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Review
Highlighted Article

Genetic regulation of central synapse formation and organization in *Drosophila melanogaster*

Juan Carlos Duhart, Timothy J. Mosca *

Department of Neuroscience, Vickie and Jack Farber Institute of Neuroscience, Thomas Jefferson University, Philadelphia, PA 19107, USA

*Corresponding author: Department of Neuroscience, Vickie and Jack Farber Institute of Neuroscience, Thomas Jefferson University, Bluemle Life Sciences Building, Philadelphia, PA 19107, USA. Email: timothy.mosca@jefferson.edu

Abstract

A goal of modern neuroscience involves understanding how connections in the brain form and function. Such a knowledge is essential to inform how defects in the exquisite complexity of nervous system growth influence neurological disease. Studies of the nervous system in the fruit fly *Drosophila melanogaster* enabled the discovery of a wealth of molecular and genetic mechanisms underlying development of synapses—the specialized cell-to-cell connections that comprise the essential substrate for information flow and processing in the nervous system. For years, the major driver of knowledge was the neuromuscular junction due to its ease of examination. Analogous studies in the central nervous system lagged due to a lack of genetic accessibility of specific neuron classes, synaptic labels compatible with cell-type-specific access, and high resolution, quantitative imaging strategies. However, understanding how central synapses form remains a prerequisite to understanding brain development. In the last decade, a host of new tools and techniques extended genetic studies of synapse organization into central circuits to enhance our understanding of synapse formation, organization, and maturation. In this review, we consider the current state-of-the-field. We first discuss the tools, technologies, and strategies developed to visualize and quantify synapses *in vivo* in genetically identifiable neurons of the *Drosophila* central nervous system. Second, we explore how these tools enabled a clearer understanding of synaptic development and organization in the fly brain and the underlying molecular mechanisms of synapse formation. These studies establish the fly as a powerful *in vivo* genetic model that offers novel insights into neural development.

Keywords: *Drosophila*; synapse; development; olfaction; visual system; mechanosensory; genetic tools; active zone; synaptogenesis; central nervous system

Introduction

Synaptic connections represent the fundamental functional unit of the nervous system. Every event that transpires in the brain requires transmission of information across a synapse at some point. Chemical synapses are asymmetric cell–cell junctions specialized for neurotransmission that utilize the trafficking of chemical messengers across a cleft to drive information flow and neural processing in the nervous system. Broadly, synapses are comprised of presynaptic sites from which neurotransmitter (NT) is released and postsynaptic sites that are specialized with receptors for specific NTs. Every computation that underlies behavior, cognition, or emotion requires robust and reliable synaptic transmission (Mayford *et al.* 2012; Ploski and McIntyre 2015; Chou *et al.* 2020). Due to the critical importance of synapses in nervous system function, attaining a deeper understanding of how and when synapses assemble, how they are organized in 3D space, and the molecular mechanisms that regulate their function is essential. Understanding synapse function and development is also a critical translational question as many neurodevelopmental, neuropsychiatric, and even neurodegenerative diseases specifically impair synaptic function and organization (Marcello *et al.* 2018; Taoufik *et al.* 2018). A firmer grasp of how synaptic dysfunction and errors in development contribute to neurological disorders is thus key to understanding how neural circuits operate and how to treat neurological disease.

Drosophila has long stood as a powerful model system for understanding the genetic basis of cellular development, including formation of the nervous system. The short life-cycle, tractable genetics, plethora of available tools for mutant analysis, single-cell resolution for labeling and genetic perturbation, and specific access to many distinct classes of cells via binary expression systems like GAL4/*UAS*, QF/*QUAS*, and lexA/*LexAop* (Venken, Simpson, *et al.* 2011) have allowed a steady reduction of the frontiers of knowledge with regards to how synapses form and function. In the last 20 years alone, a suite of immunohistochemical and genetic tools has been developed to visualize *Drosophila* synapses by light microscopy. These tools allow genetic analysis of synaptogenesis in intact tissues or whole organ preparations. Despite this rich history of understanding the molecular and cellular mechanisms of synapse formation and the underlying logic of circuit organization, a thorough understanding of synaptic

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development, particularly in the central nervous system (CNS), remains incomplete. Work in *Drosophila* has predominantly focused on the neuromuscular junction (NMJ) for its accessibility, high cellular resolution, and ready supply of genetic tools. The NMJ has thus served as the primary model to investigate the mechanisms that govern synaptic architecture and organization (Keshishian *et al.* 1996; Kraut *et al.* 2001; Packard *et al.* 2002; Keshishian and Kim 2004; Liebl *et al.* 2006; Kurusu *et al.* 2008; Bayat *et al.* 2011; Koles and Budnik 2012; Menon *et al.* 2013). At the NMJ, a presynaptic motoneuron interacts with (in many cases) a single postsynaptic target—the muscle. This allows for studies with high spatial resolution to understand cell biological mechanisms that regulate synapse formation. Studies in the CNS, however, have historically been more challenging. In the brain, neural circuits form between multiple classes of neurons in a densely packed brain consisting of over 100,000 neurons. The density, small size, and vastly increased complexity of the CNS over the NMJ have made analysis of central synaptic features challenging—particularly when focusing on select neurons or circuits. The density and the lack of cellular resolution, in contrast to the NMJ, have stymied progress in understanding critical questions regarding CNS synaptic development. For example, are the mechanisms that control NMJ development shared with the CNS? Do peripheral and central synapses have different modes of synapse formation? When the increased levels of complexity of the brain are introduced to the problem of synaptic development, how does a genetic system organize such development spatially, temporally, and molecularly? Furthermore, central synapses have far more diversity in terms of class, function, and NT identity than neuromuscular synapses, increasing the complexity of the system and requiring additional levels of cell-type-specific synaptic organization (Meinertzhagen and O'Neil 1991; Chen *et al.* 2014; Mosca and Luo 2014; Urwyler *et al.* 2015; Schlegel *et al.* 2021). In the last decade, however, the final frontier of CNS synaptic biology is slowly becoming more accessible due to several advances in genetic manipulation and imaging techniques that allow the specific and reliable manipulation of genetically identifiable neuronal populations and their subsequent quantification. Technical advancements led to multiple studies that established distinct brain regions as powerful models for studying synaptogenesis. Fundamental parameters of synaptic organization are now known for distinct neuronal classes of the antennal lobe (AL; Mosca and Luo 2014), mushroom body (MB; Christiansen *et al.* 2011; Elkahlah *et al.* 2020), optic lobe (Chen *et al.* 2014), and in mechanosensory neurons (MSNs) that innervate large dorsocentral sensory bristles (Urwyler *et al.* 2015). In each of these model systems, visualization of active zone (AZ) markers using light microscopy and genetic tools allows both quantitative and qualitative analysis of synaptic organization and distribution in 3D space. In each case, light-level analyses through confocal, expansion, and super-resolution microscopy are in agreement with high-resolution studies of synaptic organization carried out by electron microscopy (Chen *et al.* 2014; Mosca and Luo 2014; Urwyler *et al.* 2015). Though unlike EM, light microscopy allows considerable genetic analysis and assessment due to its high-throughput nature. This is especially important as EM studies necessarily provide immense datasets for small numbers of animals, sometimes only 1–2 sides of a brain for 1–2 animals (Zheng *et al.* 2018; Scheffer *et al.* 2020); light microscopy studies allow for more facile analysis of larger numbers of samples to study genetic perturbations, different environmental conditions, and even developmental variation among populations (Honegger *et al.* 2020; Kiral *et al.* 2021). Thus, the 2 approaches serve as an essential complement to one another.

The marked explosion in both technology and molecular understanding has finally granted access to distinct CNS circuits to understand first, the basic principles of synaptic organization at the cell-type-specific level, and second, the molecular mechanisms that govern synapse formation, development, and structure. In the first part of this review, we will present the current state-of-the-art of genetic tools and labels commonly used to visualize synapses in the fly CNS. Subsequently, in part II, we discuss how these synaptic labeling strategies have been leveraged to assess synaptogenesis at multiple developmental stages and investigate the genetic basis of synaptic development, organization, and plasticity.

Part I: The tools of the trade: genetically encoded synaptic labeling allows the study of central synapses by light microscopy

The synapse is a multifunctional subcellular compartment specialized for cell–cell neurotransmission, adhesion, and contact coordination. Thousands of proteins work together to support synaptic assembly and function (Burré and Volknandt 2007; Wilhelm *et al.* 2014; Pazos Obregón *et al.* 2015; Kittel and Heckmann 2016; Cizeron *et al.* 2020). Historically, visualization of many of these proteins by light microscopy is readily achieved by immunostaining endogenous or epitope-tagged proteins (Table 1) in fixed tissues or through live imaging of fusion proteins bearing a fluorescent tag. In each case, imaging of synaptic proteins or markers provides important details of synaptic parameters; for example: which cells express specific NTs or NT receptors; how many synapses are assembled by a specific neuron and where they are located in 3D space; whether a particular AZ or synaptic contact is populated by many or few synaptic vesicles; or, when visualized in live tissues, when in development synaptic labels accumulate at cell-to-cell contacts. Thus, the ensemble of tools and strategies available to visualize synaptic proteins enables studies that seek to understand synaptic development, organization, and how these synaptic features impinge on circuit function and computation.

Until recently, the complex cellular environment in the CNS presented several technical challenges that precluded mechanistic studies of central synaptic development and organization. In *Drosophila*, immunostaining of endogenous proteins of interest is relatively straightforward in less complex synaptic systems, like the peripheral NMJ. There, the low density of synaptic connections (typically 1–4 easily discernible motoneurons onto a single muscle fiber) and relatively large size of synaptic boutons provide high spatial resolution that allows for facile genetic analysis. A rich history of study enabled by antibodies to many endogenous pre- and postsynaptic markers revealed key features of synaptic architecture, subcellular organization, and mechanisms underlying the cell biology of synaptic development (Nose 2012; Harris and Littleton 2015). In the fly brain however, over 100,000 neurons (Raji and Potter 2021), containing nearly 100 million synapses are highly intermingled (Scheffer *et al.* 2020), creating a more complex density problem than the NMJ. Instead of 40 presynaptic motoneurons making stereotyped synaptic connections with 31 postsynaptic muscle targets, circuits can have far more connections and intricate wiring compositions. Moreover, the spatial resolution of central synapses is made more difficult by the increase in average synaptic density due to the size of the fly brain. Therefore, staining for endogenous synaptic markers fails to provide the same cell-type-specific spatial information as an NMJ synapse. Instead, this approach reveals all or most synaptic

Table 1. Synaptic labeling toolkit in *Drosophila*.

Synaptic compartment	General class	*Drosophila* protein	Vertebrate ortholog (murine)	Labeled reagent	Expression approach	BDSC Stock ID	References
Presynaptic	AZ	Bruchpilot	Erc2	Brp-FL::GFP	Inducible (STaR): FLP/FRT	BDSC: 55759	Chen *et al.* (2014)
				Brp-FL::smV5	Inducible (STaR): FLP/FRT	n/a	Peng *et al.* (2018)
				Brp-FL::V5	Inducible (STaR): FLP/FRT	BDSC: 55760	Chen *et al.* (2014)
				Brp-FL::GFP	Binary expression: Gal4/UAS	BDSC: 36291	Wagh *et al.* (2006)
				Brp-Short::GFP	Binary expression: Gal4/UAS	n/a	Fouquet *et al.* (2009)
				Brp-Short::mStrawberry	Binary expression: Gal4/UAS	n/a	Fouquet *et al.* (2009)
				Brp-Short::mStrawberry	Binary expression: QF/QUAS	BDSC: 80571	Mosca and Luo (2014)
				Brp-Short::CFP	Binary expression: Gal4/UAS	n/a	Schmid *et al.* (2008)
				Brp-Short::Cherry	Binary expression: Gal4/UAS	n/a	Fouquet *et al.* (2009) and Kremer *et al.* (2010)
				Brp-Short::Cherry	Binary expression: LexA/*LexAop*	n/a	Berger-Müller *et al.* (2013)
		Cacophony	Cacna1a	Cac-1::EGFP	Binary expression: Gal4/UAS	BDSC: 8582	Kawasaki *et al.* (2004)
		Liprin-α	Ppfia2	GFP::Liprin-α	Binary expression: Gal4/UAS	n/a	Fouquet *et al.* (2009)
		RhoGAP100F (Syd-1)	Syde1	GFP::Syd-1	Binary expression: Gal4/UAS	n/a	Owald *et al.* (2010)
		RBP	Tspoap1	GFP::DRBP	Binary expression: Gal4/UAS	n/a	Liu *et al.* (2011)
		Unc-13	Unc13a	Unc-13A::GFP	Binary expression: Gal4/UAS	n/a	Böhme *et al.* (2016)
				Unc-13B::GFP	Binary expression: Gal4/UAS	n/a	Böhme *et al.* (2016)
	Synaptic Vesicle	neural Synaptobrevin	Vamp2	nSyb::EGFP	Binary expression: Gal4/UAS	BDSC 9263	Estes *et al.* (2000)
		Rab3	Rab3c	GFP::Rab3	Inducible: B2R/B2RT recombination (gene edit)	BDSC: 81502	Williams *et al.* (2019)
				mCherry::Rab3	Inducible: B2R/B2RT recombination (gene edit)	BDSC: 81503	
				3xFLAG::Rab3	Inducible: FLP/FRT recombination (gene edit)	BDSC: 81501	
				2xHA::Rab3	Inducible: FLP/FRT recombination (gene edit)	BDSC: 81500	
		Synaptotagmin 1	Syt I	Syt-1::EGFP	Binary expression: Gal4/UAS	BDSC 6926	Zhang *et al.* (2002)
		Vesicular Glutamate Transporter	vGlut	smFLAG::vGlut	Inducible: RSR/RSRT recombination (gene edit)	n/a	Certel, Ruchti, *et al.* (2022)
		Vesicular GABA Transporter	Slc32a1	9xV5::VGAT	Inducible: RSR/RSRT recombination (gene edit)	n/a	Certel, McCabe, *et al.* (2022)
Postsynaptic	Dendritic compartment	No homolog	Icam5	Icam5::mCherry (DenMark)	Binary expression: Gal4/UAS	BDSC: 33062	Nicolaï *et al.* (2010)
	NT receptor	GluClα	Glra1	GluClα::GFP	Binary expression: Gal4/UAS	BDSC: 92149	Fendl *et al.* (2020)
				GluClα::GFP	Inducible (FlpTag): FLP/FRT	BDSC: 92145	
		GABA-B receptor 1	Gabbr1	$GABA_BR1$::GFP	Inducible (FlpTag): FLP/FRT	BDSC: 92148	
		Acetylcholine Receptor α7 (Dα7)	Chrna7	Dα7::GFP	Binary expression: Gal4/UAS	BDSC: 39692	Leiss *et al.* (2009)
		Ort (Ora transientless)	Unclear	OLLAS::Ort	Inducible (STaR): FLP/FRT	BDSC: 55761	Chen *et al.* (2014)
		Rdl	Gabrp	Rdl::HA	Binary expression: Gal4/UAS	BDSC: 29035	Sánchez-Soriano *et al.* (2005)
				Rdl::GFP	Binary expression: Gal4/UAS	BDSC: 92150	Fendl *et al.* (2020)
	Glutamatergic postsynapse	Drep-2	Cidea	Drep-2::EGFP	Binary expression: Gal4/UAS	n/a	Andlauer *et al.* (2014)
				Drep-2::mStrawberry	Binary expression: Gal4/UAS	n/a	

A selection of these tools is highlighted in Parts I and II of this review. This table is intended as a general aid, but should not be considered an exhaustive list of available reagents.

contacts indiscriminately, rendering specific analysis of individual neurons or neuron classes technically challenging.

The coupling of conditional expression systems and inducible mosaic techniques (Germani *et al.* 2018) has enabled genetic manipulation and analysis in single cells or specific cell types and facilitated detailed descriptions of single neuron architecture (Nern *et al.* 2015) and synaptic organization. In *Drosophila*, the *GAL4/UAS* system is the most widely known tool for conditional gene expression (Fig. 1; Brand and Perrimon 1993; Duffy 2002) and consists of 2 components: the yeast transcriptional activator, GAL4, and its cognate promoter, the *upstream activating sequence* (*UAS*). When both components are present in the same cell, GAL4 drives expression of any *UAS*-regulated transgene. Thousands of GAL4 lines with defined expression patterns are available, allowing for labeling or genetic manipulations to be carried in specific cells or tissues (Hayashi *et al.* 2002; Venken, Simpson, *et al.* 2011; Jenett *et al.* 2012). The versatility of the GAL4/UAS system is complemented by 2 analogous binary expression systems: LexA/*lexAop* and QF/QUAS (Lai and Lee 2006; Potter *et al.* 2010). Each system uses a distinct transcriptional activator (GAL4, LexA, or QF) that exclusively recognizes its cognate promoter (*UAS*, *LexAop*, or *QUAS*) to achieve expression. Thus, all 3 expression systems can

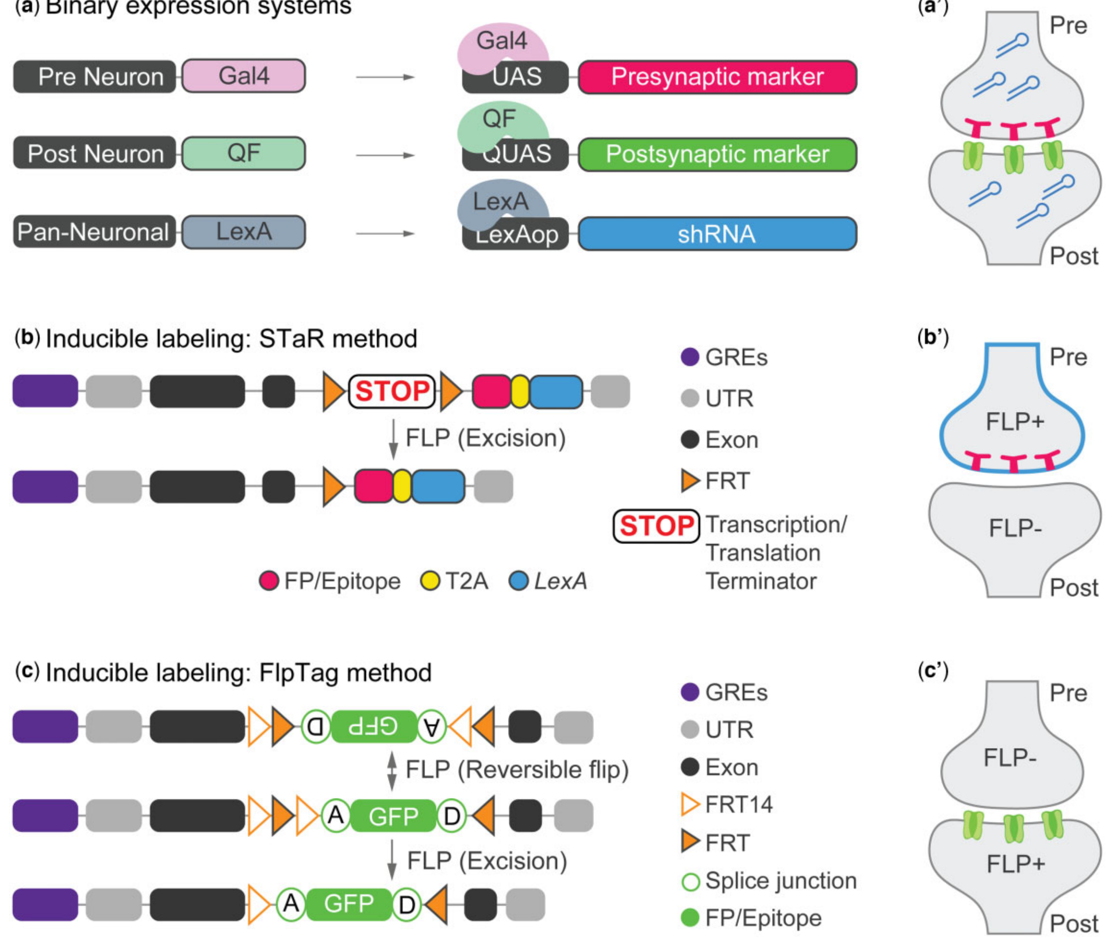

Fig. 1. Approaches for conditional and inducible synaptic transgene expression in *Drosophila*. a) The GAL4/UAS, QF/QUAS, and LexA/LexAop binary expression systems are each comprised of 2 components: a transcription factor (Gal4, QF, or LexA) and its cognate promoter (UAS, QUAS, or LexAop, respectively). Specific promoters (left) regulate the expression of Gal4, QF, and LexA, which in turn drive expression of their responder transgenes in specific cells or tissues. a') Example application of multiple binary expression systems in the CNS. The synaptic compartment is labeled in the presynaptic neuron via expression of a fluorescent protein- or epitope-tagged AZ protein under Gal4/UAS control; expression of a postsynaptic compartment marker (e.g. a fluorescently labeled NT receptor) in the postsynaptic neuron is under QUAS control; pan-neuronal expression of a short hairpin RNA, under LexA/LexAop control, knockdown expression of the gene of interest in all neurons. b, c) Cell-type-specific strategies that use FLP recombinase to label a protein only in specific cells (GRE, gene regulatory elements; UTR, untranslated region; FP, fluorescent protein). Flippase recombinase (FLP) induces site-specific recombination between matching FRTs. When tandem FRT sites are arranged in the same orientation, FLP recombination excises the intervening sequences, eliminating one of the FRT sites as shown in (b) and (c). When tandem FRT sites are arranged in opposing orientations, FLP recombination inverts the orientation of the intervening sequences as shown in (c). Each of these FLP/FRT approaches has been leveraged to generate inducible systems for synaptic labeling: (b) In the STaR method, conditional FLP expression in presynaptic neurons leads to FLP-mediated excision of the *brp* transcriptional terminator allowing transcription of an engineered cassette containing a fluorescent protein or epitope tag, thus producing a tagged version of Bruchpilot (Brp), which labels presynaptic AZs (Chen *et al.* 2014). This cassette also features a ribosomal skipping sequence, T2A, followed by LexA ORF (Daniels *et al.* 2014), which effectively couples inducible synaptic labeling with activation of the LexA/LexAoP binary expression system. The LexA/LexAoP system can be used to drive expression of additional transgenes only in FLP-expressing cells, for example, a membrane marker as diagrammed in (b'). c) In the FlpTag method, conditional FLP expression in a postsynaptic neuron inverts the orientation of an artificial exon inserted into, for example, an NT receptor gene. The artificial exon encodes GFP, which is only spliced into the mature mRNA when the donor and acceptor sites are in the correct orientation. The resultant NT receptor::GFP fusion protein labels the postsynaptic compartment in FLP expressing cells. Similar approaches have also been used to generate inducible Rab3-, vGAT-, and vGlut-based synaptic vesicle markers (Williams *et al.* 2019; Certel, McCabe, *et al.* 2022; Certel, Ruchti, *et al.* 2022).

be used in combination to manipulate up to 3 distinct genetic elements in the same animal (Fig. 1, a–a"). Further increasing the versatility of these expression systems, conditional expression of site-specific recombinases (e.g. FLP, Cre, ΦC31, RSR, B2R) allow targeted DNA rearrangements *in vivo* (Fig. 1b and c; Simpson 1993; Kilby *et al.* 1993; Siegal and Hartl 2000; Groth *et al.* 2004; Nern *et al.* 2011; Weasner *et al.* 2017). These manipulations can be used, for example, to generate loss-of-function mosaic tissue or cell-type restricted protein labeling (Lee and Luo 2001; Lai and Lee 2006; Chen *et al.* 2014; Urwyler *et al.* 2015; Fendl *et al.* 2020).

The combination of binary expression systems, conditional expression strategies, and genetically encoded synaptic labels has circumvented the limitations of CNS studies by enabling the examination of specific synaptic contacts in genetically identifiable neurons (e.g. in the CNS: Fouquet *et al.*, 2009; Kremer *et al.*, 2010; Christiansen *et al.*, 2011; Chen *et al.*, 2014; Mosca and Luo, 2014). Thus, instead of using an antibody to the endogenous protein that recognizes all synapses in the brain, with no cell-type-specific delineation, this approach allows visualization of only one population of synaptic contacts. Moreover, the ability to reliably label synapses in specific, genetically accessible neurons enables direct comparisons of synaptic features from animal to animal in wild type, mutant, or otherwise perturbed conditions. Currently, a wide range of validated genetically encoded markers is available and amenable for use in the fly CNS (Fig. 2; Table 1). While the specific design of each construct varies to some extent and can offer distinct insights depending on the protein, genetically encoded markers typically consist of a synaptic protein or a portion of a synaptic protein fused to a fluorescent label or epitope tag (as in Zhang *et al.* 2002). Broadly useful synaptic markers should meet the following criteria in that they (1) are a common, often essential, synaptic component; (2) are expressed at a level that allows robust detection by light microscopy; and (3) do not significantly affect synaptic structure or function when expressed. Combined with conditional expression of genetically encoded markers by one of 3 common binary expression systems in *Drosophila* or by inducible, recombination-based approaches, the repertoire of synaptic labels allows for complex dissection of the genetic basis underlying synaptogenesis.

Genetically encoded presynaptic labeling strategies

Synaptic vesicle (SV) labeling

A broadly accessible synaptic marker should take advantage of a protein that is expressed at most, if not all synapses, to ensure its physiological relevance. All chemical synapses are united in their requirement for vesicular release of NT to enable communication between neurons. Because of this, the first generation of genetically encoded synaptic labels was based on integral synaptic vesicle proteins, including Synaptotagmin and Synaptobrevin/VAMP fused to GFP—Syt-1::GFP and n-Syb::GFP (Estes *et al.* 2000; Zhang *et al.* 2002; Fig. 2). Both proteins are common to most, if not all synapses, as they represent critical SNARE proteins needed for all vesicle fusion (Sauvola and Littleton 2021). Labeled versions of Syt-1 and n-Syb provide information about the location and magnitude of synaptic vesicle accumulation and function as a proxy for presynaptic NT release sites. Restricted neuronal expression of Syt-1::GFP or n-Syb::GFP via binary expression systems is frequently used to label presynaptic terminals (Brand and Perrimon 1993; Lai and Lee 2006; Potter *et al.* 2010) and has been a fruitful tool for analysis of neuronal circuits (Ramaekers *et al.* 2005; Otsuna and Ito 2006; Helfrich-Förster *et al.* 2007; Zhang *et al.* 2007; Gorostiza *et al.* 2014; Goyal *et al.* 2019; Guo *et al.* 2019). For example, mapping the pre- and postsynaptic terminals of a neuron of interest is often accomplished by expression of Syt-1::GFP, to identify the presynaptic compartment in 1 neuron, and the dendritic marker DenMark, to identify the postsynaptic region in its downstream target (see below; Nicolaï *et al.* 2010; Ni *et al.* 2019). Such approaches can also be used concurrently in multiple classes of cells (using multiple binary expression systems) to examine potential regions of apposition, differentiate pre- and postsynaptic regions within single neurons, and begin to infer circuit-level connectivity (Kennedy and Broadie 2018; Lamaze *et al.* 2018; Chen *et al.* 2019; Jung *et al.* 2020; Zhang and Simpson 2022).

Important caveats exist with vesicular markers, however, that may limit their utility. First, domains of synaptic vesicle (SV) protein enrichment do not always overlap precisely with presynaptic active zones (AZ)s (Urwyler *et al.* 2015), especially during development (e.g. Urwyler *et al.* 2015). Second, some vesicular proteins, particularly members of the Synaptotagmin family, function postsynaptically (Barber *et al.* 2009; Harris *et al.* 2016, 2018; Wu *et al.* 2016; Quiñones-Frías and Littleton 2021), thus limiting their ability to differentiate pre- from postsynaptic terminals in certain circumstances. Finally, overexpression of any protein may lead to ectopic enrichment if the overexpression conditions exceed the ability of the cell to localize it properly. It is therefore possible that overexpression of SV markers may produce artifactual labeling, thus limiting their fidelity as synaptic markers (Williams *et al.* 2019). Recently, however, alternative approaches for SV labeling were generated using CRISPR/Cas9 genomic editing. These tools ensure cell-type-specific labeling via conditional incorporation of a label (under the control of a site-specific recombinase), but because they retain endogenous promoter control, they circumvent complications associated with protein overexpression as they are expressed at normal physiological levels (Williams *et al.* 2019; Certel, McCabe, *et al.* 2022; Certel, Ruchti, *et al.* 2022). For example, 3 markers, based on the synaptic vesicle proteins Rab3, vGAT, and vGlut, were generated by inserting an N-terminal epitope- or fluorescent protein-tag immediately downstream of a transcriptional stop cassette, which can be conditionally excised by expression of a site-specific recombinase (reminiscent of the strategies outlined in Fig. 1b). In the absence of the recombinase, the unlabeled endogenous protein is expressed. When a recombinase is provided in select cells using a binary expression system, the stop cassette is removed and the protein from the endogenous gene is tagged, enabling the visualization of specific populations of SVs in target neurons using immunocytochemistry for the indicated tag (Williams *et al.* 2019; Certel, Ruchti, *et al.* 2022). By not relying on vesicular protein overexpression, the strategy circumvents overexpression caveats.

Synaptic vesicle markers serve as powerful tools for marking vesicle populations largely associated with presynaptic release sites. When vesicle markers are applied in live tissues, these labels can be used to track dynamic features including SV trafficking, depletion, or accumulation (Zhang *et al.* 2002; Poskanzer *et al.* 2003; Christiansen *et al.* 2011). When coupled to pH-sensitive fluorophores like pHlourin (Miesenböck *et al.* 1998) or with features of the GFP-reconstitution across synaptic partners (GRASP) technique (Feinberg *et al.* 2008), vesicle markers like n-Syb may be able to report connections in an activity-dependent fashion (Macpherson *et al.* 2015) in live imaging, adding to the utility and power of synaptic vesicle markers. When coupled with sequences from the synaptic proteins Neurexin and Neuroligin (Südhof 2018), this enables precise synaptic targeting (Shearin *et al.* 2018) of GRASP markers to assess connectivity. Despite the incredible utility of vesicle markers as synaptic tools, proteins like Syt-1 and

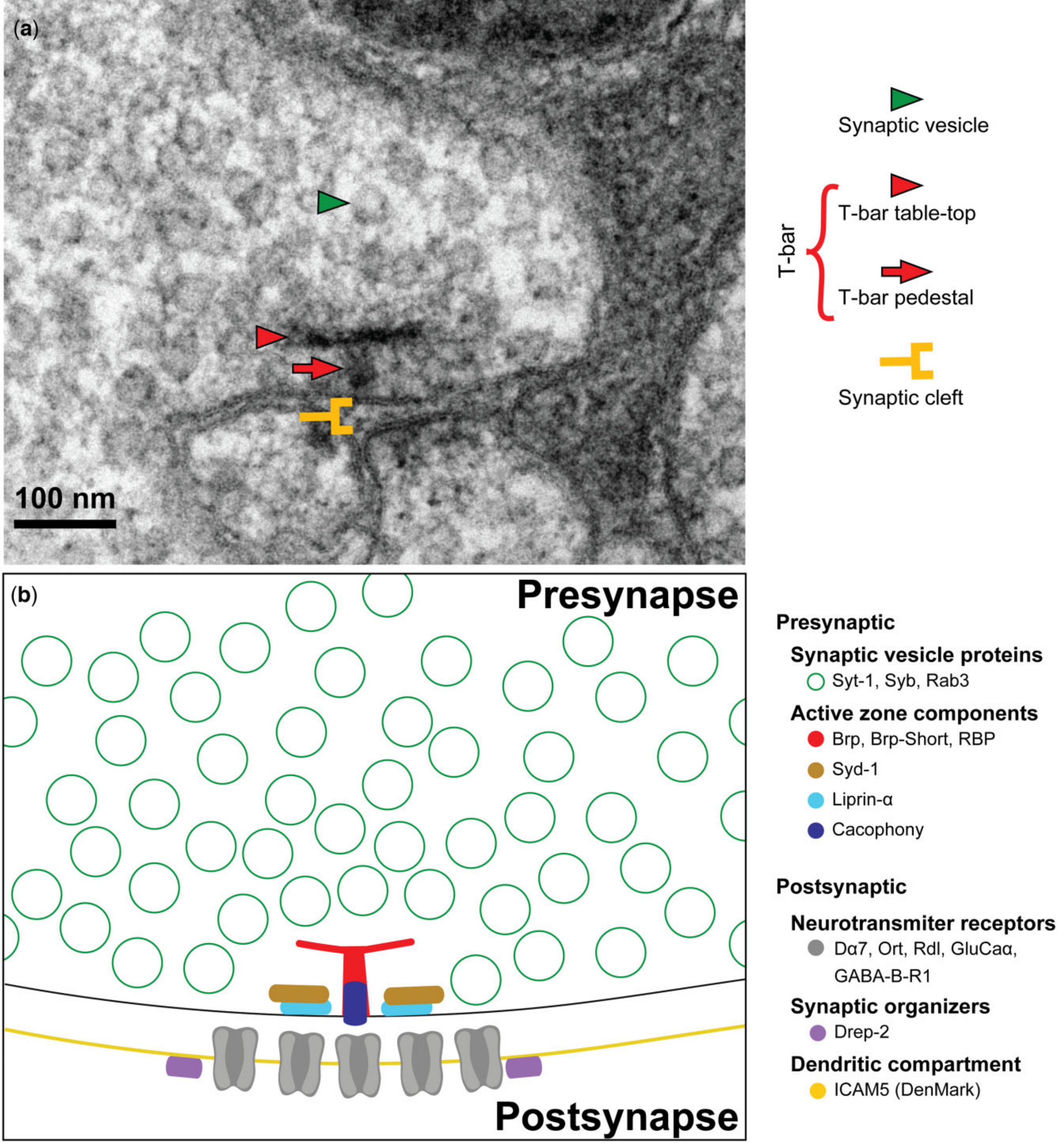

Fig. 2. A repertoire of pre- and postsynaptic markers for studying synaptic organization in *Drosophila*. a) Electron micrograph of an ORN axon terminal. The *Drosophila* AZ is readily identified by an electron-dense T-bar structure that is composed of a Brp-rich table-top (red arrowhead) and an RBP-rich pedestal (red arrow). The T-bar is surrounded by synaptic vesicles (green arrowhead) and abuts the synaptic cleft (yellow bracket). b) Cartoon schematic of *Drosophila* synapse. Synaptic proteins accumulate in distinct subcellular compartments at the synapse. Synaptic markers for visualization are generally recombinant proteins consisting of the full-length protein sequence of a synaptic protein (or an interacting portion of that protein) fused to a fluorescent protein or epitope tag. The resultant product can then be followed in one specific cell or set of cells using inducible expression strategies and imaged using commercially available antibodies to the epitope or fluorescent tags with immunohistochemistry or live imaging. A selection of published pre- (top) and postsynaptic (bottom) labels are based on the proteins highlighted (Right; color coding reflects their general location at the synapse as diagrammed).

n-Syb do not report other critical structural features of synaptic organization, such as the precise location and distribution of AZs or ion channels. Additional strategies, including those used to label AZs, synaptic ion channels, or other mechanistic synaptic proteins serve as an excellent complement to SV markers to identify functional presynapses in neurons of interest. Such studies that integrate multiple presynaptic markers can markedly advance our understanding of physiological synaptic properties. For example, the physical distance between synaptic vesicles and voltage-gated Ca^{2+} channels, or coupling distance, varies across synapses and influences release probability (Wadel *et al.* 2007; Fulterer *et al.* 2018; Ghelani and Sigrist 2018). Visualizing synaptic

vesicle proteins and AZ components by super-resolution microscopy enables the assessment of synaptic architecture at the nanometer scale, enabling assessment of critical structural features (Ehmann *et al.* 2014; Spühler *et al.* 2016; Fulterer *et al.* 2018). Thus, the suite of genetically encoded synaptic markers coupled with the versatility of expression systems in *Drosophila* serves as an excellent entrée into visualizing specific synaptic populations in *Drosophila* but must be complemented with additional synaptic markers and tools to enable a thorough genetic dissection of the synaptic architecture that underlies function.

Active zone (AZ) labeling

The AZ is a subcellular, presynaptic specialization that provides the scaffolding for NT vesicle release and calcium channel localization, ultimately serving to regulate NT exocytosis (Südhof 2012). AZs can be identified ultrastructurally as electron-dense projections that abut the presynaptic membrane, lie apposed to postsynaptic densities, and are decorated by synaptic vesicles (Koenig and Ikeda 1996; Zhai and Bellen 2004). In *Drosophila*, AZs adopt a characteristic "T" shape (Fig. 2a) and are called T-bars (Meinertzhagen 1996; Prokop and Meinertzhagen 2006; Hamanaka and Meinertzhagen 2010); the T-bar structure is shared by both peripheral and central synapses (Wichmann and Sigrist 2010). In the absence of the T-bar, synaptic transmission is severely impaired in *Drosophila* (Kittel *et al.* 2006; Wagh *et al.* 2006; Liu *et al.* 2011), highlighting its essential role in neurotransmission. Further underscoring the importance of the T-bar to synaptic analysis, connectomics analyses and EM-based studies identify synapses based on the presence or absence of T-bars (Scheffer *et al.* 2020). The *Drosophila* T-bar is comprised of 2 major molecular components, the ERC (ELKS/Rab-interacting/CAST) protein Bruchpilot (Brp) which forms the T-bar tabletop and RIM-binding protein (RBP) which forms the pedestal (Fig. 2a; Wagh *et al.* 2006; Kittel *et al.* 2006; Fouquet *et al.* 2009; Hallermann *et al.* 2010; Liu *et al.* 2011; Acuna *et al.* 2016; Scholz *et al.* 2019). As Brp and RBP are essential structural components of most, if not all fly synapses, multiple labeling strategies and genetically encoded transgenic approaches target these proteins to label the presynaptic AZ (Kawasaki *et al.* 2004; Fouquet *et al.* 2009; Sugie *et al.* 2015). Unlike synaptic vesicle markers that label large pools of synaptic vesicle proteins and often span multiple AZs, AZ markers accumulate in a punctate manner that allows for quantification of distinct parameters of synaptic organization including the number of presynaptic AZs and their organization in 3D space (Figs. 2 and 3c). To date, Brp is the most widely utilized presynaptic protein for genetically encoded AZ labeling strategies, but RBP as well as other strategies based on proteins like the auxiliary AZ proteins Syd-1 (Owald *et al.* 2010), Liprin-α (Kaufmann *et al.* 2002), and the Ca^{2+} channel Cacophony (Kawasaki *et al.* 2004) function analogously to assess both CNS and PNS synaptic organization (Table 1). Here, we highlight each of these tools in concert with unique genetic labeling strategies to understand how AZs are visualized in *Drosophila*.

Brp-based labeling strategies

The most widely used approaches for cell-type-specific AZ labeling in *Drosophila* center on Brp. At the *Drosophila* NMJ, a single AZ incorporates ∼137 Brp molecules (Ehmann *et al.* 2014), allowing considerable opportunity for labeling. Brp-based synaptic labels are typically expressed using a binary expression system to label only the synapses of selected, genetically identifiable cells *in vivo*. Generally, Brp labels are recombinant proteins that fuse a fluorescent- or epitope-tag to either a full length or truncated Brp protein and can be visualized by immunohistochemistry or live imaging. We will refer to full-length Brp as Brp-FL and to the truncated form as Brp-Short (also known as Brp-D3; Fouquet *et al.* 2009). Historically, the 2 approaches (Brp-FL and Brp-Short) have been used interchangeably, though important caveats must be considered as Brp-Short and Brp-FL are functionally distinct and can behave differently when overexpressed (see below).

Two separate, and functionally distinct, methods employ Brp-FL to label synapses. First, Brp-FL can be expressed through traditional binary expression systems (Wagh *et al.* 2006; Flood *et al.* 2013) and imaged to ascertain key synaptic parameters such as AZ numbers and organization. However, though facile and at least partly reflective of endogenous AZ organization, Brp-FL can form aggregates outside of synaptic compartments when overexpressed via GAL4/*UAS* in a nonrelevant cell, resulting in artifactual punctate labeling (Wagh *et al.* 2006). Moreover, GAL4/*UAS* driven Brp-FL in photoreceptors produces a diffuse signal that does not accurately label individual presynaptic active sites (Chen *et al.* 2014). With binary expression systems, the onset and relative levels of transgene expression are determined by the specific combination of driver/responder used. As a result, induced overexpression is unlikely to accurately reflect cellular conditions. To circumvent variability from different promoters, and to achieve physiologically relevant expression levels, Chen *et al.* (2014) developed the Synaptic Tagging by Recombination (STaR) method. STaR is an inducible AZ labeling strategy that relies on the endogenous *brp* promoter to regulate expression of the labeled transgene. The STaR method consists of a genomically inserted bacterial artificial chromosome (BAC) harboring the *brp* genomic locus. The locus itself is modified to contain a V5 epitope- or GFP-tag immediately downstream of the *brp* transcription termination sequence (as diagrammed in Fig. 1b). The termination sequence is then flanked on either side by FLP-recombinase recognition target (FRT) sequences. In the absence of FLP, wild-type Brp-FL without a label is expressed from the BAC with transcriptional regulation provided by the intact endogenous promoter and is indistinguishable from endogenously expressed Brp from the native genomic region. However, in the presence of FLP (supplied in a cell-type-specific fashion using a binary expression system like GAL4/*UAS*), excision of the transcriptional stop cassette leads to the production of the tagged Brp-FL protein (Brp-FL::V5 or Brp-FL::GFP) only in those cells where FLP is expressed (Fig. 1b–b'). Thus, specific synaptic labeling is achieved by restricting the expression of FLP to the neuron(s) of interest. This fusion protein is fully functional, localizes correctly to the AZ, and is expressed at physiological levels (Chen *et al.* 2014). This strategy has been successfully used to reveal aspects of synaptic organization in multiple *Drosophila* circuits (Sugie *et al.* 2015; Akin and Zipursky 2016; Liu *et al.* 2016; Xu *et al.* 2018) and is consistent with ultrastructural data, indicating that it is a largely accurate reporter (Chen *et al.* 2014). It remains unclear, however, whether in a wild-type fly, the additional copy of Brp-FL (despite being expressed under the control of the native *brp* promoter) produces overexpression artifacts, as Brp-FL can when expressed via GAL4/*UAS* (Wagh *et al.* 2006; Chen *et al.* 2014). Recent work indicates that *brp* copy number is physiologically relevant in the context of sleep, where *brp* dosage promotes sleep (Huang *et al.* 2020). Nonetheless, in the visual system, the subcellular distribution and number of Brp-FL::GFP positive puncta is unaffected when the *brp* copy number is increased from 1 to 4 (Chen *et al.* 2014) indicating that key synaptic parameters in the visual system are unchanged by *brp* genetic dosage. Overall, studies employing Brp-FL labeling strategies have enabled circuit-

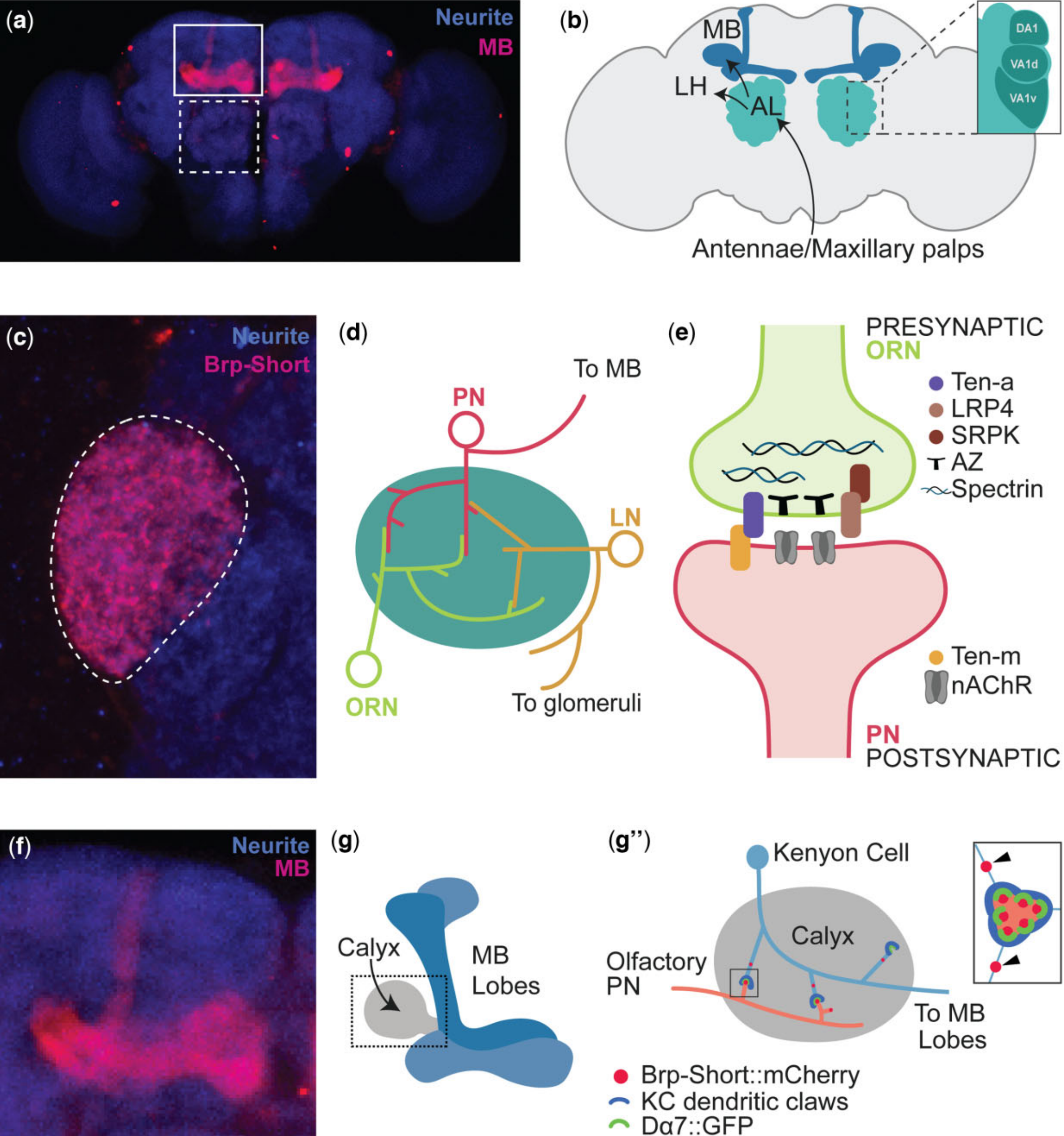

Fig. 3. Model systems of synaptic organization in the *Drosophila* olfactory circuit. a) Micrograph of an adult *Drosophila* brain stained with a general neurite label (blue) and a marker that reveals the mushroom body (MB) lobes (magenta). The anatomical locations of the antennal lobe (AL; dashed box) and MB (solid box) are indicated. b) Schematic of the fly brain with annotated olfactory circuit [figure panel modeled after Schlegel *et al.* (2021)]. Odor information flows from the antennae and maxillary palps to the first-order processing center, the AL (shown in green). Olfactory information is then transmitted to higher order brain regions, including the MB shown in blue and the lateral horn (LH). The AL is organized into discrete neuropil where 3 major neuronal classes form synaptic connections (inset shows 3 anatomically distinct neuropil: DA1, VA1d, and VA1v). c) Micrograph of AL glomeruli corresponding to the region indicated in the dashed box in (b). All ORNs that express a particular OR (Or67d) converge on a single glomerulus (Dashed; DA1). Restricted expression of Brp-Short::mStrawberry in these ORNs reveals AZ distribution. d) Three major neuronal classes reside in each AL glomeruli [figure modeled after Cachero and Jefferis (2008)]. A single glomerulus is diagrammed in (d) where ORN are presynaptic to PNs. PNs then transmit odorant information to higher-order brain regions including the MB and LH. The LNs comprise many different classes of cells defined by morphology and form an extensive lateral network that connects most or all glomeruli. LNs form synaptic connections with ORNs, PNs, and other LNs. e) Synaptic organization in the AL is regulated by the Teneurin and LRP4 signaling pathways [figure adapted from DePew *et al.* (2019) and DePew and Mosca (2021)]. Trans-synaptic heterophilic Teneurin interactions instruct synaptic organization in the AL. Presynaptic Ten-a functions with Spectrin to promote presynaptic AZ assembly and organization. The role of postsynaptic Ten-m remains unknown. In addition to the Teneurins, LRP4 function is required to maintain normal synaptic organization in the AL. The current model of LRP4 function posits that LRP4 recruits SRPK79D (SRPK) to the synapse where these 2 regulate synaptic assembly and morphology. f) Micrograph of MB lobes (magenta). MB calyces are not discernible. g) Schematic of the MB. The MB intrinsic neurons, the Kenyon cells (KCs), concentrate their dendrites in the calyx and send axonal projections in parallel bundles to form the MB lobes. g") KC dendrites are mixed neurites that exhibit both pre- and postsynaptic specializations [figure adapted from Christiansen *et al.* (2011)]. KCs are postsynaptic to olfactory PNs and form specialized "dendritic claws" that can be labeled with the acetylcholine receptor subunit Dα7::GFP (Green in inset; cartoon modeled after; Kremer *et al.* 2010). KC-derived presynaptic AZs form outside of the dendritic claws (arrowheads in inset).

level and molecular analyses that have contributed significantly to our understanding of synaptic organization.

The second Brp-related strategy that has been successfully used to determine synaptic organization involves a truncated version of Brp, Brp-Short (Schmid *et al.* 2008; Fouquet *et al.* 2009), that comprises the central 473–1,226 amino acids of the full-length protein (1,740 aa; GenBank: AAF58930). Brp-Short alone is nonfunctional and causes no discernible effects on cell morphology, synaptic organization, or neuronal function when overexpressed (Schmid *et al.* 2008; Fouquet *et al.* 2009; Mosca and Luo 2014; Urwyler *et al.* 2015). Though nonfunctional, Brp-Short can interact with endogenous Brp and is thus recruited to presynaptic AZs (Fouquet *et al.* 2009; Mosca and Luo 2014). When fused to a fluorescent protein or epitope tag, Brp-Short serves as a proxy label of endogenous presynaptic AZs, where it accumulates in discrete puncta (Wagh *et al.* 2006; Fouquet *et al.* 2009). Immunoelectron microscopy confirms that Brp-Short labels T-bars (Mosca and Luo 2014) and colocalizes with other known synaptic and AZ-related proteins like Syt1, DSyd-1, D-Liprin-α, and Cac (Fouquet *et al.* 2009; Mosca and Luo 2014; Urwyler *et al.* 2015). When Brp-Short is expressed in neurons with relatively sparse synaptic organization, the number of Brp-Short puncta and their subcellular distribution agrees with analogous T-bar counts and distribution data from EM studies (Takemura *et al.* 2008; Berger-Müller *et al.* 2013). A striking example comes from MSNs that innervate the dorsocentral bristles of adult flies (Urwyler *et al.* 2015). In this system, inducible approaches such as mosaic analysis with a repressible cell marker (MARCM; Lee and Luo 2001) or FLP-based removal of termination STOP cassettes (Urwyler *et al.* 2015) allows for reproducible generation of single-cell clones that selectively express Brp-Short::GFP and mCD8::mCherry. The ability to restrict labeling to a single neuron enables one to image the same neuron by 3D correlative light and electron microscopy (CLEM; (Bishop *et al.* 2011; Urwyler *et al.* 2015). In MSNs, CLEM analysis revealed that the location of Brp-Short::GFP puncta maps to the same cellular coordinates as T-bars and SVs, and further, that Brp-Short::GFP does not accumulate ectopically outside of presynapses (Urwyler *et al.* 2015).

In neurons with more dense synaptic organization such as in the antennal and optic lobes, Brp-Short labels accurately detect fold changes in AZ number in response to genetic or environmental perturbations (Mosca and Luo 2014; Sugie *et al.* 2015). In response to prolonged light exposure, synapses are remodeled in select photoreceptor neurons (Sugie *et al.* 2015), a process involving partial AZ disassembly and removal of Brp. Light-induced plastic changes are reversible and indeed, can be studied using both Brp-Short and the STaR methods. Both approaches yield comparable results in terms of labeled Brp puncta numbers, distribution under steady state conditions, and response to extended light exposure (Sugie *et al.* 2015). Intriguingly, Brp-Short (but not Brp via STaR) becomes diffuse following prolonged light exposure; this may reflect differences in processing of the Brp-Short fragment vs the full-length label though this remains unclear. However, the propensity of Brp-Short to become diffuse in response to prolonged light has recently been exploited as the phenotypic basis for a genetic screen seeking to identify regulators of activity-dependent synaptic remodeling (Araki *et al.* 2020).

In the *Drosophila* AL, measurements taken using Brp-Short and confocal microscopy in the 3 major component neurons [olfactory receptor neurons (ORNs), projection neurons (PNs), and local interneurons (LNs)] are consistent with analogous ultrastructural reconstructions that show similar results regarding the proportion of total synapses made by each class of cells (Rybak *et al.* 2016; Tobin *et al.* 2017; Horne *et al.* 2018; Coates *et al.* 2020). Taken together, these studies indicate that Brp-Short is a powerful AZ marker that can be used to quantitatively measure synaptic AZ organization with high fidelity in a diverse array of neurons in *Drosophila*; including the olfactory system (Mosca and Luo 2014; Coates *et al.* 2017; Mosca *et al.* 2017; Fulterer *et al.* 2018), the mushroom body (Kremer *et al.* 2010; Christiansen *et al.* 2011), the visual system (Berger-Müller *et al.* 2013; Sugie *et al.* 2015; Özel *et al.* 2019), the larval ventral nerve cord (Hu *et al.* 2017; Tenedini *et al.* 2019), and the ellipsoid body (Xie *et al.* 2019). In MSNs, GAL4-driven Brp-Short AZ labeling produces indistinguishable results when compared directly to Brp-FL AZ labeling via the STaR method, indicating that these 2 strategies are viable synaptic labeling alternatives (Urwyler *et al.* 2015). Taken together, both Brp-Short and inducible Brp-FL labeling approaches (STaR) successfully measure synaptic organization across diverse circuits, often serving as complementary techniques that yield great flexibility in experimental design (Table 1).

Complementary AZ labels

In addition to Brp-based labels, epitope- or fluorophore-tagged versions of ancillary AZ proteins can also report synaptic organization in concert with binary expression systems. The AZ scaffolding protein, RBP (Sugie *et al.* 2015), the voltage-gated Calcium channel Cacophony (Liu *et al.* 2011; Fulterer *et al.* 2018; Sugie *et al.* 2018), synaptic seeding factors such as Liprin-α and Syd1 (Fouquet *et al.* 2009; Mosca *et al.* 2017; Özel *et al.* 2019), synaptic vesicle release factors Unc13A (Reddy-Alla *et al.* 2017; Fulterer *et al.* 2018), and others (Fig. 2b) have all been employed to study synapse formation, development, and organization (Table 1). Like Brp-based tools, these labels accumulate in a punctate pattern at the presynaptic membrane and can be quantified to define synapse number, subcellular synaptic distribution, or dynamic events such as recruitment to or removal from the synapse when imaged in living tissues (Fouquet *et al.* 2009; Sugie *et al.* 2015; Mosca *et al.* 2017; Fulterer *et al.* 2018; Özel *et al.* 2019). As each label is a functionally distinct component of the presynaptic AZ, they offer unique advantages and disadvantages as synaptic labeling reagents. For example, in some cases, accumulation of Liprin-α and Syd1 may precede accumulation of Brp labels or synaptic vesicle markers which may increase the temporal resolution of synaptogenesis (e.g. Özel *et al.* 2019). Alternatively, in other circumstances, plastic synaptic remodeling in response to environmental stimuli (Sugie *et al.* 2015) may be detectable using some AZ markers (e.g. Brp-Short::mCherry, GFP::Liprin-α, and GFP::RBP), but not others (e.g. GFP::Syd1, Cac::GFP). The available repertoire of high-fidelity presynaptic labeling strategies, coupled with approaches for conditional or inducible labeling in *Drosophila*, allows for straightforward identification of the presynaptic compartment and qualitative and quantitative analyses of synaptic organization. A combinatorial approach by the field, utilizing multiple different AZ labels, is a powerful strategy that can yield a deeper understanding of AZ assembly and synaptic organization.

Genetically encoded postsynaptic labeling strategies

Presynaptic labels necessarily constitute half of a visualized, functioning synapse—for every active presynapse, a postsynaptic apparatus must exist to receive those signals and effect a response. Without postsynaptic labeling strategies, a proper analysis of circuit partners is incomplete. Additional insights into

neural logic and information processing become accessible when paired with postsynaptic labels. Currently, however, postsynaptic labeling strategies in general are limited and have lagged behind presynaptic strategies for a number of reasons. Presynaptic AZs are specialized for NT secretion and share a core secretory machinery for multiple different kinds of NTs (Südhof 2012) that can be exploited for labeling strategies. Postsynaptic specializations, on the other hand, exhibit greater functional diversity. Broadly, postsynaptic specializations differ significantly from one another in terms of their molecular composition depending on whether they support excitatory or inhibitory neurotransmission (Sheng and Kim 2011). NT specialization is further differentiated by expression of distinct classes of NT receptors (depending on the nature of the synapse and the NT needed to promote signaling), as well as structural, regulatory, and signaling molecules consistent with those subtypes of NT receptor. As a result, it has been challenging to identify postsynaptic markers suitable for a general, genetically encoded synaptic labeling strategy because there are fewer shared components across all postsynapses than presynaptic release sites. To date, the most successful and broadly used postsynaptic labeling strategy to study synaptic organization in *Drosophila* relies on epitope- or fluorescent-tagged NT receptors (Fig. 2b). Though limited to specific types of receptors, labeled receptors are excellent markers for postsynaptic architecture allowing further study. Despite this complexity and challenge, though, a growing repertoire of postsynaptic markers is emerging. These markers (Nicolaï *et al.* 2010; Andlauer *et al.* 2014; Chen *et al.* 2014; Mosca and Luo 2014) are suitable for use in multiple neuronal classes regardless of NT receptor identity and label the somatodendritic compartment, postsynaptic structural proteins, or synaptic organizers (Fig. 2b). The field of postsynaptic marker development is burgeoning in *Drosophila*, and all postsynaptic labeling strategies have contributed to our understanding of postsynaptic development, quantification of NT receptor clusters in adult circuits, and 3D synaptic organization in the brain.

NT receptor labeling

Postsynapses are specialized to respond to the specific NTs released by their presynaptic partners. As a result, a common labeling strategy uses genetically encoded NT receptors featuring epitope- or fluorescent protein-tags that are either overexpressed using binary expression systems or expressed at approximately physiological levels via conditional recombination strategies. Each strategy has contributed significantly to the understanding of synaptic organization.

In the CNS, epitope-tagged or fluorescently labeled individual subunits of various NT receptors have enabled identification of distinct postsynaptic regions and studies of synaptic organization in multiple circuits. In the *Drosophila* brain, acetylcholine functions as the major excitatory NT (Gundelfinger and Hess 1992; Kondo *et al.* 2020; Rosenthal *et al.* 2021). In studies of the olfactory system (Wilson 2013) including olfactory PNs in the AL that receive cholinergic input from ORNs and in the MB Kenyon cells (KCs) which receive cholinergic input from olfactory PNs (Yasuyama *et al.* 2002; Ramaekers *et al.* 2005; Gu and O'Dowd 2006), a GFP-tagged Dα7 subunit of the acetylcholine receptor has been used with conditional expression via GAL4/*UAS* (Leiss, Koper *et al.* 2009). Dα7-GFP accumulates at the synaptic membrane and directly apposes the presynaptic AZ (Leiss, Groh, *et al.* 2009; Kremer *et al.* 2010; Christiansen *et al.* 2011; Mosca and Luo 2014; Mosca *et al.* 2017). Quantification of Dα7::GFP expressed specifically in PNs or KCs yields measurements of synapse number and spatial organization consistent with the matching parameters from studies involving presynaptic labeling of Brp (Christiansen *et al.* 2011; Mosca and Luo 2014; Mosca *et al.* 2017). This approach has also been validated and extended with diverse other postsynaptic NT receptors including the GABA receptor Resistant to dieldrin (Rdl; Sánchez-Soriano *et al.* 2005; Fendl *et al.* 2020) and GluClα glutamate receptors (Fendl *et al.* 2020), among others. In the optic lobe especially, HA epitope- or GFP-tagged versions of the GABA receptor subunit Rdl have been used to study synaptic organization in motion sensing T4/T5 neurons and in the lobula plate tangential cells in the optic lobe using binary expression systems (Sánchez-Soriano *et al.* 2005; Raghu *et al.* 2007; Fendl *et al.* 2020). Importantly, though, conditional expression of NT receptor transgenes has essential caveats. First, introducing an epitope or fluorescent tag to an NT receptor may alter receptor function, resulting in a gain or loss of function of the receptor in question. Second, as transgene expression levels are constrained by the strength of the GAL4/QF/lexA driver, labeled NT receptors may affect synaptic physiology when overexpressed. Conversely, low-level expression below a certain threshold of the transgene may stymy reliable detection by light microscopy. Finally, as with Brp-FL-based strategies, ectopic expression that exceeds the cell's natural ability to process and correctly target overexpressed protein may lead to ectopic accumulation at nonphysiological postsynaptic sites. Thus, though powerful tools, results from these strategies must be carefully interpreted.

More recent strategies have sought to circumvent the deleterious effects associated with protein overexpression by relying on endogenous transcriptional regulation to drive expression of postsynaptic markers. Though general approaches like MiMIC (Venken, Schulze, *et al.* 2011) facilitate epitope- or fluorescent-labeling of endogenously expressed proteins, by themselves, they lack the tissue-specific expression needed to make assessments in specific cells of interest. Even genome-wide resources for the ∼113 postsynaptic NT receptors (Kondo *et al.* 2020) show general expression patterns even with tagged receptors, but lack cell-type-specific control. Two strategies in particular, however, have been pioneered to combine postsynaptic labeling with cell-type-specific expression at roughly endogenous levels. In the visual system, the STaR method (Chen *et al.* 2014) that was successfully applied with Brp, has also been applied to produce an OLLAS-tagged version of Ort, the histamine receptor, and used to study postsynaptic organization. In laminar neurons that are postsynaptic to photoreceptors (R1–R6) in the optic lobe, OLLAS-Ort is encoded via a BAC containing the Ort promoter and ORF and localizes directly to postsynaptic sites that directly appose presynaptic AZs labeled by Brp-FL (Chen *et al.* 2014). Though powerful, this method still requires introduction of a BAC containing the genetic locus, which introduces an additional copy of the gene in an otherwise wild-type condition, potentially producing overexpression artifacts. As approaches for genomic engineering (Baena-Lopez *et al.* 2013; Gratz *et al.* 2013, 2014; Zirin *et al.* 2022) or site-specific genetic manipulation (Venken, Schulze, *et al.* 2011) have matured and become widely adopted, the field has moved toward inducible systems for conditional NT receptor labeling. Fendl *et al.* (2020), developed an inducible labeling strategy named FlpTag, in which a GFP protein-tag is conditionally spliced into the mature mRNA encoding either GluClα and $GABA_BR1$ (Fig. 1c–c'). This takes advantage of concepts like FLPStop (Fisher *et al.* 2017), which originally permitted conditional removal of genes from specific cell types, and replaces it with the ability to conditionally tag a gene only in cells where FLP is present. Incorporation of the GFP protein-tag requires FLP recombinase

activity to invert the cassette from a nonproductive orientation to one that facilitates splicing into the mature mRNA (Fendl *et al.* 2020). In its current iteration, the FlpTag approach is optimized as a general strategy for use in combination with MiMIC libraries to generate inducible synaptic labels by recombination mediated cassette exchange (Venken, Schulze, *et al.* 2011). Necessarily, such an approach is constrained by the exact location of the MiMIC landing site. In some cases, the MiMIC site may not be suitable for tagging, be in a position that results in suboptimal presentation of the tag, or result in a tagged version of the protein that compromises receptor function or localization. Available FlpTag labels based on GluClα and $GABA_BR1$ for example, localize correctly to the expected subcellular postsynaptic compartment, but receptor function and impact on cell physiology have not been characterized. Therefore, care must be taken with this approach to ensure that the resultant product is a functional, correctly localized receptor protein. The advancement of gene-editing technology like CRISPR/Cas9, however, allows for targeted manipulations to generate inducible labels modeled after STaR, FlpTag, or other approaches in order to ensure that the synaptic label remains subject to endogenous transcriptional regulation with the goal of eliminating overexpression artifacts. Despite their tremendous utility, each of these approaches still requires *a priori* knowledge of the postsynaptic NT receptor at the synapse of interest and careful functional validation, highlighting the growing need for general postsynaptic labels for when that information is not available, when commonplace labeling approaches for specific NT receptors fail, and when different receptor subtypes may be organized differently from one another.

General postsynaptic labeling approaches

Each neuron in the *Drosophila* CNS expresses approximately 22 NT receptors, complicating selection of the appropriate NT label between specific pre- and postsynaptic partners (Kondo *et al.* 2020). Moreover, for many neuronal classes and synapses of interest, the relevant NT and concomitant receptor are not known. To analyze those neurons and synapses, general postsynaptic labels are needed. A major strategy to examine general postsynapses in *Drosophila* has involved the dendritic marker system, DenMark (Nicolaï *et al.* 2010). DenMark is a general somatodendritic marker in all fly neurons consisting of a heterologously expressed, mCherry-labeled, mammalian ICAM5. ICAM5 has no obvious homology to any fly genes but localizes appropriately to dendritic membrane when expressed in specific cells under the control of a binary expression system (Nicolaï *et al.* 2010). Furthermore, ICAM5::mCherry overexpression in fly neurons has no deleterious effects, unlike prior dendritic labeling strategies using a specific isoform, *Dscam[17.1]*, of the *Dscam1* gene (Wang *et al.* 2004). When used in concert with Syt-1::EGFP as a presynaptic label, the combination of Syt and DenMark is very productive in mapping presumptive pre- and postsynaptic sites of novel neurons of interest (Nicolaï *et al.* 2010; Flood *et al.* 2013; Kennedy and Broadie 2018; Lamaze *et al.* 2018; Chen *et al.* 2019; Jung *et al.* 2020). However, it is not known if DenMark labels postsynaptic regions that are not in dendritic compartments, as might be observed with postsynaptic muscles or in mixed neurites that feature both pre- and postsynaptic specializations (e.g. Christiansen *et al.* 2011). Therefore, the use of DenMark as a truly general postsynaptic label remains unclear. Moreover, as DenMark labels the entirety of postsynaptic dendritic compartments and lacks the specificity of a marker that would label a postsynaptic specialization, it would not be suitable for studies that wish to assay detailed parameters of postsynaptic apparatus organization, including density and distribution. To circumvent the lack of subcellular specificity of a postsynaptic marker like DenMark, the CIDE-N protein Drep-2 (Andlauer *et al.* 2014) has more recently emerged as a postsynaptic label with notable utility. Drep-2 is expressed throughout the adult *Drosophila* brain specifically at glutamatergic synapses and enriched at the postsynaptic membrane where it colocalizes with glutamate receptors where it directly apposes presynaptic Brp-labeled AZs (Andlauer *et al.* 2014). Conditional expression of labeled Drep-2 yields clear punctate postsynaptic labeling (Andlauer *et al.* 2014) that facilitates quantitation of postsynaptic parameters including density and organization at glutamatergic synapses (Andlauer *et al.* 2014; Spühler *et al.* 2016; Fulterer *et al.* 2018; Tenedini *et al.* 2019; Pooryasin et al. 2021). In all, however, despite the utility of tools like DenMark and Drep-2, a more general label of postsynaptic specializations remains elusive.

The quest for comprehensive postsynaptic label options with cell-type specificity is ongoing. The immediate next goals for the field include development of general excitatory and inhibitory postsynaptic labels that have cell-type specificity and precise subcellular postsynaptic localization. However, despite a need for such tools, the currently available suite of pre- and postsynaptic labels, coupled with the many avenues for cell-type-specific expression of these tools, has allowed extensive exploration of normal synaptic development and organization in the fly brain. The facility with which synaptic organization can be assessed by light microscopy, using the synaptic tools we have discussed, allows for genetic dissection of the mechanisms that underlie the development, maturation, and plasticity of synaptic architecture.

Part II: Toward understanding the genetic basis of central synapse development and organization

The cell-type-specific expression of synaptic markers like Syt-1 enabled considerable advances in understanding synaptic biology, wiring, and neural circuit assembly through the last 20 years of *Drosophila* research (Venken, Simpson, *et al.* 2011). As the synaptic toolkit expanded, high-resolution studies of synaptic organization and development, specifically in the *Drosophila* CNS, became possible using the tools and methodologies detailed in Part I. In the last decade, Brp-based approaches have sprung to the forefront of *Drosophila* CNS research, contributing integral components of many pioneering studies. More recently, the development of additional synaptic labeling strategies beyond AZ machinery supplemented Brp-based assays, providing the most detailed view yet of synaptic cell biology across distinct regions in the fly brain. We focus on those studies in the second part of this review, as such studies have provided unprecedented information regarding the mechanisms of synapse formation, assembly, and organization.

The antennal lobe (AL)

Drosophila olfaction is a well-studied model system for understanding the molecular, genetic, and circuit concepts underlying learning and memory, neuronal organization and patterning, axon guidance, and behavioral coordination (Hummel and Rodrigues 2008; Wilson 2013; Grabe and Sachse 2018). In the fly olfactory system (Fig. 3), the AL is the first-order processing center for olfactory information and also more recently emerged as a powerful model (Fig. 3a–c) to investigate the mechanisms of synapse development and organization in central neurons (Mosca and Luo 2014; Coates *et al.* 2017; Mosca *et al.* 2017). The AL is

divided into distinct subregions called glomeruli that represent odorant information channels (Fig. 3b–d; Wilson 2013a). Early morphological studies of the AL mapped its glomerular architecture using the nc82 monoclonal antibody (Laissue *et al.* 1999), which recognizes Bruchpilot (Brp), the critical AZ scaffolding protein (Wagh *et al.* 2006). Brp localization revealed synapse dense neuropils within all AL glomeruli (Laissue *et al.* 1999) but monoclonal antibody staining alone was unable to discern key aspects of synaptic organization including how each class of AL neurons contributed to the general synaptic profile of each glomerulus, where those synapses localized in a cell-type-specific manner, and how synaptic organization varies over development or from neuron class to neuron class.

The discovery of *Drosophila* odorant receptors (ORs; Vosshall *et al.* 1999) and the subsequent mapping of their glomerular targets (Vosshall *et al.* 2000; Couto *et al.* 2005) enabled the creation of genetic reagents to directly manipulate genetically identifiable ORNs via binary expression systems (Vosshall *et al.* 2000). Subsequent analyses (Ito *et al.* 1998; Tanaka *et al.* 2008; Chou *et al.* 2010) expanded this technical repertoire to distinct classes of PNs and LNs, providing genetic access to most of the neurons that comprise each glomerulus (Fig. 3d). The combination of such genetic access along with new tools for cell-type-specific synaptic labeling (Fig. 2b) uniquely positioned the olfactory system as a powerful system for the genetic dissection of cell biological mechanisms involved in synapse formation and organization (Kremer *et al.* 2010; Christiansen *et al.* 2011; Mosca and Luo 2014).

Hallmark features of synaptic organization in the AL obey 3 rules

The *Drosophila* AL is comprised of ~50 glomeruli (Fig. 3b and c) that contain projections from ORNs, PNs, and LNs that synapse with each other (Fig. 3d; Hummel and Rodrigues 2008). Despite being an outstanding model for studying axon guidance and wiring decisions (Jefferis *et al.* 2002), synaptic studies lagged behind this progress due to the complexity and density of projections within glomeruli and the absence of synapse-specific labels for cell-type-specific study. The advent of tools like Brp-Short and genetic access to distinct classes of ORNs, PNs, and LNs finally enabled access to the AL for high-resolution synaptic study. Using a Brp-Short labeling strategy, Mosca and Luo (2014) studied synapse organization in the AL to determine how the mature synaptic landscape arose and what genes influenced formation and development.

Synapses from AL neurons follow a set of morphological and developmental rules. First, synaptic density in ORNs appears invariant across the AL. When synapse number was measured in multiple classes of ORNs that have sex-specific variability in glomerular volume, show similar glomerular volume across sexes, or are either responsible for sensing food-based odorants or pheromone-based odorants, the ORN synaptic density in each glomerulus was the same (~0.5 synapses/μm^3 of neurite volume). This occurred despite marked differences in glomerular volume, ORN neurite volume, and the aggregate total number of synapses made by ORNs in 5 different glomeruli (Mosca and Luo 2014). Thus, ORN synapse number scales with ORN neurite volume, which both scale with glomerular volume. Specifically, the volumes of the DA1 and VA1lm glomeruli are approximately 50–60% larger in males than in females (Stockinger *et al.* 2005) but despite males and females having different total synapse numbers as a result, synapse density scaling ensures that their density is identical (Mosca and Luo 2014; Grabe *et al.* 2016). Second, each individual ORN within a distinct class contributes an equal number of synapses to the aggregate average. The total number of synapses made by a class of ORNs represents the contributions of 20–25 cells: in such a scenario, each neuron can contribute an equal number of synapses, or there can be marked variation between cells, leading to "major" contributors and "minor" contributors. Using MARCM, Mosca and Luo (2014) examined small (1–4 cells) clones of DL4 and DM6 ORNs expressing Brp-Short-mStraw and a neurite mCD8-GFP marker. As clonal size increased, total synapse number in the clone increased quantally, indicating that each DL4 and DM6 ORN makes a similar number of synapses and each cell has the same synaptic density as the entire ORN population. This occurs despite differences in the absolute number of synapses made by each DL4 and DM6 ORN (19 and 29 Brp-Short puncta/ORN, respectively). These data indicate that a mechanism exists to ensure synapse number scales proportionately with neurite volume at the single cell and the ORN class level. Third, ORN, LN, and PN synapses exhibit distinct spatial organizational themes at glomerular and subglomerular scales. In the DA1 glomerulus, ORN and PN synapses are more generally distributed across the entire glomerulus, though each class has characteristic focal regions in the glomerulus that lack synapse and neurite labeling. These voids in ORN/PN synaptic labeling were instead largely filled by LN neurites (Hummel and Zipursky 2004) that were enriched with LN AZs (Mosca and Luo 2014). These LN synapses likely represent LN-LN and LN-PN synapses, It is likely that the LN-derived AZs that fill these voids represent synapses to other LNs or PNs though there is also some limited overlap between LN and ORN neurites, consistent with previously reported bidirectional signaling (Olsen *et al.* 2007; Root *et al.* 2007, 2008; Olsen and Wilson 2008; Chou *et al.* 2010; Huang *et al.* 2010; Yaksi and Wilson 2010; Wilson 2011, 2013; Kind *et al.* 2021). Quantitatively, each of the 3 neuronal classes (ORN, PN, and LN) exhibit distinct synaptic organization with respect to their own neurites. ORNs have the highest level of AZ clustering and the shortest mean distance between synapses. PNs show a slightly larger mean distance between synapses while LNs provide the most space between their connections. With regards to clustering, LNs follow closely behind ORNs while PNs have a clustered percentage of nearly half that of LNs. These indicate that there are additional mechanisms to control the precise 3D spatial organization of synapses in each class of AL neurons. Recent connectomics work showed that ~75% of ORN output is split evenly between downstream LNs and PNs (Schlegel *et al.* 2021). Considering that LN distribution in the DA1 glomerulus is quite limited, it is tempting to speculate that local Brp clustering is exploited to increase ORN:LN connectivity given these spatial restrictions. Overall, genetically encoded synaptic labeling through Brp-Short suggests that distinct rules exist to govern qualitative and quantitative synaptic organization in the component neurons of the AL.

The rule-breakers: Teneurin and LRP4 are required to maintain invariant synaptic density

If distinct rules exist to regulate synapse density and synaptic organization, this suggests that there must be mechanisms to enforce those rules, ensuring normal development of the synapse and proper circuit function. What is the nature of these rules? Are they genetic? Activity-dependent? Are they general modes for synapse formation or do they function similarly in multiple synapses? Moreover, are the rules generalizable across multiple types of synapses or does each system follow its own set of

developmental rules? The field is in the early stages of addressing these questions. Thus far, 2 main signaling systems that alter synaptic density in the AL have been identified: the Teneurin and LRP4 signaling systems.

The Teneurins represent a conserved family of transmembrane proteins with defined roles in synaptic partner matching and synaptogenesis in *Drosophila* (Hong *et al.* 2012; Mosca *et al.* 2012; Mosca 2015; DePew *et al.* 2019) and mammalian systems (Chand *et al.* 2013; Woelfle *et al.* 2015, 2016; Berns *et al.* 2018; Sando *et al.* 2019; del Toro *et al.* 2020; Pederick *et al.* 2021). In the *Drosophila* AL, *ten-a* and *ten-m* are expressed at a basal level in all glomeruli. In select glomeruli, elevated levels during development are responsible for partner matching in select ORN–PN pairs (Hong *et al.* 2012). At all glomerular synapses, though, heterophilic transsynaptic interactions (Fig. 3e) maintain normal ORN::PN synapse numbers (Mosca and Luo 2014). Specific perturbation of presynaptic ORN *ten-a* or postsynaptic PN *ten-m* resulted in a ∼25% decrease in AZs or NT receptor clusters visualized cell-autonomously using Brp-Short::mStrawberry and Dα7::GFP, respectively. Further underscoring the importance of this transsynaptic pair, postsynaptic PN *ten-m* knockdown non cell-autonomously impaired presynaptic ORN synapse number, suggesting that PN Ten-m is the valid postsynaptic partner required for synaptic interaction. Taken together, this work shows that ORN Ten-a and PN Ten-m form a transsynaptic pair that regulates synapse organization, like its role at the NMJ (Mosca *et al.* 2012). Ultrastructurally, Ten-a is also required for normal AZ morphology: in *ten-a* mutants, nearly ∼50% of AZs are impaired, revealing misshapen, detached, or otherwise abnormal T-bar structures (Mosca and Luo 2014). Consistent with AZ defects, olfactory behavior in response to attractive odorants is notably impaired in *ten-a* mutants (DePew *et al.* 2019). The mechanisms through which *ten-a* and *ten-m* instruct synaptic assembly across synaptic partners are not well understood (Mosca 2015) though candidate effectors are beginning to emerge. In ORNs, *ten-a* functions by regulating levels of α- and β-spectrin in the AL (Mosca and Luo 2014), consistent with known roles for Teneurin proteins in cytoskeletal regulation (Nunes *et al.* 2005; Mörck *et al.* 2010; Mosca *et al.* 2012; Suzuki *et al.* 2014), which in turn regulates AZ number (Mosca and Luo 2014). Though *ten-a* and *spectrin* function in the same genetic pathway to support presynaptic AZ assembly in ORNs, they do not account for all Teneurin function at central synapses. Future work will be needed to understand how transsynaptic Ten-a: Ten-m signaling influences spectrin organization at the synapse, how spectrin organization specifically facilitates normal synaptogenesis, and what other downstream interactors function with the Teneurins to regulate synaptic organization.

A second cell surface receptor, LRP4, functions as another enforcer of the synaptic density rule in the fly brain. LRP4 is best known for its role as a postsynaptic organizer at the mouse NMJ where it functions as the receptor for Agrin, an essential synaptogenic signal secreted from presynaptic motoneurons (McMahan 1990; DeChiara *et al.* 1996; Gautam *et al.* 1996; Hopf and Hoch 1998; Weatherbee *et al.* 2006; Kim *et al.* 2008; Zhang *et al.* 2008; DePew and Mosca 2021). The *Drosophila* LRP4 homolog is expressed broadly throughout the brain and localizes to AZs and peri-AZs at axon terminals (Mosca *et al.* 2017). LRP4 is enriched in excitatory neurons (cholinergic and glutamatergic) but is scarcely expressed in inhibitory GABAergic neurons; consistent with this, loss of *lrp4* in ORNs results in a 35% reduction in excitatory synapse number (as measured by both ORN Brp-Short and PN Dα7-GFP assays) in the AL and the MB but does not alter inhibitory synapse number in either the AL or the lateral horn. LRP4 thus functions presynaptically and cell-autonomously to regulate synapse organization. As with the Teneurins, loss of *lrp4* results in ultrastructural impairments to most AZs and in the near-complete loss of odorant attractive behavior (Mosca *et al.* 2017). In all cells (excitatory and inhibitory), however, LRP4 overexpression increases synapse number, suggesting (1) that LRP4 plays an instructive role in synapse organization and (2) all neurons share a core downstream machinery necessary for LRP4 to instruct synapse formation. Such a core pathway may be responsive to multiple upstream activating inputs, of which LRP4 is one. Downstream, however, LRP4 functions via SRPK79D, a serine-arginine protein kinase to regulate synapse organization (Mosca *et al.* 2017). LRP4 is required for the proper synaptic localization of SRPK79D and the 2 proteins colocalize at synapses. Moreover, loss of SRPK79D phenocopies the loss of *lrp4* and expression of an activated SRPK79D can suppress the synaptic defects of an *lrp4* mutant (Mosca *et al.* 2017). Resembling the relationship between Teneurin and Spectrin, it is unlikely that SRPK79D is the only downstream effector of LRP4 at the synapse. Future work will further explore the interaction between LRP4 and SRPK79D and identify additional downstream effectors. Importantly, though, the analyses of the Teneurins and LRP4 establish the first major players in olfactory synapse organization in *Drosophila*, highlight their downstream mechanisms, and establish the AL as a model synapse for assessing cell-autonomous and non cell-autonomous factors in central synaptogenesis in *Drosophila*.

The mushroom body (MB)

In the *Drosophila* brain, olfactory information that is processed in the AL is next conveyed to higher olfactory centers including the MB and the lateral horn (Fig. 3, a, b, f, and g). In many insect species, associative learning takes place in the MB (reviewed in Schürmann 2016; Modi *et al.* 2020), making it analogous to the vertebrate hippocampus and cerebellum (Davis and Han 1996; Elkahlah *et al.* 2020; Scaplen *et al.* 2021). The MB is made up of ∼2,000 intrinsic neurons called KCs whose organization gives rise to the 3 main anatomical features that characterize the fly MB (Aso *et al.* 2014). Though the MB receives inputs from multiple sensory modalities (Kirkhart and Scott 2015; Li, Lindsey, *et al.* 2020; Li, Mahoney, *et al.* 2020; Schlegel *et al.* 2021), the majority of KC inputs are made by olfactory PNs from the AL onto KC dendrites in the calyx (Fig. 3g–g″). KC axons are then bundled in parallel and project out from the calyx, giving rise to the peduncle (or stalk) and the lobes of the MB (Technau and Heisenberg 1982; Ito *et al.* 1997; Kunz *et al.* 2012). At this point, KCs synapse onto MB output neurons and receive modulatory inputs from dopaminergic neurons, forming an intricate synaptic network (Li, Lindsey, et al. 2020). Recent studies in this circuit have made significant inroads into understanding physiological responses to sensory input (Kremer *et al.* 2010; Cohn *et al.* 2015; Pech *et al.* 2015; Sugie *et al.* 2018; Bilz *et al.* 2020) and behavioral output (Modi *et al.* 2020; Li, Lindsey, et al. 2020; Scaplen *et al.* 2021) that are beyond the scope of this review. However, the molecular aspects of synaptic architecture and organization in the calyx and how they are influenced by genetic perturbations of activity are beginning to be understood, using genetically encoded synaptic labels (Leiss, Groh, *et al.*, 2009; Kremer *et al.*, 2010; Christiansen *et al.*, 2011).

A survey of presynaptic AZs in MB KCs identified unexpected synaptic organization

The classical view of synaptic organization posits that presynaptic specializations reside exclusively in axonal neurites while postsynaptic specializations reside in dendritic neurites.

However, it is increasingly appreciated that in many cells, especially in sensory systems, pre- and postsynaptic specializations can both reside in the same neurite (Carden and Bickford 2002; Chou *et al.* 2010; Grimes *et al.* 2010; Mosca and Luo 2014; Morgan and Lichtman 2020). However, the function of such dendritic presynapses as well as how they are organized with respect to other, more classical, synaptic contacts from other neurons, are not well understood. In the MB, axon terminals from olfactory PNs (whose dendrites terminate in the AL) innervate the calyxRe (Leiss, Groh *et al.* 2009; Butcher *et al.* 2012), concentrating their presynapses in structurally defined neuropils, termed microglomeruli (Fig. 3g''). Conditional expression of Brp-short::GFP in KCs, however, revealed dendritic presynaptic AZs (Christiansen *et al.* 2011), termed KCACs (KC-derived AZs residing in the calyx). KCACs are functional and account for ~20% of the AZs that reside in the calyx (Ng *et al.* 2002; Owald *et al.* 2010; Christiansen *et al.* 2011). KCACs are enriched in a pattern complementary to the incoming PN projections, outside of the defined microglomeruli (Christiansen *et al.* 2011). Using MARCM analysis of single neurons, Christiansen *et al.* (2011) showed that expression of Brp-Short accumulates outside of the morphologically distinct, claw-like, neurite termini where Dα7 is enriched. Dendritic claws form part of the microglomeruli and surround the PN axons with which they synapse (Fig. 3g''). KCACs on the other hand were rarely observed at these claw-like specializations, indicating that mechanisms must exist to exclude or otherwise prevent the spatial overlap of pre- and postsynapses in the calyx, despite them residing in the same neurite. The genetic and/or cellular requirements that define and enforce the calycal microglomerular architecture, spatially segregate pre- and postsynaptic specializations in KC dendrites or regulate key synaptic parameters such as the number of synapses formed by KCs are not well understood. Recent work, though, is beginning to shed light on mechanisms governing the more canonical PN to KC synapses (see below). It will be intriguing to determine whether manipulations that affect PN to KC synapses affect KCAC features such as their subcellular distribution or their total numbers.

Presynaptic activity influences microglomerular architecture at the MB calyx

How are input synapses from PNs to the MB calyces organized? What cellular processes influence the sparse wiring of MB circuits and the microglomerular organization of the MB? Work from Kremer *et al.* (2010) first indicated a role for neuronal activity in regulating microglomerular and synaptic organization in olfactory PNs and MB KCs. When the PN inputs to the MB are electrically silenced using $K_{IR}2.1$ (Nitabach *et al.* 2002), both the number of microglomeruli and Brp-Short labeled AZs are increased (Kremer *et al.* 2010). Concomitantly, the relative size of the microglomeruli measured by postsynaptic Dα7::GFP localization in KCs also increased. This suggests that the MB as a system responds to decreased input by increasing the number and size of synaptic regions as a compensatory mechanism. Further compensatory mechanisms exist at the level of PN boutons onto KCs. More recent work showed that PNs scale bouton number to the number of KCs present (Elkahlah *et al.* 2020) and individual PNs make fewer boutons onto KCs when there are more PNs present. This suggests that there is a distinct presynaptic plasticity that influences connectivity while the postsynaptic regions set by the KCs are reasonably fixed (Elkahlah *et al.* 2020). Intriguingly, though, ablation of 50% of PNs using diphtheria toxin did not alter the number of KC microglomeruli, in contrast to electrical silencing (Kremer *et al.* 2010; Elkahlah *et al.* 2020). Future work is needed to resolve this apparent discrepancy. It could indicate the combination of activity-dependent and activity-independent processes that rely more directly on cell number as a checkpoint for synaptic development and organization. A tempting hypothesis is that functional connections that achieve a certain threshold (i.e. are not impaired by electrical silencing) between PNs and KCs are required for PNs activity to have a non cell-autonomous effect on KCs postsynaptic structure. It will be illuminating to determine if PN electrical activity influences the function of synaptogenic regulators in the CNS. Moreover, recent work has shown that long-term memory consolidation alters circuit organization at the MB calyx, whereby additional microglomeruli form (Baltruschat *et al.* 2021); this finding suggests that classical genes involved in learning and memory may also function to organize synaptic architecture. Synaptic reorganization is also reminiscent of the circadian regulation of neurite projections and AZ material in clock neurons like the LNvs (Gorostiza *et al.* 2014; Petsakou *et al.* 2015; Fernandez *et al.* 2020) though the specific mechanisms related to adjustment of AZs are not well understood. Furthermore, levels of Brp and other AZ components in the fly brain cycle along with sleep and wakefulness states (Gilestro *et al.* 2009; Huang *et al.* 2020; Weiss and Donlea 2021) along with circadian rhythms, with further implications for how this mechanism influences MB learning (Weiss and Donlea 2021). By combining high-resolution imaging, behavioral studies, and the exquisite genetic access provided in *Drosophila*, genetically encoded synaptic labels are poised to open a new forefront of determining mechanisms underlying synaptic organization.

The visual system

Beyond the olfactory system, the *Drosophila* visual system has also emerged as a highly tractable and advantageous model for studying mechanisms of synaptogenesis, owing largely to a series of facets. First, a wealth of driver lines are available that allow direct genetic manipulation of small populations of specific neurons with identified roles or neuronal classes in the visual circuit (Meinertzhagen and Sorra 2001; Morante and Desplan 2008; Jenett *et al.* 2012; Nern *et al.* 2015; Wu *et al.* 2017; Davis *et al.* 2020; Scheffer *et al.* 2020). Second, *Drosophila* has a rich repertoire of visually evoked behaviors to test synaptic function and an array of technologies for examining the in vivo physiology and response of diverse visual neurons. Third, there is a wealth of information about visual system structure (Fig. 4): the compound eye of *Drosophila melanogaster* consists of 700–800 ommatidia (Kumar 2012), each containing 8 photoreceptor neurons, which send projections into the optic lobe, where 4 main neuropil reside [Fig. 4a'; lamina, medulla, lobula, and lobula plate; reviewed in Nériec and Desplan (2016)], providing unique stereotypy to examine. Fourth, for many neuronal classes in the optic lobe, including photoreceptors which we focus on here (Fig. 4a'–c), key synaptic parameters including the aggregate number of synapses and their spatial organization are known from EM reconstructions and light microscopy studies (Meinertzhagen and Sorra 2001; Takemura *et al.* 2008, 2013; Rivera-Alba *et al.* 2011; Berger-Müller *et al.* 2013; Chen *et al.* 2014). Coupled with genetically encoded synaptic labels, and in recent years, genetic analyses using light microscopy, the advantages of the visual system have begun to uncover the developmental features and genetic regulation underlying synaptogenesis.

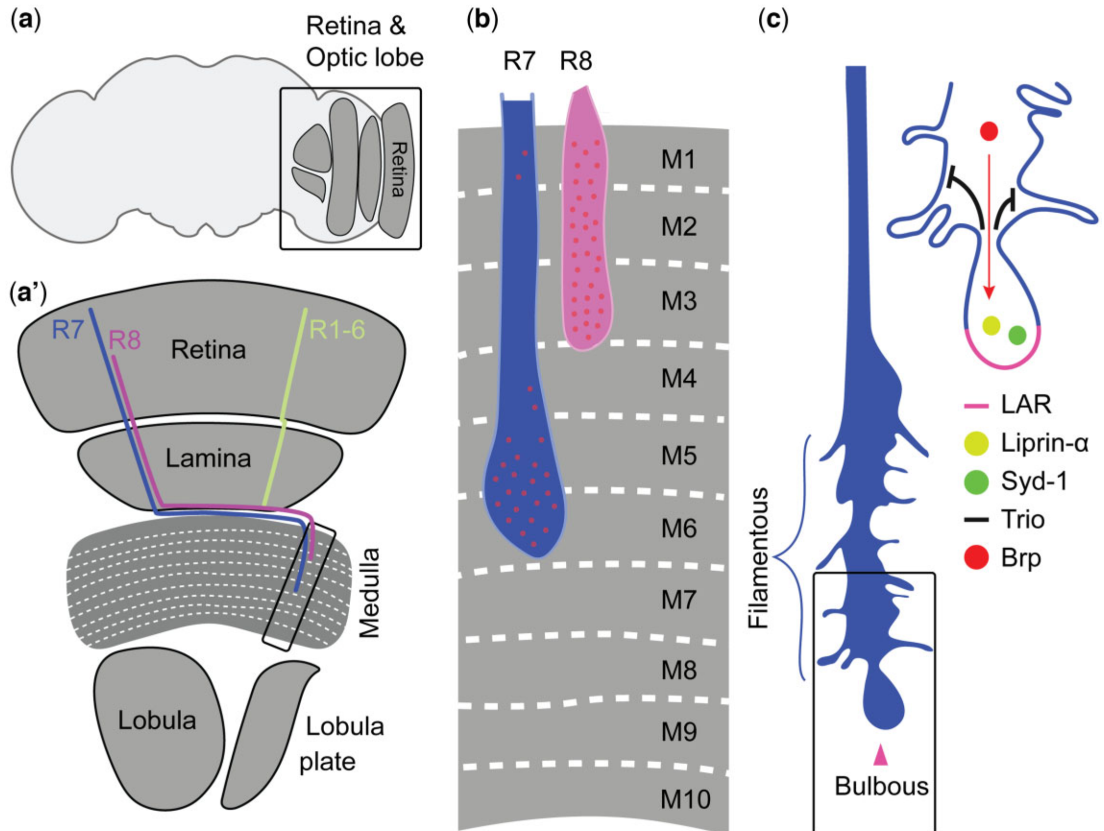

Fig. 4. The *Drosophila* visual system exhibits stereotyped connectivity and synaptic organization. a) Schematic of the *Drosophila* brain. Boxed region corresponds to the retina and optic lobe diagrammed in (a'). a') Schematic of the 4 main optic lobe neuropils [figure panel modeled after; Nériec and Desplan (2016)]. R1–R6 photoreceptors innervate the lamina. R7 and R8 photoreceptor neurons innervate distinct layers in the medulla and form synaptic connections with different postsynaptic targets (not diagrammed). Boxed region in the medulla is enlarged in (b). b) Photoreceptor neurons exhibit class-specific synaptic numbers and organization [figure panel based on Berger-Müller *et al.* (2013) and Chen *et al.* (2014)]. R8 photoreceptors assemble approximately twice as many AZs in the medulla (~50) as R7 photoreceptors (~25). Moreover, synaptic organization is distinctly different in these 2 neuronal classes. In R8 photoreceptors, presynaptic AZs are distributed uniformly along the axonal terminal whereas, in R7 photoreceptors, they are concentrated near the distal tip of the axonal terminal. c) Synaptic addition in R7 photoreceptors takes place in a stepwise fashion, at bulbous filopodia (arrowhead), which are morphologically distinct from filamentous filopodia [figure panel based on Özel *et al.* (2019)]. In the current model, the cell surface receptor LAR initiates formation of bulbous filopodia, likely through local attachment at the presumptive synaptic site. Recruitment of the synaptic seeding factors, Liprin-α and Syd-1, stabilize the bulb while the RhoGEF Trio antagonizes formation of supernumerary bulbous filopodia. This pathway ensures that no more than 1–2 bulbous filopodia are formed throughout the synaptogenic period, limiting the number of partners that are competent to form a connection. Bulbous filopodia are long-lived (>8 min), but eventually retract. Following retraction, Brp is recruited to the nascent synapse where Liprin-α and Syd-1 reside.

Asynchronous pre- and postsynaptic assembly in photoreceptors and their postsynaptic targets

In the fly eye (Fig. 4), each ommatidium contains 8 photoreceptor neurons that comprise one of 2 general categories. The outer 6 photoreceptors (R1–R6) are specialized for motion detection and innervate the lamina, while the inner photoreceptors (R7 and R8) are specialized for color vision and innervate the medulla (Kumar 2012). The STaR method for labeling pre- and postsynapses was initially optimized for *Drosophila* photoreceptor synapses (Chen *et al.* 2014) and has contributed greatly to our understanding of first, how synapses in the visual system form and organize, and second, what genetic mechanisms promote synapse formation. The concurrent labeling of pre- and postsynapses first enabled a developmental analysis of synaptic assembly in photoreceptors (R1–R6) and their postsynaptic partners, the L3 lamina neurons (Chen *et al.* 2014). At the photoreceptor::laminar synapse, Brp-FL::V5 puncta begin accumulating at 40 h after puparial formation (APF) and continue to the end of pupal development at 100 h APF. This predates postsynaptic accumulation, as OLLAS-Ort puncta are not visible until 77 h APF. This reveals that the development of pre- and postsynaptic specializations in the visual system is asynchronous and likely to be determined and driven by photoreceptor neurons. Intriguingly, time course studies suggest that visual system synapse formation may differ from the normal progression of synaptogenesis at the *Drosophila* NMJ (Rasse *et al.* 2005; Fouquet *et al.* 2009; Owald *et al.* 2010). However, it remains possible that postsynaptic assembly (as measured by other postsynaptic adaptors or seeding factors) may begin concomitantly with Brp accumulation while receptor recruitment is delayed. The expansion of techniques like STaR and indeed, of all genetically encoded synaptic labels, into additional, more general postsynaptic labels will be needed to differentiate between these possibilities and provide a more precise and elaborate delineation of the temporal dynamics of synaptic development.

Genetically encoded AZ labels allow live tracking of synaptogenesis in developing photoreceptors

In *Drosophila* photoreceptors, axon growth and synaptogenesis occur during overlapping time periods and require extensive, stochastic formation and retraction of filopodial cellular extensions

(Langen *et al.* 2015; Özel *et al.* 2015). However, whether these filopodia play a direct role in synapse formation remained unclear (Özel *et al.* 2015, 2019; Nériec and Desplan 2016). Work from the Hiesinger and Altschuler labs developed revolutionary time-lapse intravital imaging of pupae to study growth cone morphology and dynamics during synaptic development (Langen *et al.* 2015). This work demonstrated that a set of simple rules governed the organization of R1–R6 axon terminals in the lamina during development ensuring that axon sorting and target recognition occurred without fault. Time-lapse imaging of R7 photoreceptor cells in intact pupal brains also revealed previously unappreciated filopodial features, suggesting an instructive role in synapse assembly following target recognition (Langen *et al.* 2015; Nériec and Desplan 2016; Özel *et al.* 2019). By combining intravital imaging along with multiple genetically encoded synaptic labels, Özel *et al.* (2019) found that 1 or 2 long-lived filopodia with characteristic bulbous tips are always present during the period of bulk synaptic addition (Fig. 4c). Notably, GFP-tagged Liprin-α and Syd-1 accumulate in these bulbous filopodia, but never in the more numerous, short-lived, filamentous filopodia. In the absence of either *syd-1* or *liprin-α* from the bulbous filopodia, R7 photoreceptor synaptogenesis is impaired, ultimately resulting in fewer Brp-Short labeled AZs accumulating (Özel *et al.* 2019). This is consistent with roles for Syd-1 and Liprin-α as synaptic seeding factors in a pathway that also involves LAR and Trio signaling (Dai *et al.* 2006; Astigarraga *et al.* 2010; Owald *et al.* 2010; Holbrook *et al.* 2012; Hakeda-Suzuki *et al.* 2017). Similarly, perturbation of *syd-1*, *liprin-α*, *lar*, or *Trio* alters filopodial dynamics and influences synapse organization. The current model proposes that *lar* initiates bulbous filopodial formation, which is then stabilized by synaptic seeding factors *syd-1* and *liprin-α* while *trio* suppresses assembly of additional bulbs. As a result, throughout the period of synaptic addition, a maximum of 1–2 long-lived (>40 min) synaptogenic bulbous filopodia are present at any given time (Fig. 4c). This dynamic process of serial synaptic addition restricts the number of synapses that can be formed during the normal developmental window to ∼25 synaptic AZs in R7 photoreceptor neurons and limits potential targets, suggesting that a temporal model of availability governs synaptic organization. Together with work from Chen *et al.* (2014), these studies provide a description of the developmental window within which synapses are assembled in *Drosophila* photoreceptors and a model of serial synaptic addition for how the aggregate number of AZs per photoreceptor is determined. Beyond the temporal parameters of synaptogenesis, there are several remaining aspects that remain active areas of research, including the spatial specificity of synapse formation and the mechanisms that regulate selectivity of synaptic partners in 3D space.

Neuronal mistargeting affects synaptic development in photoreceptors

Neuronal circuit assembly relies on precise matching between pre- and postsynaptic partners and formation of the correct number of synapses onto precise, subcellular locations. These 2 steps are separated temporally and while the molecular players that regulate each step can overlap (Hong *et al.* 2012; Mosca *et al.* 2012; Mosca and Luo 2014), the extent to which partner matching instructs synaptic organization remains poorly understood. In the visual system, photoreceptor classes are readily identified based on their terminal morphology; for example, R7 and R8 photoreceptor neurons both innervate the medulla, but terminate at distinct layers and synapse with distinct postsynaptic partners (Kazama and Wilson 2008; Takemura *et al.* 2008, 2013; Courgeon and Desplan 2019): R7 spans medulla layers M1-6, while R8 spans layers M1-3 (Fig. 4a'–b). Considerable work has revealed diverse molecular determinants of layer specificity in the visual system, providing methods to adjust where different neurons project (Pecot *et al.* 2013; Tan *et al.* 2015; Akin and Zipursky 2016; Peng *et al.* 2018; Xu *et al.* 2019; Sanes and Zipursky 2020; Santiago *et al.* 2021). Using overexpression of cell surface receptors to influence targeting and simultaneously quantifying synapses with Brp-Short, multiple studies have mistargeted photoreceptor neurons to ectopic medullary layers to determine how altered targeting influences synaptic organization.

The *capricious* gene encodes Caps, a leucine-rich repeat-containing protein that is necessary for correct target selection at both peripheral and central *Drosophila* synapses (Shishido *et al.* 1998; Hong *et al.* 2009). In the visual system, pan-neural expression of Caps redirects R7 terminals to the M3 layer (Berger-Müller *et al.* 2013). Despite the ectopic R7 targeting, synaptic organization remains largely unchanged (Takemura *et al.* 2008, 2013; Berger-Müller *et al.* 2013; Chen *et al.* 2014): R7 neurons still assemble their characteristic number of AZs (∼25) and do so distally in the axon terminal. These features are distinct from R8 photoreceptors which normally target the M3 layer. R8 photoreceptors assemble ∼50 AZs and distribute them evenly along the length of the axon shaft. Thus, despite incorrect targeting, the synaptic complement made by those R7 cells remains unaffected, suggesting that synaptic partner matching and synaptic organization may not be obligately linked (Berger-Müller *et al.* 2013). There may, however, be notable exceptions. When R7 is mistargeted to M3 by a different method—overexpression of the transmembrane proteins Golden Goal (Gogo) and Flamingo (Fmi), which are both required for normal R8 targeting to M3 (Hakeda-Suzuki *et al.* 2011)—R7 photoreceptors form the correct number of synapse (∼25) but these AZs are evenly distributed along the axon shaft rather at the axon terminal. This instead suggests that some manipulations can change synaptic organization and wiring simultaneously. This may be further influenced by proteins like DIP-α, which functions through the Dpr6 and Dpr10 receptors to regulate neural circuit assembly, and with it, synapse number and distribution (Xu *et al.* 2018). The complete mechanisms underlying these distinct effects on synaptic organization, in response to mistargeting, remain unknown. Taken together, though, these results indicate that synaptic organizational themes can be regulated independently of synaptic number and axon guidance or partner matching but may rely on the specific expression of cell surface proteins in that class of neurons. Many open questions remain, however, including the extent to which parameters such as synapse number or synapse organization are determined cell-autonomously, through direct cell–cell interactions, or via interactions with the local cellular environment as neurons develop. Also, what are the roles of large cell-surface families like the DIPs and Dprs (Carrillo *et al.* 2015; Tan *et al.* 2015; Cosmanescu *et al.* 2018; Xu *et al.* 2018, 2019) in regulating synapse organization and wiring development? How DIPs and Dprs influence synaptic partner selection and competency for synaptogenesis will likely influence the formation of AZs both in their dynamics as well as their spatial locations. This is an active area of study that suggests the existence of multiple pathways acting in parallel to ensure appropriate partner selection and synapse formation (Xu *et al.* 2019). Finally, whether molecular codes contribute the bulk of synaptic specificity or if synapse formation and selectivity are more linked to developmental timing and availability remains to be more deeply understood.

Activity-dependent synaptic plasticity

Beyond synapse formation in the visual system, the link between stimuli and synaptic organization is still incompletely understood. Do plastic responses to environmental stimuli rely on the same machinery that developing neurons employ to assemble synapses? How are these processes intertwined and can they influence each other? When flies are exposed to a constant light exposure schedule (LL) and synaptic organization is examined using Brp-Short, the number of AZs and T-bar structures are significantly decreased in R8 photoreceptor cells (Böhme and Sigrist 2015; Sugie *et al.* 2015). The reduction in presynaptic AZs observed under LL conditions requires synaptic transmission between R8 cells and their Ort-positive postsynaptic partners and normal excitability of either cell (Sugie *et al.* 2015). Liprin-α and RBP (but not Cacophony or Syd-1) also dissociate from the AZ upon prolonged light exposure. Changes in synaptic organization under LL conditions are reversible as normal Brp-short puncta numbers are restored when flies are reared under constant darkness (DD) or 12-h light/dark cycling (LD). How environmental exposure influences rapid changes in AZ assembly and synaptic organization remains unknown. It is possible that retention of Syd-1 and Cacophony at the synaptic membrane may facilitate AZ reassembly under permissive conditions (DD and LD), but future work will be needed to determine whether AZ are assembled de novo or if they re-assemble at Syd-1/Cacophony footprints. What remains clear is that (1) the molecular composition of mature R8 AZs can be modulated by light stimulation and (2) Ort-positive postsynaptic cells can signal to R8 cells, in an activity-dependent manner, to trigger AZ disassembly in those R8 cells. By combining genetically encoded synaptic labels with different stimuli, these findings raise the possibility that signaling mechanisms exist that acutely, and non cell autonomously, control synaptic numbers and output. What molecular mechanisms may exist to regulate synapse disassembly? Recent work identified Wnt signaling by the *Drosophila* Wnt1 homolog Wingless as a key mediator of visual synaptic plasticity, opening the door to mechanistic studies of synaptic remodeling postdevelopment. Components of the divergent canonical Wnt signaling pathway (Ciani *et al.* 2004; Salinas 2007; Miech *et al.* 2008) are required for activity-dependent AZ remodeling in R8 photoreceptors (Sugie *et al.* 2015; Kawamura *et al.* 2021). In wild-type animals reared under LL conditions, prolonged light exposure leads to Wingless pathway inactivation through endocytosis and autophagic degradation of the Wingless ligand (Kawamura *et al.* 2021) and to a reduction in AZ numbers. Ectopic activation of the Wingless pathway on the other hand, also under LL conditions, prevents the activity-dependent reduction in AZs (Kawamura *et al.* 2021). This highlights a role for Wingless signaling in the activity-dependent modulation of synaptic organization, but additional Wingless-dependent and Wingless-independent roles likely remain. Wingless pathway inactivation under LD conditions decreases Brp-Short puncta, suggesting that AZ maintenance may also require Wingless. Intriguingly, however, ectopic activation or electrical silencing of Ort-positive cells has no effect on Wingless accumulation or endocytosis in R8 photoreceptors, suggesting the feedback signaling by Ort-positive neurons may be Wingless-independent. Future work will be needed to further dissect how postsynaptic feedback influences AZ remodeling in the visual system, whether Wingless-dependent phases of synaptic remodeling occur by the phosphorylation of known downstream Wingless pathway members including Futsch/MAP1b and Shaggy/GSK3β (Gögel *et al.* 2006), and how multiple signals are integrated to ensure mature synaptic organization. The visual system provides, however, another outstanding model central synapse in the fly brain, coupled with genetically encoded synaptic labels, to understand how synapses form, function, and organize.

The dorsocentral mechanosensory neurons (dcMSNs)

The MSNs that innervate the large dorsocentral thoracic bristles of adult *Drosophila* are highly genetically accessible and represent a fourth powerful fly synapse with unique morphology to investigate the cell biological mechanisms of synaptic development. MSNs have large axonal projections, which contribute to their accessibility and form stereotyped, highly branched arborizations that target distinct areas and postsynaptic cells in the ventral nerve cord (Fig. 5a). Importantly, MSNs exhibit exquisite spatially distinct synaptic organization across their collateral branches, allowing them to serve as a unique locale for examining synapse specificity as well as formation and development (Alsina *et al.* 2001; Urwyler *et al.* 2019). MSNs extend 3 primary axon collaterals that project anteriorly, contralaterally, or posteriorly; each collateral exhibits a characteristic synaptic organization. Visualization of AZs using the genetically encoded labels Brp-Short::GFP, mCherry::Syt1, and Cacophony::GFP showed that each of these axonal compartments of MSNs varies according to local synaptic concentration (Kawasaki *et al.* 2004; Fouquet *et al.* 2009; Urwyler *et al.* 2015). The contralateral projections are highly synaptogenic and form synapse-dense terminal arbors (Urwyler *et al.* 2015, 2019). In contrast, anterior and posterior collaterals have fewer synapses and display distinct synaptic organization. Whereas the anterior branch assembles synapses along the entirety of its length, the posterior branch is largely devoid of synapses, except at 4 stereotypically spaced foci that are synapse dense (Urwyler *et al.* 2015, 2019). When genetically encoded labels for synapses are combined with single-cell analysis tools like MARCM and FLP-out, this enables high resolution of individual dcMSNs and as such, the dcMSNs are ideally suited to investigate mechanisms that control synaptic organization with precise subcellular specificity at the level of individual axonal branches.

AZ assembly and synaptic development in dorsocentral MSNs

In motoneurons, the *liprin-α/syd-1/lar* pathway functions in multiple intertwined steps of synapse development. Perturbation of any pathway components results in decreased axonal branching, aberrant T-bar organization, altered Brp distribution, and compromised recruitment of synaptic vesicles (Kaufmann *et al.* 2002; Owald *et al.* 2010; Li *et al.* 2014). How this may influence central synapse formation remains less clear. In the visual system, the *liprin-α/syd-1/lar* pathway regulates synapse formation by localizing to bulbous filopodia and ensuring their competence to form connections (Özel *et al.* 2015, 2019). In the adult MSNs, however, RNAi against or mutation of members of the *liprin-α/syd-1/lar* pathway did not affect Brp-Short accumulation, suggesting that AZ assembly was unaffected (Urwyler *et al.* 2015). Intriguingly, though, perturbation of the pathway resulted in redistribution of the synaptic vesicle marker Syt-1. Syt-1 is normally localized along the axonal branch but in mutants of the *liprin-α/syd-1/lar* pathway, it instead accumulated at distal axon termini (Urwyler *et al.* 2015). This indicates that the *liprin-α/syd-1/lar* pathway influences synaptic vesicle recruitment to presynaptic AZs but is dispensable for the actual assembly of AZs. This provides a striking contrast to its role in developing photoreceptor neurons and

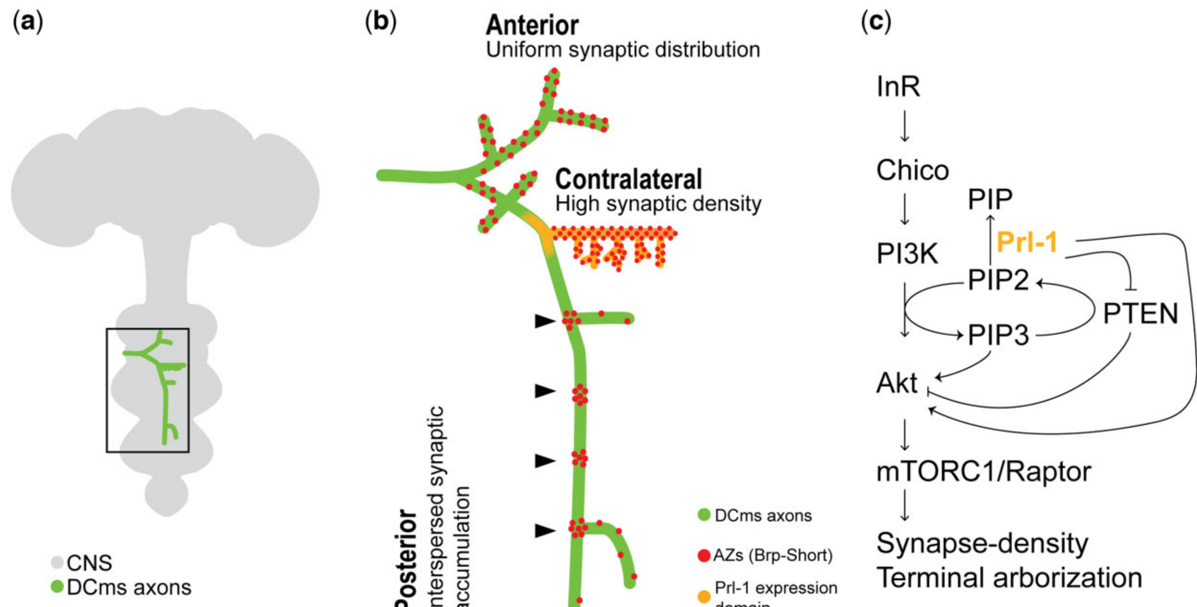

Fig. 5. Branch-restricted regulation of synaptic organization in MSNs. a) Schematic of the *Drosophila* adult CNS. A single dorsocentral MSN (in box) is diagrammed in the thoracic ganglia [figure panel modeled after Chen *et al.* (2006)]. b) MSNs exhibit a highly stereotyped branching pattern with distinct synaptic organization: the anterior branches exhibit uniformly distributed synapses while the posterior branches exhibit interspersed areas of high synaptic density [black arrowheads; figure adapted from Urwyler *et al.* (2015, 2019)]. The contralateral branch is unique in that it contains high synaptic density throughout the entire length of that specific branch. Local enrichment of the membrane-anchored phosphatase, Prl-1 (orange), in the contralateral branch is required for normal synaptogenesis, terminal arborization, and this increased local synaptic density. c) Prl-1 controls synapse formation in the contralateral branch of MSNs by antagonizing PTEN function and synergizing with the InR-Akt signaling pathway (Urwyler *et al.* 2019).

motoneurons (Owald *et al.* 2010; Özel *et al.* 2019). When taken together, these data demonstrate that while multiple types of synapses (peripheral and central) employ shared synaptogenic pathways, considerable functional divergence is evident across distinct neuronal classes. The details of how these pathways are modulated with cell-type specificity to yield different synaptic organization remain unclear but underscore the importance of comparative studies to both illuminate and investigate these differences. It is likely that these different synapses engage distinct regulatory mechanisms, effector molecules, or distinct combinations of synaptogenic pathways to achieve cell-type-specific synaptic organization.

Synapse specificity in dorsocentral MSN branches

The contralateral branches of the MSNs have a high density of AZs compared to its other collateral branches (Urwyler *et al.* 2015) that feature fewer presynapses and distinct synaptic organization (uniform vs sparse; Fig. 5b). In an RNAi knockdown screen targeting the fly kinome and phosphatome, Urwyler *et al.* (2019) identified the membrane-anchored phosphatase, Prl-1 (Phosphatase of regenerating liver-1), as an branch restricted factor that specifically controls synaptic density in the contralateral branch of dcMSNs (Urwyler *et al.* 2019). RNAi-mediated *prl-1* knockdown in MSNs results in a loss of terminal arbors and fewer synapses specifically in the contralateral branch. This effect is recapitulated in *prl-1* mutants. Moreover, *prl-1* mutant animals exhibited developmental defects in AL and MB neurons while peripheral synapses like the NMJ remained unaffected, reflecting a specific requirement for Prl-1 in central synaptogenesis. In MSNs, Prl-1 localizes specifically to the contralateral branch where it modulates Insulin Receptor (InR) signaling (Fig. 5b and c). Here, the Insulin pathway promotes formation of terminal arbors and synaptic assembly; perturbation of InR pathway signaling components like *chico, Akt*, *p110*, and *raptor* decreases both arbor complexity and synapse number. Acting locally, Prl-1 promotes synapse formation and terminal arborization specifically in contralateral branches by antagonizing PTEN (Phosphatase and Tensin homolog), a negative regulator of insulin signaling output (Urwyler *et al.* 2019). This raises the tantalizing possibility that local enrichment of other phosphatases or kinases may play similar roles to fine-tune synaptic organization with subcellular specificity. Moreover, these same phosphatase and kinases may act more broadly as general regulators of synapse assembly in other neuronal classes.

The diversity of mechanisms that control branch-restricted synaptogenesis is only beginning to emerge. In MSNs, the RNA-binding protein Musashi exhibits highly contrasting roles in distinct collateral axonal branches (Landínez-Macías *et al.* 2021). In contralateral branches, which are highly synaptogenic, *musashi* loss of function results in decreased numbers of presynaptic AZs. In posterior branches, however, which are largely devoid of synapses, *musashi* loss of function leads to ectopic Syt-1 accumulation. How Musashi promotes synaptic assembly in one axonal branch, but antagonizes it in another, is not fully understood. In MSNs, Musashi binds to the mRNA encoding the receptor protein tyrosine phosphatase Ptp69D. In *musashi* mutants, *ptp69D* mRNA poly(A)-tail length and Ptp69D protein levels are decreased. Notably, Ptp69D loss of function recapitulates the loss of presynaptic AZs from contralateral branches seen in *musashi* mutants but does not result in ectopic assembly of presynapses in the posterior branch. Thus, this study suggests that Musashi may function broadly to maintain physiological protein levels of select synaptogenic regulators, such as Ptp69D, which promotes synapse formation in contralateral MSN branches, and presumably of negative regulators of synaptogenesis that inhibit synapse formation in posterior branches. The identity of these regulators, and how they restrict their activity to distinct axonal branches remain unknown and an exciting area of research. Central

Drosophila synapses like the MSNs are well suited to dissect these mechanisms of both synapse specificity and synapse assembly to more deeply understand how connections in the fly brain are controlled by genetic mechanisms.

Discussion/concluding remarks

Central synapses exhibit remarkable organizational themes that operate at all levels of complexity, from cellular and subcellular to the level of multicellular neuropil. Visualization of central synapses by light microscopy coupled with an explosion in the last 10 years of genetically encoded synaptic labels and imaging strategies allows for a unique leverage of the awesome power of *Drosophila* genetics in determining the mechanisms that contribute to the assembly and organization of synapses in 3D space. Current work in the field is revealing both conceptual elements of synaptic organization in distinct central circuits, elaborating the molecular mechanisms of known synaptogenic genes in mediating synapse organization, and identifying novel regulators of synaptogenesis, synapse assembly, and synaptic specificity. Furthermore, while both central and peripheral synapses rely on some shared core machinery (Featherstone *et al.* 2001; Kaufmann *et al.* 2002; Miller *et al.* 2005; Pielage *et al.* 2005, 2006; Mosca *et al.* 2012; Mosca and Luo 2014; Urwyler *et al.* 2015; Özel *et al.* 2019) to build and organize synapses (e.g. Teneurins, LAR, Liprin-α, Syd-1, Spectrin), it is becoming increasingly evident that the functions of synaptogenic proteins are largely influenced by the cellular context in which they are expressed. As such, whether the synaptic mechanisms discovered at powerhouse peripheral synapses like the NMJ function analogously at different synapses in the CNS remains an open and fascinating question for the field to tackle. Beyond this, many key questions remain—we highlight 4 that stem from the discoveries discussed in this review.

- *What factors determine synapse number in a neuron?* Distinct classes of ORNs in the AL have characteristic numbers of synapses yet there is an invariant synaptic density across different ORN classes. This density is under genetic control and can be increased or decreased through genetic manipulation (Mosca and Luo 2014; Mosca *et al.* 2017). How does synapse number scale with neurite volume in such a way to maintain that synaptic density? How do individual neurons set their synapse number capacity and achieve it without variation? How do cell-autonomous and non cell-autonomous interactions cooperate to produce synapse number? How do other circuit partners influence the number of synapses made by a neuron? And how much of synapse number in a single cell or class of cells is hard-wired within distinct populations and how much is regulated by experience or activity? By understanding how different neurons control their synapse density while still remaining capable of scaling with size, changing in response to different stimuli or conditions that invoke neural plasticity, and adjusting this with the speed necessary to maintain adequate circuit processing, we will have a better grasp of how the nervous system matures. Answers to these questions will begin to unravel the mystery of how synapses form, how their 3D architecture is achieved in the mature adult state, and how these processes are influenced by genetic programs, by experience, and how they go awry in neurodevelopmental, psychiatric, and neurodegenerative disease models.
- *How are dendritic compartments organized to contain both pre- and postsynaptic specializations?* Some KC dendrites assemble both pre- and postsynaptic specializations in the same neurite that are physically segregated from each other with exquisite subcellular precision (Christiansen *et al.* 2011). Furthermore, PNs in the AL contain postsynaptic specializations to receive ORN input and dendrodendritic synapses that connect PNs with other PNs, and to LNs (Ramaekers *et al.* 2005; Huang *et al.* 2010; Liu and Wilson 2013; Mosca and Luo 2014). LNs in the AL also lack the traditional axon/dendrite structure and instead have neurites containing mixed presynaptic AZs and postsynaptic receptors (Ng *et al.* 2002; Chou *et al.* 2010; Wilson 2013). The physiological function of dendrodendritic presynaptic AZs is not well understood nor is it known how these domains are organized to ensure their functional roles and to prevent crosstalk in neuronal transmission. From a cell-level perspective, it is further unclear how cell polarity systems that underlie early synaptogenesis events are modulated to support the development of presynapses in the dendritic compartment (Wiggin *et al.* 2005). Finally, at the subcellular scale, what are the mechanisms that physically segregate pre- and postsynaptic specializations within the same neurite? Uncovering the mechanisms that drive stereotyped organization of synaptic inputs and outputs in the same neuron will help clarify the role these mixed neurites play in information flow and processing.
- *Are the same core molecular players that underlie synaptogenesis also required for synaptic plasticity and maintenance?* In photoreceptors, synaptic assembly is tightly coupled with axonal pathfinding and growth cone dynamics (Özel *et al.* 2019) and requires coordinated brain-wide activity (Bajar *et al.* 2022), but may occur independently of light-stimulated neuronal activity (Akin *et al.* 2019). However, in adults where axon pathfinding has been completed, synapses are dynamically assembled and disassembled in response to light stimulus (Sugie *et al.* 2015). Furthermore, how does spontaneous neural activity regulate synapse formation and organization during development? Given the striking difference in cellular state, it is unclear whether the same synaptogenic machinery is used during development (for synaptic assembly or pruning) as is used to assemble or disassemble synapses in response to varying light stimuli. In mammalian systems, developmental mechanisms like Netrin signaling through the Deleted in Colorectal Cancer (DCC) receptor play a clear role in axon guidance (Dickson 2002) and are later repurposed to regulate adult synaptic plasticity (Glasgow *et al.* 2018, 2020). How often does this concept of "adaptive reuse" link the mechanisms of synapse formation with those of maintenance and plasticity? Understanding how protein roles are shared between processes will provide a wealth of information linking different stages of neurodevelopment with adult function and enable a better grasp of the coordination of neuronal functions.
- *How do complex neurons locally modulate synaptic organization?* In neurons like the dorsocentral MSNs (Urwyler *et al.* 2019), there are elegant mechanisms for local modulation of synaptogenesis in a single branch of an otherwise complex neuron. What other proteins function in the MSNs and in other circuits to locally restrict kinase, phosphatase, or some other cellular activity to influence synaptogenesis? How broad is this mechanism? Do analogous processes function at finer scales, for example, to constrain 3D synaptic organization in a neurite (i.e. dendrodendritic or pre- vs postsynaptic in the same neurite)? And finally, more broadly, how are the mechanisms of synapse specificity (where in space to form synapses) connected to the processes of actual synaptic assembly? By first

grasping how the exquisite subcellular specificity is achieved in complex neurons, we can better understand the developmental events that lead to and promote synapse formation.

By combining genetically encoded synaptic labels (Chen *et al.* 2014; Mosca and Luo 2014; Urwyler *et al.* 2015) and a myriad of genetic tools, connectomics libraries, and molecular strategies in flies, *Drosophila* is well poised to continue making landmark contributions to the field of central synaptogenesis. The above-noted questions (as well as many other outstanding mysteries in the field) may be answered through continued work leveraging tools like single-cell sequencing, genetic screens, and high-throughput microscopy including confocal, EM, and light-sheet microscopy. Now that key stereotyped features of synaptic organization are known in multiple brain regions [AL, Mosca and Luo (2014); MB calyx, Kremer *et al.* (2010) and Christiansen *et al.* (2011); optic lobe, Chen *et al.* (2014); MSN, Urwyler *et al.* (2015)], the "normal" state is beginning to be well described. With essential baseline data in hand, the field is ready to exploit the power of fly genetics to carry out forward genetic screens and RNA sequencing to identify novel synaptogenic pathways and regulatory strategies used by diverse sets of neurons to organize synapse location and 3D structure. Small-scale candidate screens in the optic lobe (Sugie *et al.* 2015; Kawamura *et al.* 2021) and in MSNs (Urwyler *et al.* 2019) have already demonstrated that imaging of synaptic organization by light microscopy can reliably identify mutant phenotypes with the advantage of a semi-high-throughput genetic screen. Furthermore, once candidate synaptogenic genes have been discovered, cell-type-specific transcriptomic and proteomic assays can be used as complementary approaches to identify additional components of synaptogenic pathways (Davis *et al.* 2020). As our understanding of the mechanisms that regulate synaptogenesis in central neurons increases, we will be better equipped to investigate how synapse dysfunction (e.g. malformation, reduced number of connections, impaired 3D organization) results in altered behavior and circuit function. With a firmer grasp of how distinct neuronal classes modulate synaptogenic processes to ensure a cell-type-specific output, we will further understand the molecular basis for clinically relevant synaptopathies and develop more informed therapeutic strategies to treat these disorders.

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Conflicts of interest

None declared.

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