C under a 12:12 h light-dark cycle for all experiments. All flies used for experiments are 3-7 days old adult males. Flies were transferred to fresh food vials after hatching and flipped every two days before experiments. The GAL4-UAS system was used to express transgenes, and balancers were removed for animals used in all experiments. Fly strains and resources used in this study are as follow: MB008B-GAL4 (BDSC 68291) (*21*); MB009B-GAL4 (BDSC 68292) (*21*); MB370B-GAL4 (BDSC 68319) (*21*); MB504B-GAL4 (BDSC 68329) (*21*) ; VT64246-GAL4 (VDRC 204311) (*53*); GH146-GAL4 (BDSC 30026) (*54*); R58E02-GAL4 (BDSC 41347) (*55*); R13F02-GAL4 (BDSC 48571) (*55*) ; UAS-CD4::tdTomato (BDSC 35841, by Dr. Yhu Nung Jan & Chun Han); UAS-Rut-RNAi (BDSC 80468) (*56*) ; *brp::GFP11* (*34*); UAS-GFP1-10 (*57*).

Sample preparation

Animals used in experiments involving comparisons between control and experimental groups were dissected on the same day. Data were collected from multiple batches of experiments performed on different days. Flies were anesthetized by ice and placed on ice before dissection. The dissection was performed in cold PBS to collect the brain without the ventral nerve cord. The brains were subsequently fixed by 2% paraformaldehyde for 1 h and then washed 3×20 min by 0.1% PBT (0.1% Triton X-100 in PBS) in a PCR tube, typically 5-6 brains in one tube. Samples were mounted on microscope slides using mounting medium SeeDB2S (*58*).

Confocal microscopy image acquisition

The image acquisition was performed using an Olympus FV1200 confocal microscope platform equipped with both PMT and GaAsP high sensitivity detectors and a 60×/1.42 NA oil immersion objective (PLAPON60XO, Olympus). The Kalman filter was turned on to 2× averaging while scanning. For Brp::rGFP samples in all GAL4 types, scanning speed: 2 µs/pixel with a voxel size of lateral 0.079 × 0.079 × axial 0.370 µm. For experimental point spread function (PSF) imaging, SeeDB2S immersed beads were scanned in a setting that is 60×/1.42 NA oil immersion objective; 473 nm laser power: 2.0%; 559 nm laser power: 1.5%; voxel size: 0.079 µm × 0.079 µm × 0.370 µm; scanning speed: 4 µs/pixel, to produce multiple bead images.

PSF acquisition and image deconvolution

Sub-resolution fluorescent beads (Tetraspeck Microspheres 0.1 µm, Thermo Fisher Scientific, T7279) were imaged to generate the PSF. The bead solution was diluted with distilled water and sonicated several times to eliminate aggregation. PSF images acquired were processed using Amira software (Thermo Fisher Scientific) with the Extract Point Spread Function module. The PSFs extracted from each image were averaged to create a single PSF for later image deconvolution (Resizing voxel size: 0.079 µm × 0.079 µm × 0.370 µm; 32 pixels × 32 pixels × 21 slices of image size). Image deconvolution was performed by using the Richardson-Lucy iterative non-blind algorithm in a Fiji plugin DeconvolutionLab2 (*59*), or by CLIJ2 GPU-based Richardson-Lucy deconvolution (*60*). Images were deconvolved 20 times iteratively to improve image quality.

Brp clusters segmentation and data analysis

For Brp::rGFP cluster detection, the image stack size was adjusted to double the XY plate pixel number using Fiji to increase the detection precision. The images were subsequently processed using the 3D maxima finder and the 3D spot segmentation function of the 3D suite package in Fiji. 3D maxima were identified for each cluster and used as the starting point for pixel clustering (*31*). An intensity threshold (threshold) and the noise tolerance were set to reduce noise and adjust the sensitivity of 3D maxima detection respectively. The threshold set the value of all pixels that have values below the threshold to NaN (not a number). The noise tolerance is set in the 3D maxima finder. To be noticed, the threshold is only applied to the image used to find 3D maxima. The 3D maxima finder generates the 3D maxima. The 3D segmentation function then requires the 3D maxima and the raw image, so that there is no intensity cut affecting the measurement of cluster volume (used deconvolved image). The local thresholding method for the 3D segmentation was set to Gaussian fit, with the "SD value" set to 2. Cluster volume was measured as the voxel count within the 3D ROI.

The above procedures may include a tiling and stitching step for images and data because of the limited computer power. Typically, an image was tiled into 25 sub-stacks with overlapped margins. After being processed separately, the data acquired from all tiled sub-stacks was stitched back with a deletion of overlapping data points to generate the complete detection result. Nearest neighbors were identified for each punctum and spatial distances in between were calculated using Python. For each sample, mean NND was calculated separately. For a particular Brp::rGFP cluster, the number of surrounding clusters within r (r = 3 × mean NND) spherically including itself is referred as the "AZ density". The "local intensity" of a particular Brp::rGFP cluster is the average of Brp::rGFP intensity of all clusters surrounding within r (r = 3 × mean NND) including itself. The plot function of Python was used to plot the color-coded 3D reconstructed maps.

F-score calculation

The 3D detection parameters were optimized by comparing the 3D maxima detection results to manually defined ground truths. The F-score was used to assess detection precision. F-score is the harmonic mean of a system's precision and recall value, calculated by the following formula: 2 × [(Precision × Recall) / (Precision + Recall)]. The precision is the number of true positives divided by the total number of identified positives, including incorrect identification. The recall is the number of true positives divided by the total number of true data points defined as the ground truth. F-scores shown in Fig. 1 were automatically calculated using a customized Fiji Marco that aligned 3D maxima with ground truth. Different combinations of noise threshold and noise tolerance were tested for the test images cropped from raw images.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8, 9 and 10 or Python. For statistical comparisons, Kruskal-Wallis test with original False Discovery Rate method of Benjamini and Hochberg correction was applied. A desired false discovery rate of 0.05. Pearson's correlation coefficient (R) was calculated using Python.

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Further information and requests on resources and reagents should be directed to and will be fulfilled by the corresponding author, Dr. Hiromu Tanimoto (hiromut@m.tohoku.ac.jp). This study did not generate any unique code. All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Figures and Tables

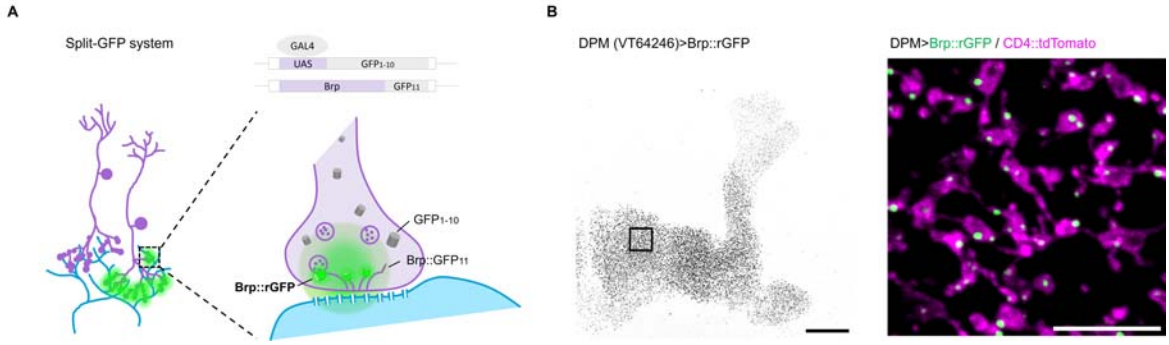

Fig. 1. Cell-type-specific visualization of endogenous presynaptic scaffold. (**A**) Schematic of the split-GFP tagging strategy. The GFP11 fragment is inserted by CRISPR/Cas9 just prior to the stop codon of *brp*, while GFP1-10 is expressed in specific cell types via GAL4-UAS system. (**B**) Brp::rGFP signals in DPM. The entire MB lobe structure is shown. The black box in the left panel indicates the zoom-in area (right panel). GFP1-10 and CD4::tdTomato (magenta) were co-expressed using VT64246-GAL4. Scale bar, 20 µm (left); 5 µm (right).

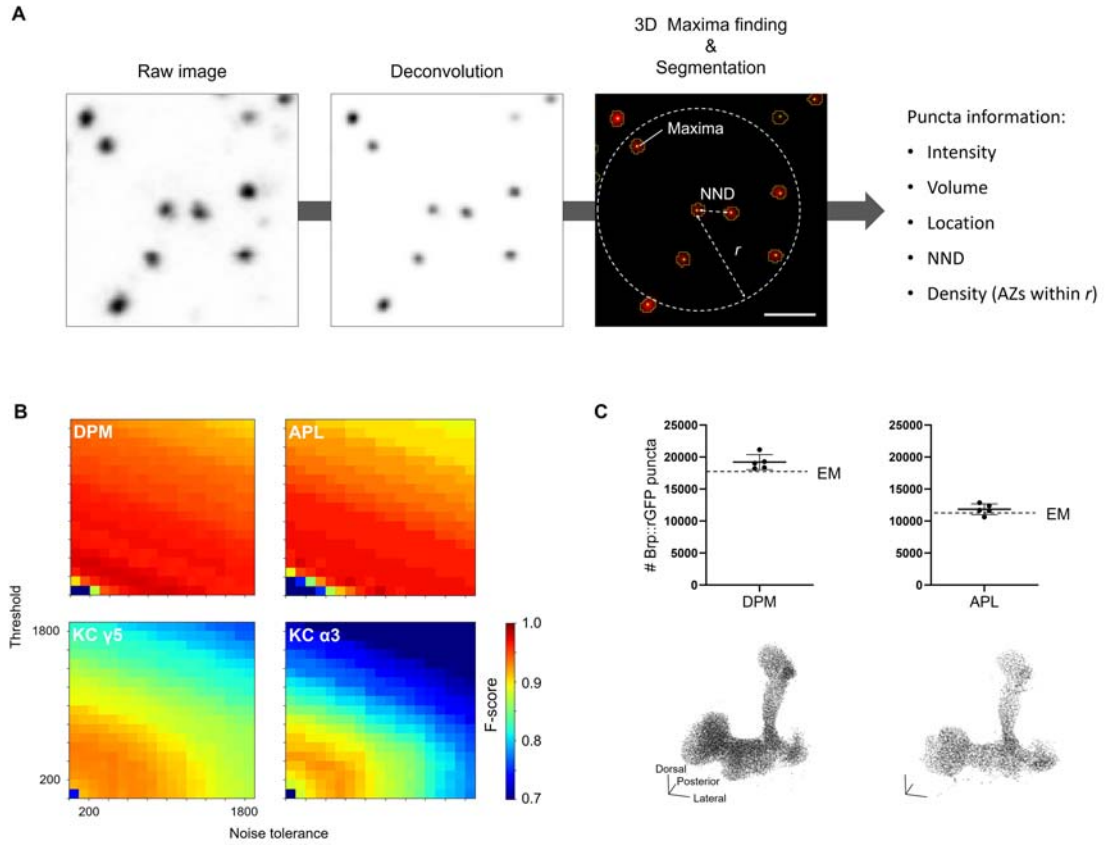

Fig. 2. Spatial characterization of Brp::rGFP clusters at single-AZ level. (**A**) Image processing pipeline. Raw images were processed with image deconvolution to improve the signal quality. 3D maxima (green pixels) are detected for each Brp::rGFP cluster. Pixels around the maxima were clustered and used to created 3D ROIs, indicated by yellow circles. Brp::rGFP intensity, cluster volume and location were recorded. The mean nearest neighbor distance (NND) was determined for each sample, with *r* set to 3 × mean NND. The "AZ density" is the number of Brp::rGFP clusters within *r*. (**B**) Heatmaps of F-scores showing the detection performance across in different cell types. An intensity threshold (threshold) is applied to reduce the background noise when detecting 3D maxima. The noise tolerance is the parameter that adjusts the sensitivity of 3D maxima detection. The color represents the F-score value. (**C**) Number of Brp::rGFP clusters detected in DPM and APL. The dashed lines indicate the number of electron dense projections identified in the hemibrain connectome for DPM (17530) and APL (10789) on the MB lobes and peduncle (cite the resource here). For both DPM and APL, n = 5. Lower panels show the representative 3D reconstructions of Brp::rGFP clusters in DPM and APL. Scale bars, 20 μm. Data were presented as mean ± s.e.m.

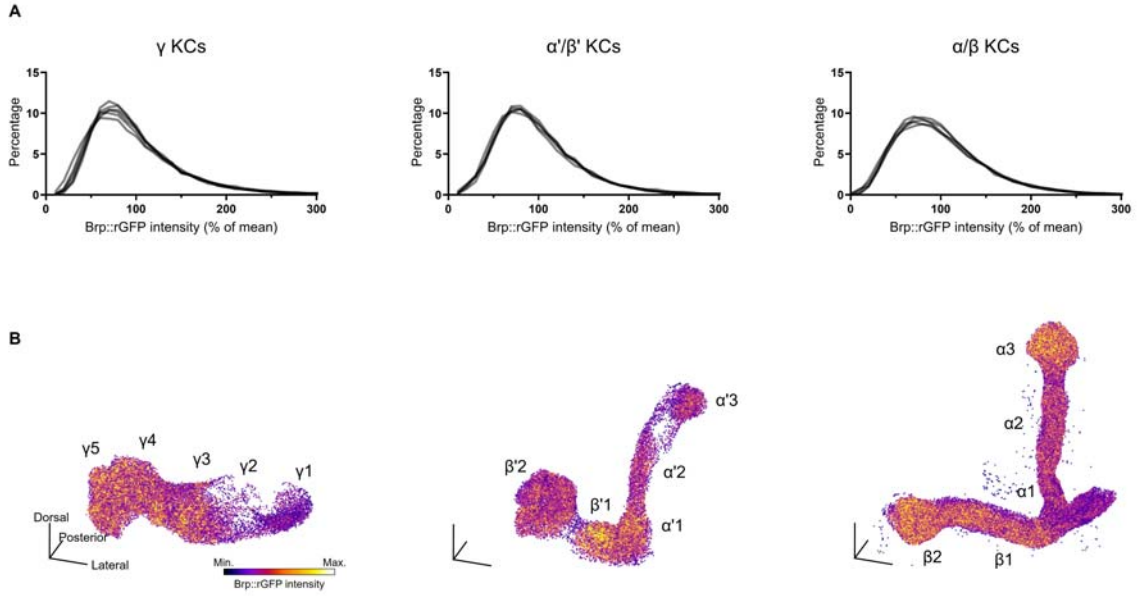

Fig. 3. Compartmentalized AZ structures of KCs. (**A**) Histograms of Brp::rGFP intensity of individual AZs from three KC subtypes. The intensity value is normalized by the average value of each data set (each sample). Each line represents the histogram of one independent sample. γ KCs (MB009B-GAL4), n = 6; α/β KCs (MB370B-GAL4), n = 5; α'/β' KCs (MB008B-GAL4), n = 5. (**B**) 3D reconstructions of Brp::rGFP clusters in three KC subtypes, colored by the Brp::rGFP intensity. The approximate locations of compartments are as indicated. Min. and Max. were set to represent the lowest and highest 5% of Brp::rGFP intensity value in the dataset respectively. Scale bars, 20 μm.

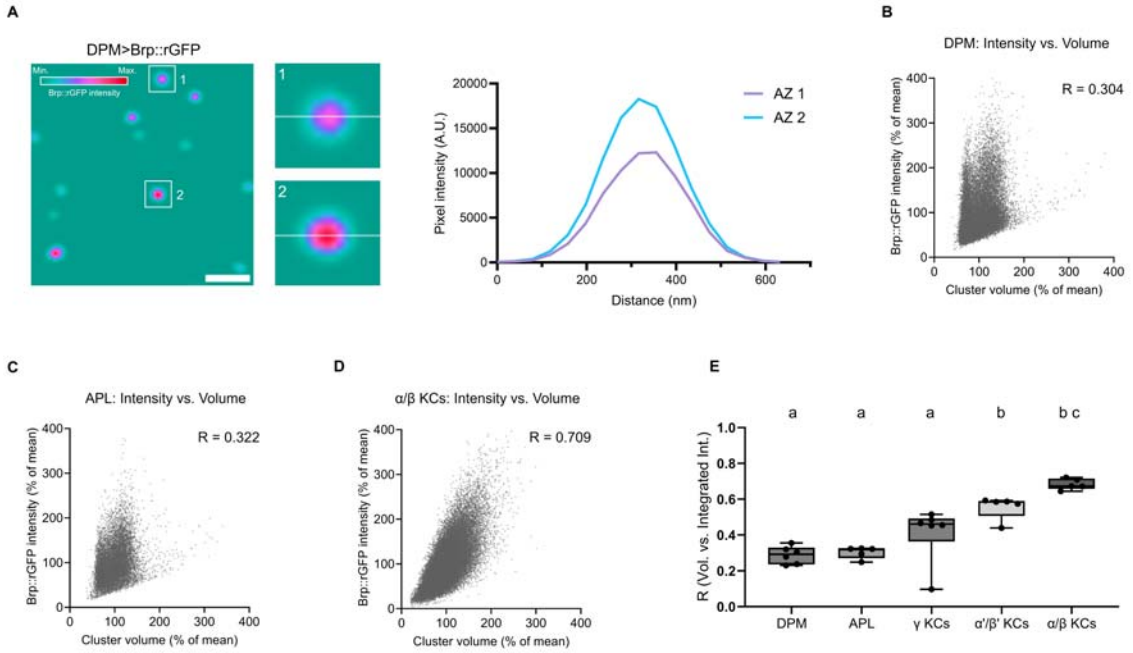

Fig. 4. The variability of Brp::rGFP concentrations depends on cell type.
(**A**) Brp::rGFP clusters with distinct intensities but similar size in DPM. The left panel shows a cropped image of Brp::rGFP in DPM. White boxes indicate areas zoomed-in in panels 1 and 2. Transparent white lines in panels 1 and 2 show the lines on which the intensity profiles were plotted. Intensity profiles were plotted for AZ1 (magenta) and AZ2 (blue) respectively. (**B-D**) Scatter plots showing the correlation between the Brp::rGFP intensity and cluster volume in DPM, APL and α/β KCs. Data of three representative samples were shown. Pearson correlation coefficient R were calculated for each sample. (**E**) Correlations between Brp::rGFP intensity and cluster volume in different cell types. DPM (n = 6), APL (n = 5), γ KCs (n = 6, MB009B-GAL4), α'/β' KCs (n = 5, MB370B-GAL4), α/β KCs (n = 5, MB008B-GAL4). Kruskal-Wallis test with original False Discovery Rate (FDR) method of Benjamini and Hochberg correction. DPM vs. α'/β' KCs: P = 0.0253; DPM vs. α/β KCs: P = 0.0009; APL vs. α/β KCs: P = 0.0035; γ KCs vs. α/β KCs: P = 0.0418. Kruskal-Wallis test. Values marked with different lowercase letters represent significant difference ($P < 0.05$); Data were presented as box plots showing center (median), whiskers (Min. to Max.).

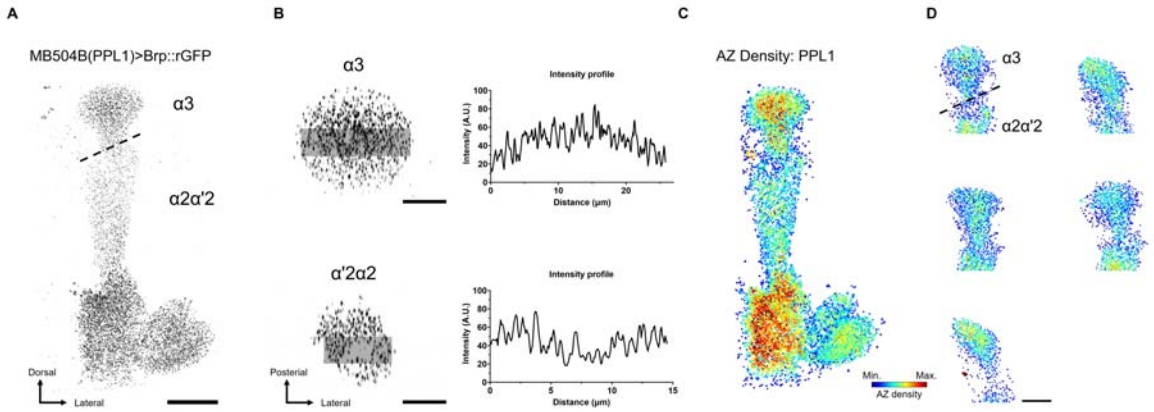

Fig. 5. Stereotyped AZ distribution of PPL1-α3 DAN. (**A**) Brp::rGFP in PPL1 DANs. UAS-GFP1-10 was expressed using MB504B-GAL4. Dashed line marks the rough boundary between α3 and α2α'2 compartments. Scale bar, 20 μm. (**B**) Brp::rGFP intensity profiles of α3 and α2α'2 compartments. Left panels show the max-projection images of α3 and α2α'2 optical coronal sections. Transparent gray stripes indicate areas where intensity profiles are plotted in the right panels. Scale bars, 10 μm. (**C**) 3D reconstruction colored by the AZ density in PPL-1 DANs. Color scale: Min. = 0, Max. = 40. Dashed line indicates the rough boundary between α3 and α2α'2 compartments. Scale bar, 20 μm. (**D**) Stereotyped AZ distribution pattern in PPL1-α3 across individuals. 3D reconstructions show the AZ density across different brain samples. Scale bar, 20 μm.

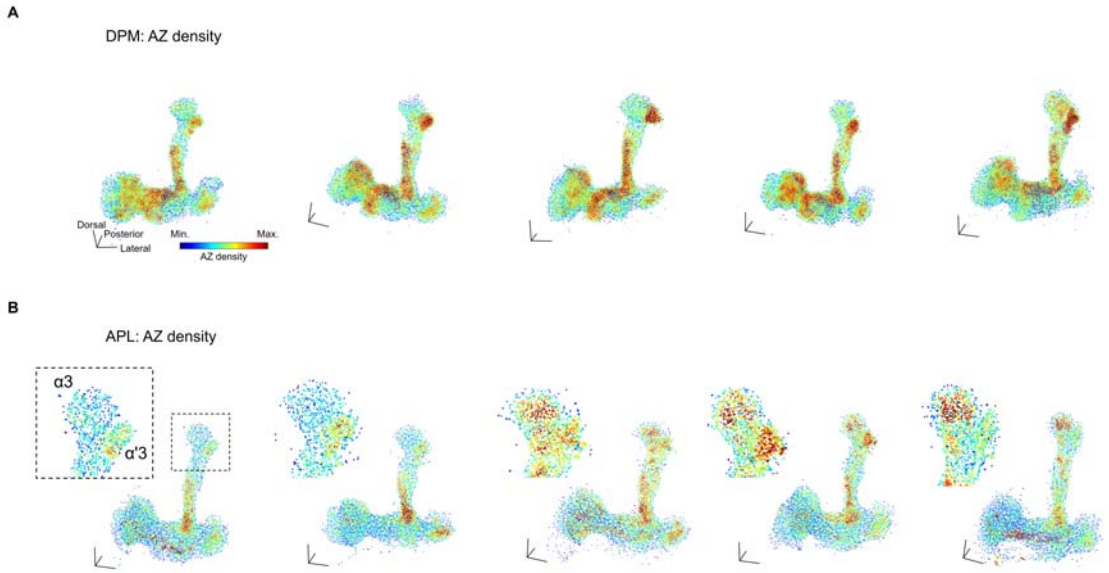

Fig. 6. Cell-type-specific stereotypy of AZ spatial distributions.
(**A**) 3D reconstructions of Brp::rGFP clusters in DPMs, colored by AZ density. Color scale: Min. = 0, Max. = 35. Black arrows indicate consistently high AZ density regions across brain samples. Scale bars, 20 μm. (**B**) 3D reconstructions of Brp::rGFP clusters in APLs, colored by AZ density. Color scale: Min. = 0, Max. = 30. Black dashed square indicates the area zoomed-in. APL reconstructions are arranged from left to right according to the overall AZ density in α3.

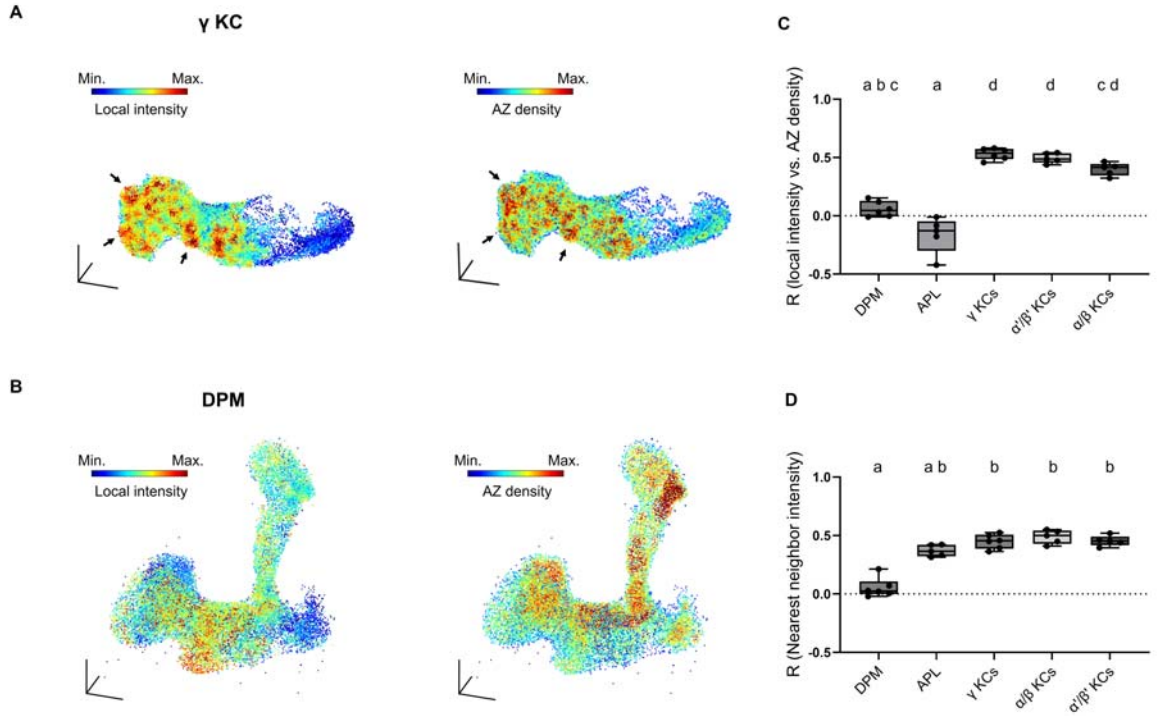

Fig. 7. Local intensity analysis revealed sub-compartmental AZ structures. (**A**) 3D reconstructions of Brp::rGFP clusters in a γ KC sample, colored by local intensity and AZ density. Black arrows indicate areas with both high local intensities and high AZ densities. Min. and Max. were set to represent the lowest and highest 5% in local intensity value respectively. Color scale of AZ density: Min. = 0 and Max = 35. (**B**) 3D reconstructions of Brp::rGFP clusters in a DPM neuron, colored by local intensity and AZ density. Min. and Max. are set to represent the lowest and highest 5% in local intensity value respectively. Color scale of AZ density: Min. = 0, Max. = 35. (**C**) Correlations between AZ density and local intensity in different cell types. DPM (n = 6), APL (n = 5), γ KCs (n = 6, MB009B-GAL4), α'/β' KCs (n = 5, MB370B-GAL4), α/β KCs (n = 5, MB008B-GAL4). DPM vs. γ KCs: P = 0.0171; DPM vs. α'/β' KCs: P = 0.0171; APL vs. γ KCs: P = 0.0014; APL vs. α'/β' KCs: P = 0.0014; APL vs. α/β KCs: P = 0.0171; (**D**) Correlations of Brp::rGFP intensities between nearest neighbor AZs in different cell types. Brp::rGFP intensities were log transformed. DPM vs. γ KCs: P = 0.0066; DPM vs. α'/β' KCs: P = 0.0027; DPM vs. α/β KCs: P = 0.0066; Scale bar: 1 μm.

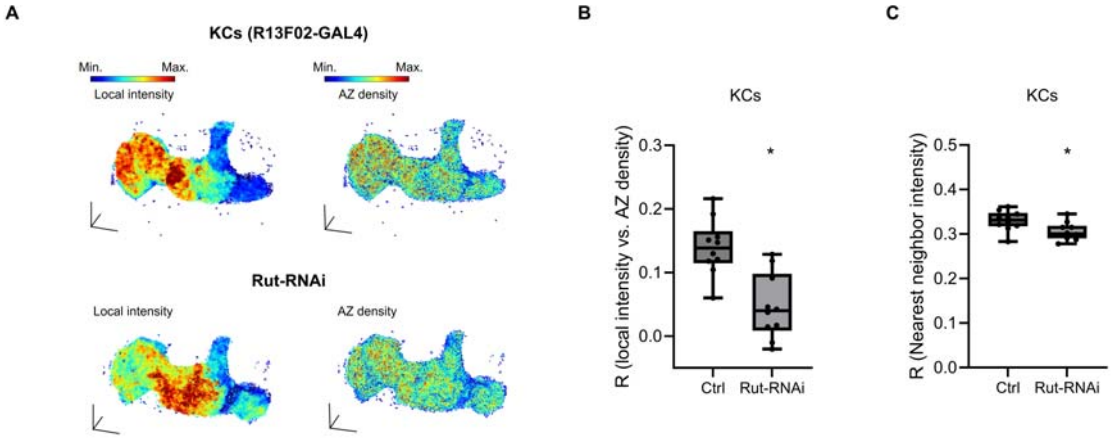

Fig. 8. Local AZ structures in KCs are Rut-dependent. (**A**) Decorrelation of AZ hot spots by knocking-down Rut. (**B**) Knocking-down Rut decreased the correlation between AZ density and local intensity in KCs. P = 0.0007. Brp::rGFP was visualized using R13F02-GAL4. Signals were sampled from all MB compartments. (**C**) Knocking-down Rut decreased the correlation of Brp::rGFP intensities between nearest neighbor AZs in KCs. P = 0.0355. Mann Whitnet test *P < 0.05. Data were presented as box plots showing centre (median), whiskers (Min. to Max.).