Investigating CNS synaptogenesis at single-synapse resolution by combining reverse genetics with correlative light and electron microscopy

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ABSTRACT

Determining direct synaptic connections of specific neurons in the central nervous system (CNS) is a major technical challenge in neuroscience. As a corollary, molecular pathways controlling developmental synaptogenesis in vivo remain difficult to address. Here, we present genetic tools for efficient and versatile labeling of organelles, cytoskeletal components and proteins at single-neuron and single-synapse resolution in Drosophila mechanosensory (ms) neurons. We extended the imaging analysis to the ultrastructural level by developing a protocol for correlative light and 3D electron microscopy (3D CLEM). We show that in ms neurons, synaptic puncta revealed by genetically encoded markers serve as a reliable indicator of individual active zones. Block-face scanning electron microscopy analysis of ms axons revealed T-bar-shaped dense bodies and other characteristic ultrastructural features of CNS synapses. For a mechanistic analysis, we directly combined the single-neuron labeling approach with cell-specific gene disruption techniques. In proof-of-principle experiments we found evidence for a highly similar requirement for the scaffolding molecule Liprin-α and its interactors Lar and DSyd-1 (RhoGAP100F) in synaptic vesicle recruitment. This suggests that these important synapse regulators might serve a shared role at presynaptic sites within the CNS. In principle, our CLEM approach is broadly applicable to the developmental and ultrastructural analysis of any cell type that can be targeted with genetically encoded markers.

KEY WORDS: Drosophila, Synapse formation, Sensory neuron, CNS, 3D CLEM

INTRODUCTION

Developmental synaptogenesis is a complex multifactorial process (Chia et al., 2013; Jin and Garner, 2008; Sheng and Kim, 2011; Yamagata et al., 2003), and many of the molecular mechanisms controlling synapse specification, formation, maintenance/elimination and plasticity in vivo are yet to be elucidated. This is especially true in the central nervous system (CNS), where synaptic partner selection within a highly complex local environment containing hundreds to thousands of cellular processes remains a key question in both normal development and under pathological conditions.

Invertebrate genetic model systems provide a good starting point for deciphering factors involved in synapse formation and specificity. Several genetic screens at synapses of hermaphrodite-specific motoneurons and AIY interneurons in C. elegans, at Drosophila neuromuscular junctions (NMJs) and in the Drosophila visual and olfactory systems have shed light on basic principles and molecules regulating these processes (Collins and DiAntonio, 2007; Goda and Davis, 2003; Oswald and Sigrist, 2009; Sanes and Yamagata, 2009; Shen and Scheiffele, 2010). However, it is clear that these findings cannot easily be generalized, and some principles might be used differently, for example at peripheral and at central synapses.

Technical limitations in imaging small structures in the CNS make the experimental dissection of central synaptogenesis particularly complicated. While super-resolution light microscopy is difficult to apply to the three dimensions of a tissue (Huang et al., 2010), serial reconstruction 3D electron microscopy (EM) is immensely time- and labor-intensive. Even with the automation of tissue sectioning in block-face scanning EM (BF-SEM), the workload remains high in the post-imaging analysis phase, particularly for the identification of the structures of interest in the neuropil (Helmstaedter, 2013).

Here, we describe genetic tools for efficient labeling and manipulation of single sensory neuron projections in the Drosophila CNS. We present correlative light and 3D EM in the Drosophila CNS, and apply this approach to determine the ultrastructure of central projections and synapses of mechanosensory (ms) neurons. To demonstrate the power and practicality of the genetic labeling approach, we combine it with the analysis of gene function by single-cell techniques, and show its applicability for performing genetic screens by RNAi or with loss-of-function mutants. This provides a means to address the molecular and cellular mechanisms of synaptic connectivity and development in the CNS using a powerful combination of specificity, resolution and large sample numbers.

RESULTS

Efficient and reproducible genetic labeling of single mechanosensory neurons

Adult ms neurons that innervate large bristles (macrochaetae) on the Drosophila thorax are part of the somatosensory system and sense airflow and touch. Four macrochaetae, named anterior/posterior dorsocentral (aDC and pDC, respectively) and anterior/posterior scutellar (aSC and pSC, respectively), are located on each side of the central dorsal thorax (Fig. 1A,B) and are innervated by single ms neurons. Axonal projections of ms neurons in the CNS are large,
with a total branch length up to 500-600 µm and individual branches up to 100-200 µm (supplementary material Fig. S1A). During pupal stages of development, the axons of ms neurons navigate from the periphery to the CNS, where they elaborate a stereotypic arborization pattern within a highly complex target area, the ventral nerve cord (VNC) (Fig. 1F,G).

In adult flies, lipophilic dye labeling provides a simple method for visualizing axonal projection patterns in the CNS (Chen et al., 2006; Grillenzoni et al., 1998). However, it has previously not been possible to selectively and efficiently label single ms neuron projections genetically. We utilized two genomic enhancer fragments – the *pannier* (*pnr*)-gal4 transcriptional transactivator (Heitzler et al., 1996) (Fig. 1B; supplementary material Fig. S1B) and an enhancer of the *achaete-scute* locus, DC1.4 (García-García et al., 1999) (Fig. 1C) – in combination with removal of transcriptional repressors (Gordon and Scott, 2009; Stockinger et al., 2005) to label any of the neurons...
Fig. 1. Genetic labeling of single ms neurons in the Drosophila adult CNS. (A) Schematic of a fly dorsal thorax. Central macrochaetae are depicted: anterior/posterior dorsocentral (aDC/pDC) and anterior/posterior scutellar (aScP/sSc). (B) Dorsal view of a fly expressing mCD8::mCherry driven by pnr-gal4, which is active only in the central domain, encompassing the two DC bristles and the two SC bristles on each side of the thorax (arrowheads and arrows, respectively; in A and B, one side is magnified in the inset in B). The pnr domain also contains dozens of microchaetae. (C) Third instar larval wing disc containing sensory organ precursors of thoracic bristles (Hartenstein and Posakony, 1989). DC1.4 enhancer activity (visualized by a direct fusion to GFP) is restricted to precursor cells of the dorsocentral domain. (D) Flip-out clone selection on the thorax. Labeling of the right pDC ms neuron is revealed by mCD8::GFP fluorescence in the neuronal cell body (arrow) underneath and slightly anterior to the bristle socket (arrowhead). (E,E′) Schematics of the two different flip-out variants used for ms neuron labeling (see main text, Materials and Methods and supplementary material Table S1 for details). (F) Schematic of a pDC neuron (green) targeting in the VNC (gray). The different thoracic neuromeres (pro, meso, meta) and the abdominal center (ac) are labeled. Asterisk indicates site of entry of the ms axon in the CNS (as in G-K). (G) pDC neuron visualized by dye filling (green), artificially overlaid onto an image showing a random subset of VNC neurons (red, visualized by driving UAS-mCD8::GFP with an enhancer trap inserted in the CG6678 gene). This illustrates the complexity of the ms neuron target area. Blue, neuropil counterstaining (nc82 antibody detecting Brp). (H,I) A single aDC (H) and pDC (I) neuron labeled using Flp to remove the STOP cassette in the reporter counterstaining (nc82 antibody detecting Brp). (J) MARCM clones generated with DC1.4-Flp (genotype given in supplementary material Table S1). Dotted line indicates CNS midline (as in J,K). Arrowheads (I) indicate an unrelated neuron that is often also labeled in conjunction with pDCs, but does not interfere with the analysis of the pDC neuron projection, especially not in the 3D confocal image stack. (J,K) MARCM clones generated with DC1.4-Flip (genotype given in supplementary material Table S1). In addition to the pDC, the aDC neuron on the same side of the animal is also labeled in K (projections overlap closely, except where indicated by arrowheads). (L-M) Labeling of single DC axons targeting in the VNC during pupal development, using the STOP cassette flip-out setup (see E′ and supplementary material Table S1 and also Fig. 2 and main text for mCherry::Syt1 expression) at ∼31-35 h after pupariation formation (apf). Thoracic neuromeres are delineated by dashed lines and labeled as in F. Boxes indicate regions magnified in L′-L′′.M′. The growth cone of the posterior branch grows past the posterior border of the mesothoracic neuromere with filopodia aligned in a posterior direction (arrowhead in L′). (M) Sample in which the growth cone has reached the anterior border of the metathoracic neuromere, has finished posterior growth, and extends a filopodium towards the midline (arrowhead in M′) to form the posterior commissural connection. Asterisks, unrelated neurons. Anterior is top and posterior bottom in all panels except C, where anterior is to the left. Scale bars: 100 µm in C,D; 20 µm in G-L,M; 5 µm in L′-L′′.M′.

innervating dorsocentral micro- and macrochaetae with high specificity (Fig. 1E,E′; see Materials and Methods). Fortuitously, the labeling occurs stochastically and only in subsets of cells in each animal. Small clones of few or single labeled neurons (Fig. 1H,I) can be obtained efficiently (up to 50%), and clone size can be modulated (see Materials and Methods).

The DC1.4 tool can also be used for generating MARCM (mosaic analysis with a repressible cell marker) clones (Lee and Luo, 1999). Single clones were recovered with a very high efficiency of ∼40% of animals of the right genotype (Fig. 1L,K; supplementary material Table S1); most of the remaining 60% had no clone or clones encompassing only microchaetae. In order to test DC1.4 specificity, we introduced either a ubiquitous or a pan-neuronal transcriptional transactivator (tub-gal4 or nSyb-gal4). Despite the broad driver expression, the labeled clones in the VNC were still restricted to dorsocentral ms neurons (supplementary material Fig. S1C,D). Importantly, this exquisite specificity provides an easy means for the analysis of cell-intrinsic requirements of specific genes of interest selectively in ms neurons. Furthermore, these genetic recombination tools render the synaptic connectivity of ms neurons amenable to a comprehensive developmental analysis, with the option of subcellular, protein-specific or activity markers (Fig. 1L,M).

Taken together, we generated genetic tools that allow for efficient labeling of single, identified ms neurons targeting in the CNS. The high frequency of recovering small clones offers the opportunity for high-throughput/genome-wide screening approaches.

Presynaptic markers accumulate at stereotypic locations along ms neuron axon branches and sparse labeling suggests single active zone resolution

We assessed the localization of putative central presynapses using presynaptic markers. To facilitate the genetic labeling, we created a bicistronic 20xUAS-based transgenic marker, which contains an FRT-flanked transcriptional STOP cassette, the axonal marker mCD8::GFP (Lee and Luo, 1999) and synaptic vesicle-associated mCherry-tagged Synaptotagmin 1 (Syt1) (Fernández-Chacón and Südhof, 1999) (supplementary material Fig. S2 and supplementary materials and methods). This allows for labeling of membranes and synaptic vesicles of any neurons of interest in the Drosophila nervous system. We first compared localizations in ms neurons of the mCherry::Syt1 fusion protein, of Bpshort::GFP (5xUAS based) (Fouquet et al., 2009), a marker that closely colocalizes with the endogenous active zone protein Bruchpilot (Brp) (Schmid et al., 2008; Wagh et al., 2006), and of Cacophonous::GFP (5xUAS based) (Kawasaki et al., 2004), a subunit of presynaptic calcium channels.

The location of the markers suggests that ms axons form mostly terminal but also en passant synapses along their central projections (Fig. 2A-E; supplementary material Figs S2 and S3). Unexpectedly, all analyzed presynaptic markers were strongly enriched in the midline region on the main anterior commissure, with dozens of puncta located on many small terminal branches emanating from the main axon shaft. Furthermore, puncta were distributed along the whole length of the anterior projecting branch and on contralateral branches. Finally, along the posterior projecting branch of pDCs, synaptic markers were prominent only in four stereotyped locations separated by long axonal segments devoid of signal. The posterior branch of aDCs has marker accumulation only at its distal tip (Fig. 2C). Importantly, all tested presynaptic markers showed a highly similar localization pattern, supporting the notion that these sites coincide with bona fide synapses and are not sites of marker accumulation that ecotopically form due to potential overexpression. To further corroborate this, we co-expressed Bpshort::mStraw (Fouquet et al., 2009) together with Syt1::GFP (Zhang et al., 2002) in single ms neurons. Syt1 signal was always found in close proximity to Bpshort puncta (Fig. 2F-I; note that not all Bpshort puncta are visible, see figure legend), consistent with synaptic vesicles being clustered around, or in the vicinity of, but not always directly at the active zone. Furthermore, high-magnification imaging of Bpshort::GFP allowed visualization of discrete puncta in the same size range as single active zones at NMJs (Kittel et al., 2006) (Fig. 2J-O). Our results suggest that single active zones or clusters of a few active zones can be identified in ms neurons.

Collectively, the distribution of markers reveals high spatial specificity of different synaptic contact sites in distinct target regions of the VNC. Highly synaptogenic compartments are concentrated at the midline region, whereas regions along the posterior ipsilateral axon segment appear to be poorly synaptogenic.

Restricted pattern of F-actin marker accumulation in ms axons

It has been shown that filamentous actin (F-actin) accumulates at presynaptic sites of the HSN motoneurons in C. elegans, and F-actin
assembly downstream of a cell-adhesion receptor is an early step in presynapse formation in these neurons (Chia et al., 2014, 2012). In order to address links between the cytoskeleton and CNS synapse development in flies, we expressed the F-actin markers LifeAct (Hatan et al., 2011) and Utrophin actin-binding domain::GFP (Rauzi et al., 2010), respectively, as well as a more general Actin marker (Verkhusha et al., 1999). All three markers showed a discrete punctate distribution pattern that correlates closely with the location of presynaptic markers in ms neurons (Fig. 3A-H, compare with Fig. 2B-D). Furthermore, colabeling of Brp\textsuperscript{short} and LifeAct revealed a high degree of colocalization (supplementary material Fig. S4). By contrast, GFP-tagged α-Tubulin (Grieder et al., 2000)
accumulates uniformly in the main shaft of the axons, with very little signal in higher order branches (Fig. 3I).

Moreover, expression of a mito-GFP marker (Rizzuto et al., 1995) in ms axons revealed a distribution of mitochondria that is strikingly different to that of the presynaptic and F-actin markers. Mito-GFP was distributed along the entire central projections, with remarkably even spacing between individual loci with mito-GFP signal (Fig. 3J-L). This is in good agreement with the requirement for mitochondria to provide energy throughout the large volume of the axon and to presynaptic active zones (Vos et al., 2010), and is also consistent with proposed roles for mitochondria in axon branch formation (Courchet et al., 2013).
Overall, we show that our genetic labeling of ms neurons allows visualization of various subcellular components at single axon branch resolution in the CNS.

3D ultrastructural analysis of ms axon branches and synapses

EM remains a gold standard for identifying and characterizing synaptic structures. In order to use EM for the characterization of defined ms neuron axons and synapses, we sought a strategy to correlate genetically encoded marker-based labeling and 3D EM datasets (Fig. 4). We relied on the near-infrared laser branding (NIRB) technique recently developed in mice (Bishop et al., 2011), which consists of generating fiducial branding marks with a near-infrared laser around a structure of interest while imaging the tissue in a confocal light microscope. These laser-induced tissue marks are readily visible in EM and of considerable help in identifying the region of interest (ROI) in the ultrastructural dataset. We expressed the mCD8::mCherry and Brpshort::GFP markers in individual DC neurons of interest (ROI) in the ultrastructural dataset. We expressed the laser around a structure of interest while imaging the tissue in a technique recently developed in mice (Bishop et al., 2011), which datasets (Fig. 4). We relied on the near-infrared laser branding (NIRB) synaptic structures. In order to use EM for the characterization of EM remains a gold standard for identifying and characterizing synapses branch resolution in the CNS.

3D CLEM revealed the presence of both monadic and polyadic synapses in ms neurons (Fig. 5B-M). Monadic synapses are juxtaposed to a large post-synaptic process (Fig. 5D-H). Small, likely dendritic post-synaptic processes are found contacting polyadic synapses (Fig. 5I-M). In both cases, prominent T-bar-shaped electron-dense bodies and adjacent synaptic vesicles were found at the ms neuron presynapse (Fig. 5E-HJ-M). Many more synaptic vesicles seemed to be present at polyadic presynapses, suggesting a higher physiological activity (Fig. 5E-HJ-M). We often observed an unidentified organelle at ms neuron presynapses, possibly an endosomal compartment. Similar to Drosophila motoneuron synapses (NMJ) and photoreceptor synapses (Prokop and Meinertzhagen, 2006), post-synaptic densities (PSDs) were difficult to discern. A 250-300 nm wide, weakly stained PSD could however be observed at monadic ms neuron synapses, with a few thin filament-like dense projections extending into the cytoplasm (Fig. 5F).

Particularly for the small dimensions of the Drosophila CNS tissue and central neurites, transmission electron microscopy (TEM) provides images with less noise and higher resolution of subcellular structures than BF-SEM. In our approach, however, selective sections in the ROI can be subjected to TEM, should additional ultrastructural information be required beyond that provided by BF-SEM (supplementary material Fig. S5).

Using 3D CLEM, we were able to verify that Brpshort::GFP expression is a good indicator for presynaptic active zones in ms neurons. In the EM dataset of the axon segments around its first branch point, we could observe presynaptic densities (T-bars) and/ or synaptic vesicles at all sites where we had observed a punctum of Brpshort::GFP fluorescence (Fig. 5B,C, compare arrowheads). Conversely, neither T-bars nor an accumulation of synaptic vesicles was observed in any other location in these ms neuron axon segments. These results support the notion that, in ms neurons, Brpshort::GFP labels most if not all chemical synapses, that it does not accumulate ectopically outside presynapses, and that it thus can be used as a bona fide active zone marker. To further corroborate this, we sought to compare UAS-based Brpshort::GFP expression with endogenous Brg expression. We used the bacterial artificial chromosome (BAC)-based expression of epitope-tagged Brg (Chen et al., 2014), which has recently been shown to reveal the quantitative as well as temporal profile of endogenous Brg expression. We found that both markers were qualitatively and quantitatively indistinguishable (supplementary material Fig. S6; Fig. 6), indicating that UAS-based Brpshort::GFP expression does not affect synapse number and location in ms neurons, and indeed reflects the normal distribution of active zones.

We also compared marker-based light microscopy and EM data for the analysis of mitochondria localization in ms axons. This analysis revealed highly similar morphologies and densities of mitochondria at the first axon branch point in the light microscopy and EM datasets (Fig. 5N-R). We found no evidence that overexpression of the mito-GFP marker would significantly alter the shape, number or distribution of mitochondria in ms neurons.

Ms neuron-specific genetic manipulation to dissect cell-intrinsic mechanisms of central targeting and synapse formation

Loss-of-function (LOF) analysis in invertebrate model organisms provides an ideal means for dissecting molecular mechanisms of neuronal wiring. We tested the effectiveness of RNAi in ms neurons by targeting genes expected to play roles in axon branching or synaptogenesis. First, we expressed a hairpin construct targeting the Dscam (Dscam1) gene (Schmucker et al., 2000), which has essential functions in ms neuron axon branch separation and targeting in the CNS (Chen et al., 2006). Dscam knockdown caused severe axon branch targeting phenotypes similar or identical to those reported for Dscam null mutations (supplementary material Fig. S7, Fig. S8A,B, Table S2).

Next, we knocked down Liprin-α in ms neurons by RNAi and examined central branch targeting and synaptogenesis. Liprin-α acts as an important synapse organizer in both vertebrates and invertebrates (Spangler and Hoogenraad, 2007). In Drosophila, Liprin-α is also required for post-synaptic target selection in photoreceptors, but not at the NMJ (Choe et al., 2006; Hofmeyer et al., 2006; Kaufmann et al., 2002). Loss of Liprin-α did not alter ms axon branching or branch targeting, but led to a pronounced decrease of Syt1 marker along axon branches and an aberrant accumulation of signal at distal tips of axon branches. These defects were especially prominent on the main anterior commissure
Fig. 4. A highly efficient 3D CLEM approach for the Drosophila CNS. (A) Schematic illustrating the location of the first main branch point of ms neurons. ROI, region of interest. (B) Maximum intensity projection of a confocal image stack in the ROI. mCD8::mCherry was expressed with the gal80 flp-out setup (see Fig. 1E) and its intrinsic fluorescence imaged. A DC macrochaeta axon (arrowhead) and a few microchaetae axons (asterisks) are labeled. This tissue was fixed strongly with both formaldehyde and glutaraldehyde for subsequent EM, which reduces specific fluorescence and leads to more artifacts (e.g. large blobs of nonspecific signal). (C) Fiducial branding marks applied to the ROI by NIRB and visualized by their autofluorescence. Arrows indicate horizontal and vertical marks delimiting the ROI, as burned into the tissue a few micrometers more ventral than the position of the ms neuron branch point (displacement in the ventral-dorsal direction is not visible in maximum projection). Two additional horizontal marks were applied adjacent to the DC ms axon branch point, ‘clasping’ it at the same ventral-dorsal depth (outlined in green). DC ms axon branches are outlined in white; strong outlines correspond to the parts of the neuron and marks reconstructed in I, fainter outlines are the continuation of these structures. (D) The strategy for approaching and quickly finding the ROI in the resin-embedded VNC mounted for BF-SEM. A guiding line is branded from the anterior end of the tissue to close to the ROI (long yellow line in magnified inset). This mark can be followed when cutting and imaging the neuropil from anterior to posterior in transverse direction (illustrated by gray plane and arrows). Additional smaller marks are placed to provide information about progress in the anterior-posterior direction (smaller vertical lines). In total, three lines (1-3) are present close to the ROI (asterisk). (E) Branding marks visualized by autofluorescence in the red part of the spectrum, where mCherry fluorescence in the axons is also detected. Branding marks delimiting the ROI show weak fluorescence, whereas guiding lines in the anterior part of the VNC are prominent. Regions shown and labeling correspond to D. Shadows seen in the magnified image come from trachea at the surface of the tissue. (F) EM image of a transverse section during approach of the ROI, at the position corresponding to dotted lines in magnified D and E. Branding marks are promptly identified in the tissue (arrowheads, numbers as in D). (G) Single confocal section in the plane of the ms axon branch point, clasped by two horizontal branding marks (arrows). (H) EM image of a transverse section in which the ms axon (pseudocolored red) is clasped by the two horizontal branding marks (yellow). (H’) A magnification of the boxed area in H, showing that the ultrastructure of cellular components, such as axonal microtubules (arrowheads) and synaptic vesicles (asterisk), is well preserved even in close proximity to the branding mark. (I) 3D reconstruction of the ms axon (red) and the branding marks (yellow) segmented from the stack of EM images in the ROI. Identity of the neuron is confirmed by correlating its morphology and relative position to the marks with the light microscopy data (C). Body axes are indicated in B,E-I; a, anterior; d, dorsal; p, proximal. Scale bars: 10 µm in B; 50 µm in E; 2 µm in F; 5 µm in G,H; 1 µm in H’.
(Fig. 7A–C), where the otherwise strong Syt1 marker signal was lost from most of the numerous terminal branches (Fig. 7B,C; supplementary material Fig. S9). To assess potential off-target effects, we determined the LOF phenotypes of Liprin-α in ms neurons using MARCM analysis (supplementary materials and methods). In Liprin-α LOF, the Syt1 marker also revealed strong defects in synaptogenesis, particularly in the main commissure (Fig. 7D; supplementary material Fig. S9), at a similar penetrance as when Liprin-α was knocked down by RNAi (Fig. 7E). These results demonstrate that Liprin-α is required cell-autonomously in ms neurons and that most of the Syt1 marker is lost from presynapses when Liprin-α levels are reduced.

Liprin-α interacts with Leukocyte-antigen-related-like (Lar) and DSyd-1 (RhoGAP100F), and overlapping but non-identical phenotypes have been described upon disruption of these genes in Drosophila (Choe et al., 2006; Hofmeyer et al., 2006; Holbrook et al., 2012; Kaufmann et al., 2002). Consistent with a potential shared role of these three factors in synapse assembly, we found...
DISCUSSION

A new experimental system for studying synapse formation of identified neurons in vivo

We present efficient genetic labeling of single, identified sensory neurons with a complex and stereotypic targeting pattern in the Drosophila CNS. The ability to genetically label and analyze the very same neuron among different animals presents a unique advantage for addressing spatial specificity of synapse formation, stereotypy versus variability/plasticity of the process, and to compare synaptogenesis in wild-type and mutant animals. The light microscopy analysis of Brp\textsuperscript{short}::GFP localization at central ms neuron synapses reveals distinct labeling of individual puncta in the size range of single active zones. Comparison with Brp::V5 expressed from a BAC and with ultrastructural data indicates that heterologous expression of the Brp\textsuperscript{short}::GFP UAS construct does not lead to ectopic accumulation of the marker along the axon. Simple transactivating tools in ms neurons furthermore result in moderate and reliable expression levels of other synaptic, cytoskeletal and mitochondrial components, suggesting that many markers can be expressed from the large collection of available UAS constructs.

Our genetic labeling approach can be directly combined with knockdown and LOF experiments. It is important to note that our tools allow recovery of small or single-cell clones with an efficiency that is an order of magnitude higher than in current labeling techniques such as MARCM with random and broad Flp expression. The genetic tool-kit that we describe is suited for high-resolution developmental analysis of ms neuron synaptogenesis within the CNS (Fig. 1L,M). This might provide important insights into the sequence of events during central synapse assembly in wild type and mutants.

Our analysis of Lar, Liprin-\(\alpha\) and DSyd-1 functions exemplifies that synaptogenesis and primary branch formation can be investigated independently in ms neurons. Upon LOF of either gene, we observed no disruption in the central branch pattern of ms axons, but found a striking disruption of tagged Syt1 localization. High amounts of Syt1 marker accumulating at branch tips suggest that its loss from en passant synapses is due neither to reduced expression nor defective transport of the marker in mutants. Interestingly, we did not observe abnormal Brp\textsuperscript{short}::GFP localization in ms neurons upon LOF of either Lar, Liprin-\(\alpha\) or DSyd-1. Although additional experiments will be necessary to support this finding, these results raise the possibility that Lar, Liprin-\(\alpha\) and DSyd-1 are required for synaptic vesicle recruitment/tethering but not for active zone formation. This would suggest a remarkable analogy to the situation in the mammalian CNS, where loss of mammalian SYD1A (SYDE1) reduces synaptic vesicle docking at the active zone but not general synapse assembly (Wentzel et al., 2013).

CLEM at the level of single active zones in the CNS

We describe an approach in which a light microscopy image stack and a correlated 3D EM dataset with a segmented neuron of interest and its 3D reconstruction can be obtained within less than 3 weeks. As such, sample preparation and data analysis time are reduced sufficiently to make it feasible to apply 3D CLEM analysis to different developmental stages or multiple mutant strains, and to correlate different subcellular markers with ultrastructural morphologies. It is important to note that, in principle, our protocol can be applied to any region of the CNS and any type of neuron of interest.

Using CLEM, we first find that ms neurons, like many Drosophila neurons, form a classical T-bar-shaped structure at the presynaptic active zone. Second, in the region analyzed, i.e. around
the first branch point of the ms neuron axon, the majority of synapses are polyadic, with one presynapse apposed to several postsynaptic elements. Third, using conventional staining methods the PSDs at ms neuron synapses are not prominent, but rather subtle. These three characteristics also apply to photoreceptor terminals in the fly visual system, and it is worth noting that in *Drosophila* both ms neurons and photoreceptors are histaminergic (Buchner et al., 1993; Pollack and Hofbauer, 1991). Invaginations of glial processes called capitate projections, which are a hallmark of *Drosophila* photoreceptor presynapses, are not however visible in the ms neuron synapses analyzed, suggesting that they are not a general feature of *Drosophila* histaminergic synapses.

Overall, our genetic tools and CLEM protocol will allow the investigation of mechanisms of ms neuron synapse formation, tackling a wide range of biological questions pertaining to the development and plasticity of central synapses.

**MATERIALS AND METHODS**

**Drosophila strains**

Genotypes of flies used in this study are listed in supplementary material Table S1 and fly stocks are further described in the supplementary materials and methods.

**Genetic labeling and MARCM of DC ms neurons**

Details of constructs are provided in the supplementary materials and methods. The DC1.4 enhancer is active in precursors of DC neurons only (Fig. 1C)(García-García et al., 1999). Functional DC1.4-FLp insertions were recovered on the second and third chromosomes. In flip-out setups, *pnr-gal4* activity was restricted by using DC1.4-FLp to excise either an FRT-flanked *gal80* cassette (Gordon and Scott, 2009) or an FRT-flanked transcriptional STOP cassette from a direct reporter construct (supplementary material Fig. S2). Clone size depends on the cassette to be excised and on the temperature at which the flies are raised; smaller clones are obtained at 29°C than at 25°C, probably reflecting the shorter time for the FLp-mediated
cassette excision to occur when the animal develops faster. Use of the FRT-flanked gala80 repressor (Gordon and Scott, 2009) results in almost all animals having at least one neuron innervating a macrochaeta labeled at 25°C, with most animals having two to three macrochaetae neurons and several microchaetae neurons labeled (see Figs 2 and 3). At 29°C, the same repressor cassette allows recovery of single macrochaetae clones in ~10-20% of the animals, while most of the remainder have only a microchaeta neuron labeled or no neuron at all. With the transcriptional STOP cassette in the UAS construct (Fig. 1E; supplementary material Fig. S2), 10-20% of animals raised at 25°C have a clone, which usually consists of a single macrochaeta (see Fig. 1H,I). FRTG13 (Fig. 1J,K) or FR740A (Fig. 7D; supplementary material Fig. S8G,H) was used to generate MARCM clones with DC1.4-Flp. In either case, ~40% of animals with small clones were recovered at 25°C.

**In vivo RNAi**

RNAi-induced knockdown of gene expression in ms neurons was assessed using two different flip-out variants, as illustrated in Fig. 1E,F. For details, see the supplementary materials and methods.

**Immunohistochemistry and imaging**

Immunostaining of VNcs, carboxyamine dye labeling, imaging of native GFP and mCherry fluorescence and processing of fluorescence microscopy images, including segmentation and 3D reconstruction, are described in the supplementary materials and methods.

**Correlative light and electron microscopy**

VNcs of flies of genotype w tub>gal80>; UAS-Brp<short>:GFP DC1.4-Flp/+; pnr-gal4 UAS-mCD8:mCherry/TM6 were dissected in cold cacodylate buffer (0.15 M Na cacodylate buffer, pH 7.2), immediately transferred to freshly prepared fixative comprising 2% paraformaldehyde and 0.5% glutaraldehyde [both EM grade from Electron Microscopy Sciences (EMS)] in cacodylate buffer, and fixed overnight at 4°C (although weaker than with milder fixation protocols, the intrinsic fluorescence of fluorescent proteins could still be imaged under these conditions, but was almost non-existent at glutaraldehyde concentrations ≥1%). Samples were washed three times for 5 min each in cacodylate buffer and then mounted ventral side up on a slide just before the branding procedure, in the same buffer. NIRB was performed according to Bishop et al. (2011) on a Zeiss LSM710 upright confocal microscope, using the bleaching function of the ZEN 2010 software (Zeiss). Just before the branding procedure, in the same buffer. NIRB was performed according to Bishop et al. (2011) on a Zeiss LSM710 upright confocal microscope, using the bleaching function of the ZEN 2010 software (Zeiss).

**Author contributions**


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**Supplementary material**

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**References**


