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Genetic regulation of central synapse formation and organization in Drosophila melanogaster

Juan Carlos Duhart and Timothy J. Mosca†

A key goal of modern neuroscience involves understanding how connections in the brain form and function. Such a knowledge is essential to better inform how defects in the exquisitely complex steps of nervous system growth underlie neuropsychiatric and neurodevelopmental disorders. In the last 40 years, studies of the nervous system in the fruit fly Drosophila melanogaster enabled the discovery of a wealth of molecular and genetic mechanisms that drive the development of these synaptic connections – specialized cell-to-cell connections that are the essential substrate for information flow and processing in the nervous system. The major driver of knowledge focused on studies at the neuromuscular junction due to its ease of examination. Analogous studies in the central nervous system lagged behind due to a lack of genetic accessibility of specific central neuron classes, appropriate synaptic labels compatible with cell-type specific access, and high resolution, quantitative imaging strategies. However, understanding how synapses in central circuits form remains a prerequisite to understanding brain development. In the last decade, a host of new tools and techniques made possible the extension of genetic studies of synapse organization into central circuits and greatly enhanced our understanding of central synapse formation, organization, and maturation. In this review, we consider the current state-of-the-field, focusing on two major elements. We first discuss the tools, technologies, and strategies developed to visualize and quantify synapses in vivo in genetically identifiable neurons of the Drosophila CNS. Second, we explore in depth how these tools enabled a clearer understanding of synaptic development and organization in different circuits and discovered novel molecular mechanisms that underlie synapse formation. These studies establish multiple brain regions in the fly as powerful in vivo genetic models that offer novel insights into synaptogenic regulators and mechanisms of neural development.
Keywords: *Drosophila*, synapse, central nervous system, synaptogenesis, genetics, development, active zones.

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 is released and postsynaptic sites that are specialized with receptors for specific neurotransmitters. Every computation that underlies behavior, cognition, or emotion, requires robust and reliable synaptic transmission (Chou et al., 2020; Mayford et al., 2012; Ploski and McIntyre, 2015). 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 three-dimensional 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 UAS/GAL4, QUAS/QF, and lexA/LexAop (Venken et al., 2011a) 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 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 (Bayat et al., 2011; Keshishian and Kim, 2004; Keshishian et al., 1996; Koles and Budnik, 2012; Kraut et al., 2001; Kurusu et al., 2008; Liebl et al., 2006; Menon et al., 2013; Packard et al., 2002). At the NMJ, a presynaptic motoneuron interacts with (in most 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 has made

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

analysis of central synaptic features challenging—particularly when focusing on select neurons or circuits. The density and the lack of cellular resolution, comparable to the NMJ, has 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 neurotransmitter identity than neuromuscular synapses, increasing the complexity of the system and requiring additional levels of cell-type specific synaptic organization (Chen et al., 2014; Meinertzhagen and O'Neil, 1991; Mosca and Luo, 2014; Schlegel et al., 2021; Urwyler et al., 2015). 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 (Mosca and Luo, 2014), mushroom body (Christiansen et al., 2011; Elkahlah et al., 2020), optic lobe (Chen et al., 2014), and in mechanosensory neurons that innervate large dorsocentral sensory bristles (Urwyler et al., 2015). In each of these model systems, visualization of active zone markers using light microscopy and genetic tools allows both quantitative and qualitative analysis of synaptic organization and distribution in three-dimensional 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.

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; Cizeron et al., 2020; Kittel and Heckmann, 2016; Pazos Obregón et al., 2015; Wilhelm et al., 2014). Historically, visualization of many of these proteins by light microscopy is readily achieved by immunostaining endogenous or epitope tagged proteins 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 neurotransmitters or neurotransmitter receptors; how many synapses are assembled by a specific neuron and where they are located in three-dimensional space; whether a particular active zone 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 post-synaptic markers revealed key features of synaptic architecture, subcellular organization, and mechanisms underlying the cell biology of synaptic development (Harris and Littleton, 2015; Nose, 2012). In the fly brain however, over 100,000 neurons (Raji and Potter, 2021), containing nearly a hundred 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

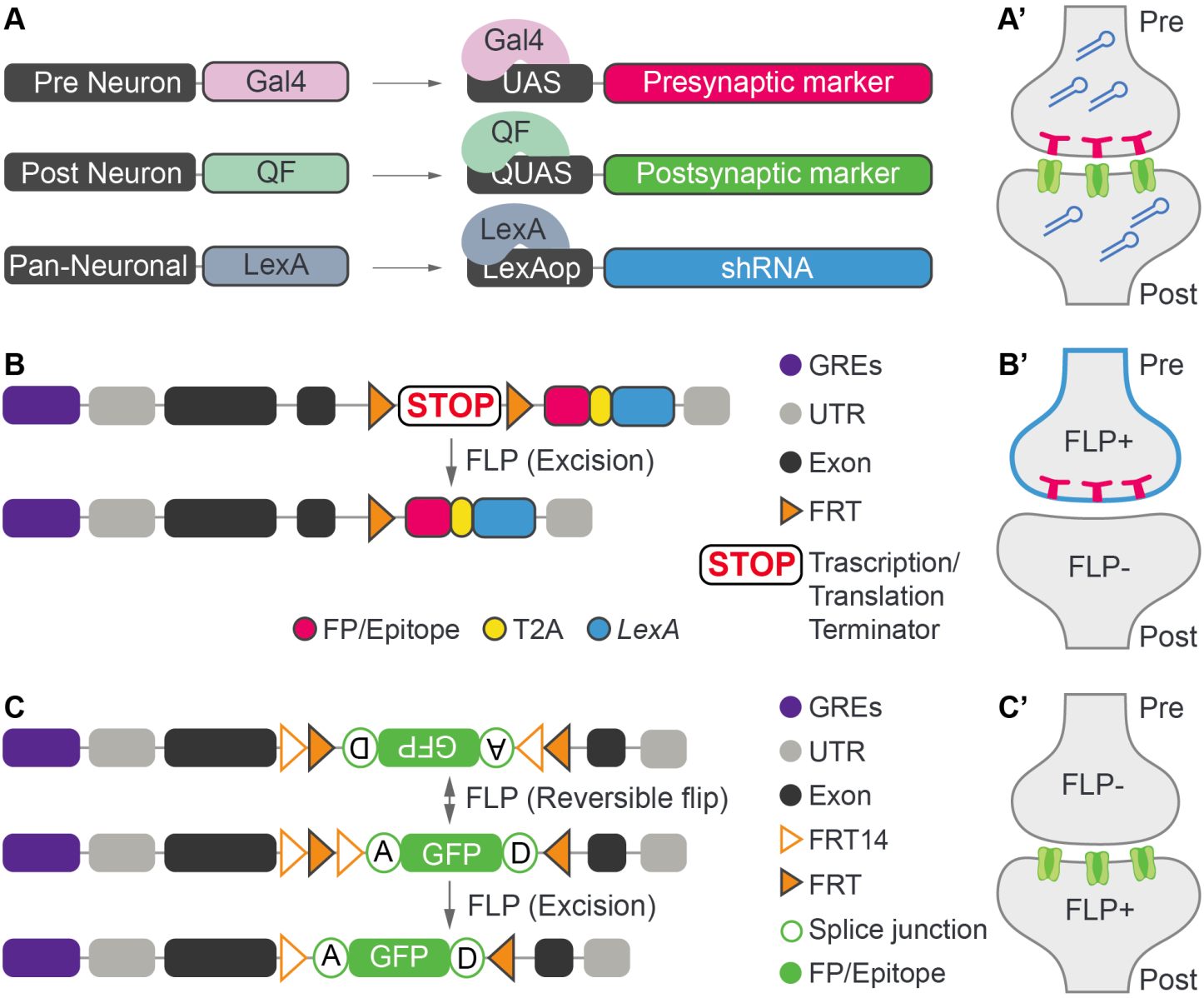

Figure 1. Approaches for conditional and inducible synaptic transgene expression in Drosophila. (**A**) The GAL4/UAS, QF/QUAS, and LexA/LexAop binary expression systems are each comprised of two components: a transcription factor (Gal4, QF, or LexA) and its cognate promoter (UAS, QUAS, or LexAop respectively). Specific promoters (left, in gray) 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 active zone protein under Gal4/UAS control; expression of a postsynaptic compartment marker (e.g. a fluorescently labeled neurotransmitter) in the postsynaptic neuron is under QUAS control; pan-neuronal expression of a short hairpin RNA, under LexA/LexAop control, knocks-down 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 FLP-recombinase recognition targets (FRT). 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 (pink), thus producing a tagged version of Bruchpilot (Brp), which labels pre-synaptic active zones (Chen et al., 2014). This cassette also features a ribosomal skipping sequence, T2A (yellow), 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 Flp-Tag method, conditional FLP expression in a postsynaptic neuron inverts the orientation of an artificial exon inserted into, for example, a neurotransmitter 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::GFP fusion protein labels the post-synaptic compartment in FLP expressing cells. Similar approaches have been used to generate inducible Rab3-, vGAT-, and vGlut-based synaptic vesicle markers (Certel et al., 2022a, 2022b; Williams et al., 2019).

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

The coupling of conditional expression systems and

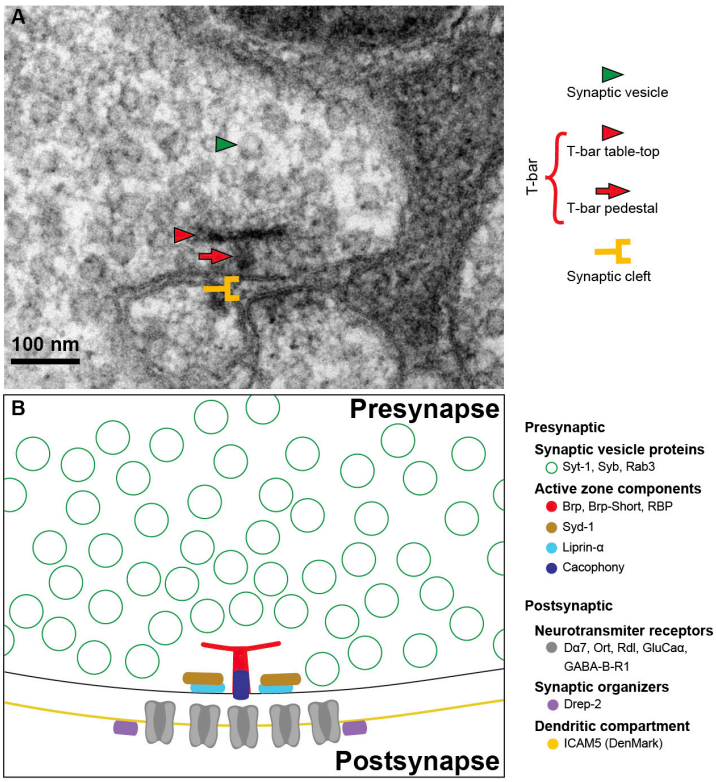

Figure 2. A repertoire of pre- and post-synaptic markers for studying synaptic organization in Drosophila. (**A**) Electron micrograph of an olfactory receptor neuron (ORN) axon terminal. The Drosophila active zone is readily identified by an electron-dense T-bar structure that is composed of a Brp-rich table-top (red arrowhead) and a 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).

inducible mosaic techniques (Germani et al., 2018) have 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 (Figure 1; Brand and Perrimon 1993; Duffy 2002), and consists of two 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; Jenett et al., 2012; Venken et al., 2011a). The versatility of the GAL4/UAS system is complemented by two 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 three expression systems can be used in combination to manipulate up to three distinct genetic elements in the same animal (Figure 1A-A"). Further increasing the versatility of these expression systems, conditional expression of site-specific recombinases (e.g. Flippase, Cre, ΦC31) allow targeted DNA rearrangements in vivo (Figure 1B-C; (Groth et al., 2004; Kilby et al., 1993; Siegal and Hartl, 2000; Simpson, 1993; Weasner et al., 2017)). These manipulations can be used, for example, to generate loss of function mosaic tissue or cell-type restricted protein labeling (Chen et al., 2014; Fendl et al., 2020; Lai and Lee, 2006; Lee and Luo, 2001; Urwyler et al., 2015).

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 are available and amenable for use in the fly CNS (Figure 2). 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 three 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 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 neurotransmitter to enable communication between neurons. Because of this, the first generation of genetically-encoded synaptic labels were 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; Figure 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 neurotransmitter 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 (Goyal et al., 2019; Guo et al., 2019; Helfrich-Förster et al., 2007; Otsuna and Ito, 2006; Ramaekers et al., 2005; Zhang et al., 2007). 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 one neuron, and the dendritic marker DenMark, to identify the postsynaptic region in its downstream target (see below; (Nicolaï et al., 2010)). 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 (Chen et al., 2019; Jung et al., 2020; Kennedy and Broadie, 2018; Lamaze et al., 2018; Zhang and Simpson, 2022).

Important caveats exist with vesicular markers, however, that may limit their utility. First, domains of SV protein enrichment do not always overlap precisely with presynaptic active zones (AZs; 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; Quiñones-Frías and Littleton, 2021; Wu et al., 2016), 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 CRIPR/Ca9 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 (Certel et al., 2022a, 2022b; Williams et al., 2019). For example, three 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 Figure 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 (Certel et al., 2022a; Williams et al., 2019). 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 (Christiansen et al., 2011; Poskanzer et al., 2003; Zhang et al., 2002). 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 can report connections in an activity-dependent fashion (Macpherson et al., 2015) in live imaging, adding to the utility and power of synaptic vesicle markers. Despite the incredible utility of vesicle markers as synaptic tools, proteins like Syt-1 and n-Syb do not report other critical structural features of synaptic organization, such as the precise location and distribution of active zones or ion channels. Additional strategies, including those used to label active zones, 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 Ca2+ channels, or coupling distance, varies across synapses and influences release probability (Fulterer et al., 2018; Ghelani and Sigrist, 2018; Wadel et al., 2007). Visualizing synaptic vesicle proteins and active zone components by super resolution microscopy enables assessment of synaptic architecture at the nanometer scale, enabling assessment of critical structural features (Ehmann et al., 2014; Fulterer et al., 2018; Spühler et al., 2016). 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 labeling

The active zone (AZ) is a subcellular, presynaptic specialization that provides the scaffolding for neurotransmitter vesicle release and calcium channel localization, ultimately serving to regulate neurotransmitter 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 (Figure 2A) and are called T-bars (Hamanaka and Meinertzhagen, 2010; Meinertzhagen, 1996; Prokop and Meinertzhagen, 2006); 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; Liu et al., 2011; Wagh et al., 2006), 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 two 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 (Figure 2A; (Acuna et al., 2016; Fouquet et al., 2009; Hallermann et al., 2010; Kittel et al., 2006; Liu et al., 2011; Scholz et al., 2019; Wagh et al., 2006)). 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 pre-synaptic active zone (Fouquet et al., 2009; Kawasaki et al., 2004; Sugie et al., 2015). Unlike synaptic vesicle markers that label large pools of synaptic vesicle proteins and often span multiple active zones, AZ markers accumulate in a punctate manner that allows for quantification of distinct parameters of synaptic organization including the number of presynaptic active zones and their organization in three-dimensional space (Figure 2 and 3C). To date, Brp is the most widely utilized presynaptic protein for genetically-encoded active zone labeling strategies, but RBP as well as other strategies based on proteins like the auxiliary active zone proteins Syd-1 (Owald et al., 2010), Liprin-α (Kaufmann et al., 2002), and the Ca2+ channel Cacophony (Kawasaki et al., 2004) function analogously to assess both CNS and PNS synaptic organization. Here, we highlight each of these tools in concert with unique genetic labelling strategies to understand how active zones are visualized in Drosophila.

Brp-based labeling strategies

The most widely used approaches for cell-type specific active zone labeling in Drosophila center on Brp. At the Drosophila NMJ, a single active zone 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 two approaches (FL-Brp and Brp-Short) can be 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 methods employ Brp-FL to label synapses. First, Brp-FL can be expressed through traditional binary expression systems (Flood et al., 2013; Wagh et al., 2006) and imaged to ascertain key synaptic parameters such as AZ numbers and organization. Though facile and at least partly reflective of endogenous active zone organization, Brp-FL can form aggregates outside of synaptic compartments when overexpressed in a non-relevant cell, resulting in artifactual punctate labeling (Wagh et al., 2006). 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. Such a caveat of overexpression led to strategies that made use of the natural brp promoter to achieve physiological expression levels, potentially removing the overexpression caveat. To circumvent variability from different promoters, Chen and colleagues (2014) developed Synaptic Tagging by Recombination (STaR) method. STaR is an inducible active zone 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 Figure 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 UAS-GAL4), 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 (Figure1B-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 active zone, 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 (Akin and Zipursky, 2016; Liu et al., 2016; Sugie et al., 2015; 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 produces overexpression artifacts, as Brp-FL can when expressed via UAS/GAL4 (Wagh et al., 2006). Overall, studies employing Brp-FL labeling strategies have enabled circuit-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 (Fouquet et al., 2009; Schmid et al., 2008), that comprises the central 473-1226 amino acids of the full-length protein (GenBank: AAF58930). Brp-Short alone is non-functional and causes no discernible effects on cell morphology, synaptic organization, or neuronal function when overexpressed (Fouquet et al., 2009; Mosca and Luo, 2014; Schmid et al., 2008; Urwyler et al., 2015). Though non-functional, 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). Immuno-electron 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 (Berger-Müller et al., 2013; Takemura et al., 2008). A striking example comes from mechanosensory neurons 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 mechanosensory neurons, 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). Further, in the Drosophila antennal lobe, measurements taken using Brp-Short and confocal microscopy in the three major component neurons (olfactory receptor neurons, projection neurons, and local interneurons) are consistent with analogous ultrastructural reconstructions that show similar results regarding the proportion of total synapses made by each class of cells (Coates et al., 2020; Horne et al., 2018; Rybak et al., 2016; Tobin et al., 2017). Taken together, these studies indicate that Brp-Short is a powerful active zone marker that can be used to quantitatively measure synaptic active zone organization with high fidelity in a diverse array of neurons in Drosophila; including the olfactory system (Coates et al., 2017; Fulterer et al., 2018; Mosca and Luo, 2014; Mosca et al., 2017), the mushroom body (Christiansen et al., 2011; Kremer et al., 2010), the visual system (Berger-Müller et al., 2013; Özel et al., 2019; Sugie et al., 2015), the larval ventral nerve cord (Hu et al., 2017; Tenedini et al., 2019), and the ellipsoid body (Xie et al., 2019). In mechanosensory neurons, GAL4-driven Brp-Short AZ labeling produces indistinguishable results when compared directly to Brp-FL AZ labeling via the STaR method, indicating that these two strategies are viable synaptic labeling alternatives (Urwyler et al., 2015). Taken together, both Brp-FL and Brp-Short approaches successfully measure synaptic organization across diverse circuits, often serving as confirmatory techniques.

Complementary active zone 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, RIM binding protein (Sugie et al., 2015), the voltage-gated Calcium channel Cacophony (Fulterer et al., 2018; Liu et al., 2011; 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 (Fulterer et al., 2018; Reddy-Alla et al., 2017), and others (Figure 2B) have all been employed to study synapse formation, development, and organization. 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; Fulterer et al., 2018; Mosca et al., 2017; Özel et al., 2019; Sugie et al., 2015). As each label is a functionally distinct component of the pre-synaptic active zone, 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 active zone labels is a powerful strategy that can yield a deeper understanding of active zone 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 post-synaptic labels. Currently, however, postsynaptic labeling strategies in general are limited and have lagged behind presynaptic strategies for a number of reasons. Presynaptic active zones are specialized for neurotransmitter secretion and share a core secretory machinery for multiple different kinds of neurotransmitters (Südhof, 2012) that can be exploited for labeling strategies. Post-synaptic specializations, on the other hand, exhibit greater functional diversity. Broadly, post-synaptic 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). Neurotransmitter specialization is further differentiated by expression of distinct classes of neurotransmitter receptors (depending on the nature of the synapse and the neurotransmitter needed to promote signaling), as well as structural, regulatory, and signaling molecules consistent with those subtypes of neurotransmitter 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 neurotransmitter receptors (Figure 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 (Andlauer et al., 2014; Chen et al., 2014; Mosca and Luo, 2014; Nicolaï et al., 2010) are suitable for use in multiple neuronal classes regardless of neurotransmitter receptor identity and label the somatodendritic compartment, postsynaptic structural proteins, or synaptic organizers (Figure 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 neurotransmitter receptor clusters in adult circuits, and three-dimensional synaptic organization in the brain.

Neurotransmitter receptor labeling

Postsynapses are specialized to respond to the specific neurotransmitters released by their presynaptic partners. As a result, a common labeling strategy uses genetically-encoded neurotransmitter (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 neurotransmitter (Gundelfinger and Hess, 1992; Kondo et al., 2020; Rosenthal et al., 2021). In studies of the olfactory system (Wilson, 2013) including olfactory projection neurons (PNs) in the antennal lobe that receive cholinergic input from olfactory receptor neurons (ORNs) and in the mushroom body Kenyon cells (KCs) which receive cholinergic input from olfactory PNs (Gu and O'Dowd, 2006; Ramaekers et al., 2005; Yusuyama et al., 2002), a GFP-tagged Dα7 subunit of the acetylcholine receptor has been used with conditional expression via UAS/GAL4 (Leiss et al. 2009). Dα7-GFP accumulates at the synaptic membrane and directly apposes the presynaptic active zone (Christiansen et al., 2011; Kremer et al., 2010; Leiss et al., 2009b; 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 Rdl (Fendl et al., 2020; Sánchez-Soriano et al., 2005) and GluRII glutamate receptors (Fendl et al., 2020), among others. In the optic lobe especially, HA epitope- or GFP-tagged versions of the GABA receptor subunit Resistant to dieldrin (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 (Fendl et al., 2020; Raghu et al., 2007; Sánchez-Soriano et al., 2005). Importantly, though, overexpression of NT receptor transgenes has essential caveats. First, NT receptor labeling may affect receptor function yielding gain or loss of function receptor variants if expressed at non-physiological levels. Second, problematic expression variability may arise based on the strength of the GAL4 / QF / lexA driver, leading to overexpressed receptor having a deleterious effect on synaptic physiology or low levels of receptor expression not surpassing a threshold for reliable detection. 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 non-physiological postsynaptic sites. Thus, though powerful tools, results from these strategies must be carefully interpreted.

More recent strategies have sought to circumvent potential overexpression caveats by expressing tagged versions of postsynaptic receptors as labels under the control of their endogenous promoter. Though approaches like MiMIC (Venken et al., 2011b) have greatly advanced abilities to tag endogenously expressed proteins for epitope- or fluorescent-labeling, by themselves they lack the tissue-specific expression needed to make assessments of individual cell populations. 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 active zones 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., 2021) or site-specific genetic manipulation (Venken et al., 2011b) have matured and become widely adopted, the field has moved towards inducible systems for conditional NT receptor labeling. Fendl and colleagues (2020), developed an inducible labeling strategy named FlpTag, in which a GFP protein tag is conditionally spliced into the mature mRNA encoding either GluClα, Rdl, or Dα7 (Figure 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 non-productive orientation to one that facilitates splicing into the mature mRNA (Fendl et al., 2020). STaR, FlpTag, and other inducible systems ensure that the synaptic label remains subject to endogenous promoter regulation including transcriptional control, trafficking, recruitment, and turnover. Moreover, as these labels are expressed at normal levels via their endogenous promoter, potential overexpression artifacts are largely reduced. Despite their tremendous utility, each of these approaches still requires a priori knowledge of the postsynaptic NT receptor at the synapse of interest, highlighting the growing need for general postsynaptic labels for when that information is not available and when different receptor subtypes may be organized differently from one another.

General post-synaptic labeling approaches

Each neuron in the Drosophila CNS expresses approximately 22 neurotransmitters, complicating selection of the appropriate NT label between specific pre and post-synaptic 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 post-synaptic 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). Further, ICAM5::mCherry overexpression in fly neurons has no deleterious effects, unlike prior dendritic labeling strategies using a specific isoform, Dscam17.1, 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 (Chen et al., 2019; Flood et al., 2013; Jung et al., 2020; Kennedy and Broadie, 2018; Lamaze et al., 2018; Nicolaï et al., 2010). However, it is not known if if DenMark labels postsynaptic regions that are not in dendritic compartments (as might be observed with postsynaptic muscles) so its use 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; Fulterer et al., 2018; Pooryasin et al., 2021; Spühler et al., 2016; Tenedini et al., 2019). 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 post-synaptic 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 post-synaptic 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.

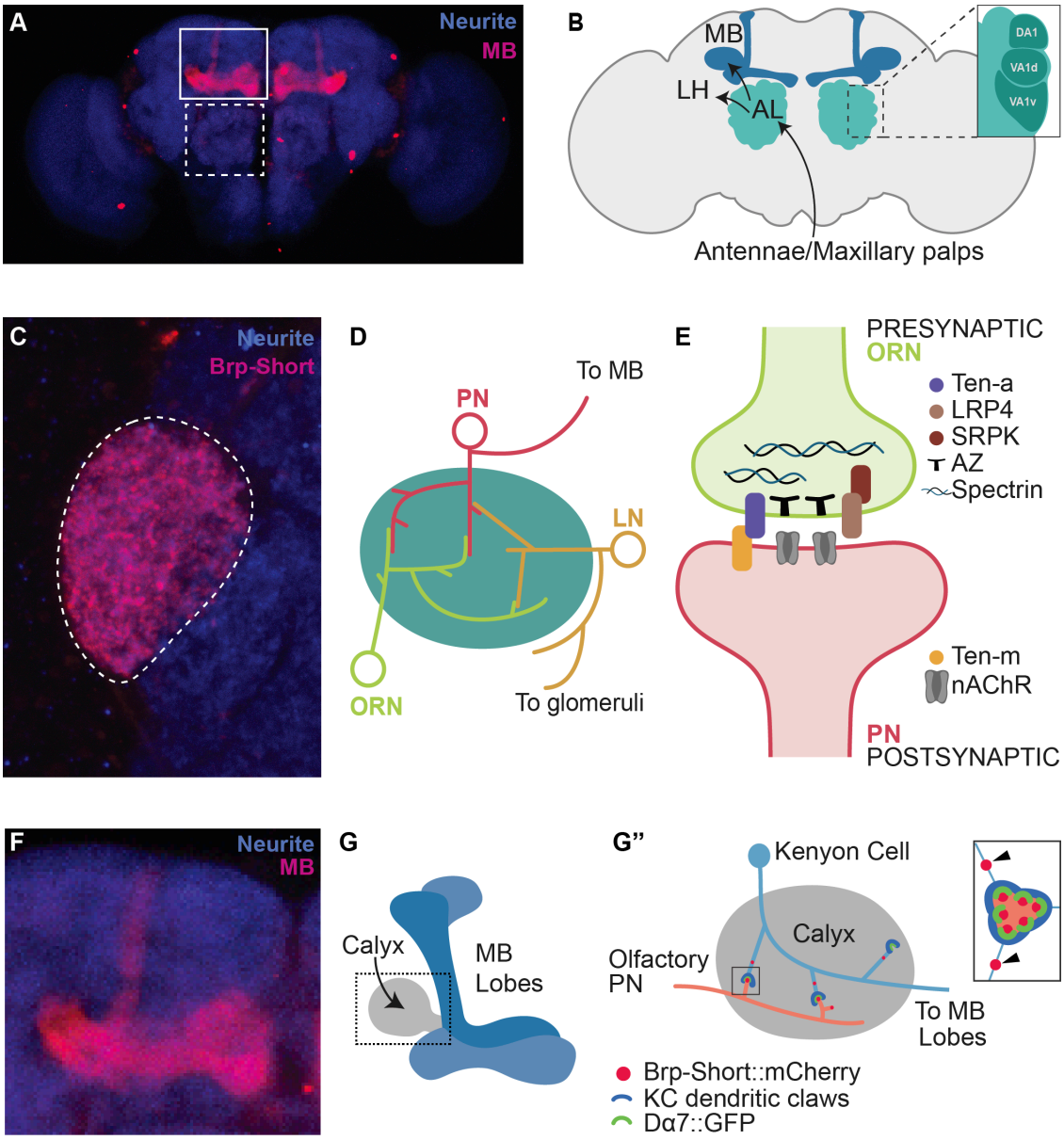

Figure 3. Model systems of synaptic organization in the Drosophila olfactory circuit. (**A**) Micrograph of an adult Drosophila brain stained with a general neurite label (blue) and a marker that reveals the mushroom body lobes (magenta). The anatomical locations of the AL (dashed box) and MB (solid 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 antennal lobe (AL; shown in green). Olfactory information is then transmitted to higher order brain regions, including the mushroom body (MB) shown in blue and the lateral horn (LH). The antennal lobe is organized into discrete neuropil where three major neuronal classes form synaptic connections (inset shows three anatomically distinct neuropil: DA1, VA1d and VA1v). (**C**) Micrograph of AL glomeruli corresponding to the region indicated in the dashed box in (**B**). All olfactory receptor neurons (ORNs) that express a particular odorant receptor (Or67d) converge on a single glomerulus (Dashed; DA1). Restricted expression of Brp-Short::mStrawberry in these ORNs reveals active zone 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 projection neurons (PNs). PNs then transmit odorant information to higher order brain regions including the MB and LH. The local interneurons (LNs) comprise many different classes of cells defined by morphology and who 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 antennal lobe is regulated by the Teneurin and LRP4 signaling pathways (figure adapted from DePew and Mosca, 2021; DePew et al., 2019). Trans-synaptic heterophilic Teneurin interactions instruct synaptic organization in the antennal lobe. Presynaptic Ten-a functions with Spectrin to promote presynaptic active zone assembly and organization. The role of post-synaptic 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 two regulate synaptic assembly and morphology. (**F**) Micrograph of mushroom body lobes (magenta). Mushroom body calyces are not discernible. (**G**) Schematic of the mushroom body. The mushroom body intrinsic neurons, the Kenyon cells (KCs), concentrate their dendrites in the calyx and send axonal projections in parallel bundles to form the mushroom body lobes. (**G"**) Kenyon cell dendrites are mixed neurites that have exhibit both pre- and post-synaptic specializations (figure adapted from Christiansen et al., 2011). KCs are post-synaptic 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). Kenyon cell-derived presynaptic active zones form outside of the dendritic claws (arrowheads in inset).

PART II: TOWARDS UNDERSTANDING THE GENETIC BASIS OF CENTRAL SYNAPSE DEVELOPMENT AND ORGANIZATION

The Antennal Lobe

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 (Grabe and Sachse, 2018; Hummel and Rodrigues, 2008; Wilson, 2013). In the fly olfactory system (Figure 3), the antennal lobe (AL) is the first order processing center for olfactory information and also more recently emerged as a powerful model (Figure 3A-C) to investigate the mechanisms of synapse development and organization in central neurons (Coates et al., 2017; Mosca and Luo, 2014; Mosca et al., 2017). The antennal lobe is divided into distinct sub-regions called glomeruli that represent odorant information channels (Figure 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 active zone 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 (Couto et al., 2005; Vosshall et al., 2000) enabled the creation of genetic reagents to directly manipulate genetically identifiable ORNs via binary expression systems (Vosshall et al., 2000). Subsequent analyses (Chou et al., 2010; Ito et al., 1998; Tanaka et al., 2008) expanded this technical repertoire to distinct classes of projection neurons (PNs) and local interneurons (LNs), providing genetic access to most of the neurons that comprise each glomerulus (Figure 3D). The combination of such genetic access along with new tools for cell-type specific synaptic labeling (Figure 2B) uniquely positioned the olfactory system as a powerful system for the genetic dissection of cell biological mechanisms involved in synapse formation and organization (Christiansen et al., 2011; Kremer et al., 2010; Mosca and Luo, 2014).

Hallmark features of synaptic organization in the antennal lobe obey three rules:

The Drosophila AL is comprised of ~50 glomeruli (Figure 3B-C) that contain projections from olfactory receptor neurons (ORNs), projection neurons (PNs), and local interneurons (LNs) that synapse with each other (Figure 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 antennal lobe. 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 / µm3 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 five 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 (Grabe et al., 2016; Mosca and Luo, 2014). 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 mosaic analysis with a repressible cell marker (MARCM), Mosca and Luo (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 active zones (Mosca and Luo, 2014). These LN synapses likely represent LN-LN and LN-PN synapses, It is likely that the LN-derived active zones 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 (Chou et al., 2010; Huang et al., 2010; Kind et al., 2021; Olsen and Wilson, 2008; Olsen et al., 2007; Root et al., 2007, 2008; Wilson, 2011, 2013; Yaksi and Wilson, 2010). Quantitatively, each of the three neuronal classes (ORN, PN, LN) exhibit distinct synaptic organization with respect to their own neurites. ORNs have the highest level of active zone 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 three-dimensional 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 antennal lobe.

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, two main signaling systems that alter synaptic density in the antennal lobe 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 (DePew et al., 2019; Hong et al., 2012; Mosca, 2015; Mosca et al., 2012) and mammalian systems (Berns et al., 2018; Chand et al., 2013; Pederick et al., 2021; Sando et al., 2019; del Toro et al., 2020; Woelfle et al., 2015, 2016). In the Drosophila antennal lobe, 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 (Figure 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 neuromuscular junction (Mosca et al. 2012). Ultrastructurally, Ten-a is also required for normal active zone morphology: in ten-a mutants, nearly ~50% of active zones are impaired, revealing misshapen, detached, or otherwise abnormal T-bar structures (Mosca and Luo, 2014). Consistent with active zone 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 antennal lobe (Mosca and Luo, 2014), consistent with known roles for Teneurin proteins in cytoskeletal regulation (Mörck et al., 2010; Mosca et al., 2012; Nunes et al., 2005; Suzuki et al., 2014), which in turn regulates active zone 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 neuromuscular junction where it functions as the receptor for Agrin, an essential synaptogenic signal secreted from presynaptic motoneurons (DeChiara et al., 1996; DePew and Mosca, 2021; Gautam et al., 1996; Hopf and Hoch, 1998; Kim et al., 2008; McMahan, 1990; Weatherbee et al., 2006; Zhang et al., 2008). The Drosophila LRP4 homolog is expressed broadly throughout the brain and localizes to active and periactive zones 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 antennal lobe and the mushroom body but does not

alter inhibitory synapse number in either the antennal lobe 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 active zones 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 (SR) protein kinase to regulate synapse organization (Mosca et al., 2017). LRP4 is required for the proper synaptic localization of SRPK79D and the two 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, highlights their downstream mechanisms, and establishes the antennal lobe as a model synapse for assessing cell-autonomous and non-cell-autonomous factors in central synaptogenesis in Drosophila.

The Mushroom Body

In the Drosophila brain, olfactory information that is processed in the antennal lobe is next conveyed to higher olfactory centers including the mushroom body and the lateral horn (Figure 3A-B, F-G). In many insect species, associative learning takes place in the mushroom body (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 ~2000 intrinsic neurons called Kenyon cells (KCs) whose organization gives rise to the three main anatomical features that characterize the fly mushroom body (Aso et al., 2014). Though the MB receives inputs from multiple sensory modalities (Kirkhart and Scott, 2015; Li et al., 2020a, 2020b; Schlegel et al., 2021), the majority of KC inputs are made by olfactory PNs from the antennal lobe onto KC dendrites in the calyx (Figure 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 (Ito et al., 1997; Kunz et al., 2012; Technau and Heisenberg, 1982). At this point, KCs synapse onto mushroom body output neurons (MBONs) and receive modulatory inputs from dopaminergic neurons (DANs), forming an intricate synaptic network (Li et al., 2020a). Significant inroads have been made into understanding mushroom body output and its relation to behavior, focusing on the MBONs and DANs (Li et al., 2020a; Modi et al., 2020; Scaplen et al., 2021) and their physiological response to sensory input (Bilz et al., 2020; Cohn et al., 2015; Kremer et al., 2010; Pech et al., 2015; Sugie et al., 2018) and 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 et al., 2009; Kremer et al., 2010; Christiansen et al., 2011).

A survey of pre-synaptic active zones in MB Kenyon cells 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; Morgan and Lichtman, 2020; Mosca and Luo, 2014). 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 antennal lobe) innervate the calyx (Butcher et al., 2012; Leiss et al., 2009a), concentrating their pre-synapses in structurally-defined neuropils, termed microglomeruli (Figure 3G"). Conditional expression of Brp-short::GFP in Kenyon cells (KCs), however, revealed dendritic presynaptic AZs (Christiansen et al., 2011), termed KCACs (KC-derived active zones residing in the calyx). KCACs are functional and account for ~20% of the active zones that reside in the calyx (Christiansen et al., 2011; Ng et al., 2002; Owald et al., 2010). 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 and colleagues (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 (Figure 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 Kenyon cell 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 mushroom body 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 and colleagues (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 KIR2.1 (Nitabach et al., 2002), both the number of microglomeruli and Brp-Short labeled active zones 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 projection neurons scale bouton number to the number of KCs present (Elkahlah et al., 2020) and individual projection neurons 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 (Elkahlah et al., 2020; Kremer et al., 2010). 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 mushroom body 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. 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 olfaction, 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 (Davis et al., 2020; Jenett et al., 2012; Meinertzhagen and Sorra, 2001; Morante and Desplan, 2008; Nern et al., 2015; Scheffer et al., 2020; Wu et al., 2017). 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 (Figure 4): the compound eye of Drosophila melanogaster consists of 700-800 ommatidia (Kumar, 2012), each containing eight photoreceptor neurons, which send projections into the optic lobe, where four main neuropil reside (Figure 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 (Figure 4A'-C)), key synaptic parameters including the aggregate number of synapses and their spatial organization are known from EM reconstructions and light microscopy studies (Berger-Müller et al., 2013; Chen et al., 2014; Meinertzhagen and Sorra, 2001; Rivera-Alba et al., 2011; Takemura et al., 2008, 2013). 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 of and genetic regulation underlying synaptogenesis.

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

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 deeper understanding first, of 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 post-synaptic partners, the L3 laminar neurons (Chen et al., 2014). At the photoreceptor::laminar synapse, Brp-FL::V5 puncta begin accumulating at 40h APF and continue to the end of pupal development at 100h APF. This predates postsynaptic accumulation, as OLLAS-Ort puncta are not visible until 77h 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. This is consistent with data from the Drosophila NMJ that indicates deposition of Brp precedes the clustering of postsynaptic glutamate receptors (Rasse et al., 2005). However, it remains possible that postsynaptic assembly (as measured by other postsynaptic adaptors or seeding factors) may begin concomitantly with

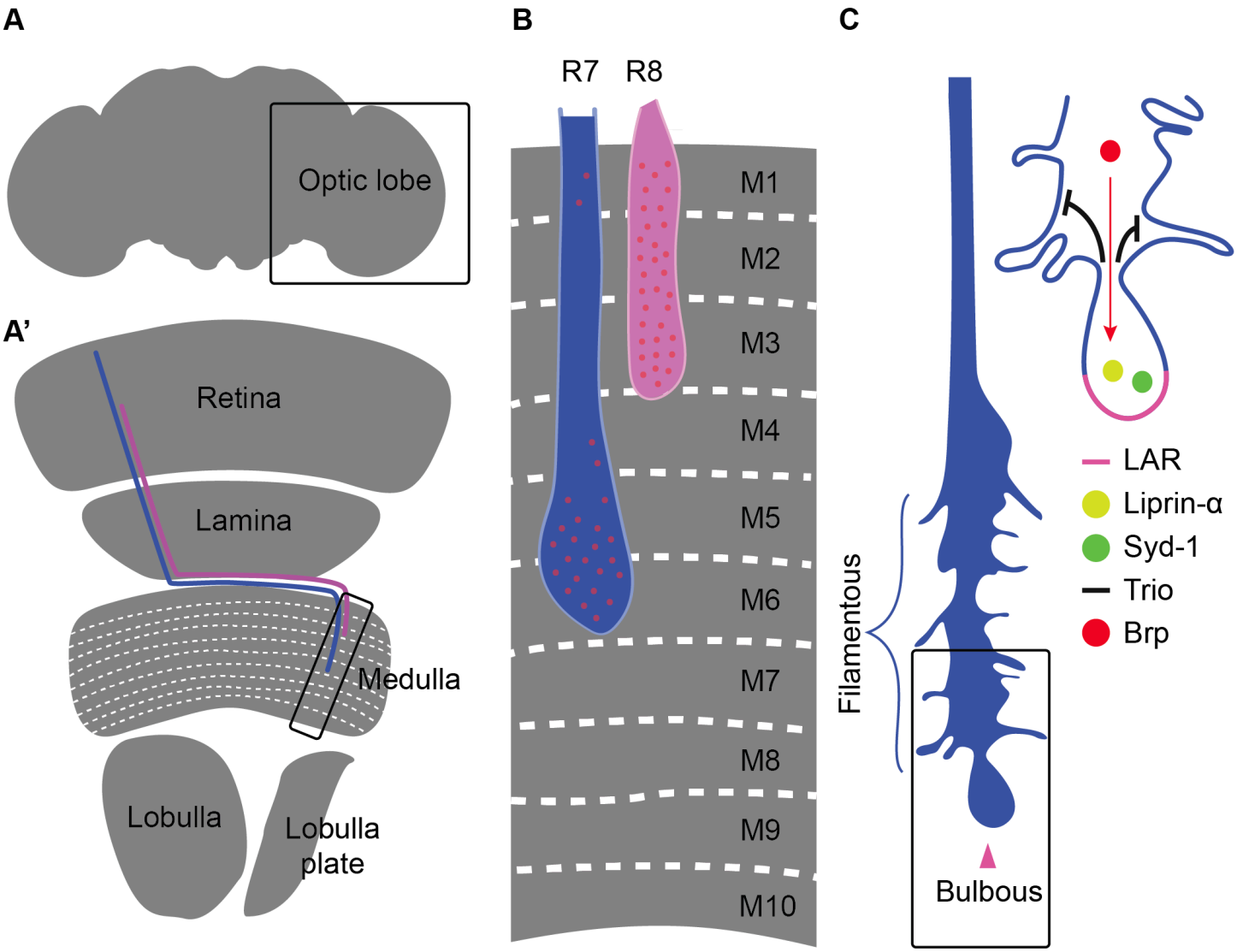

Figure 4. Mechanisms of synaptic organization in the Drosophila optic lobe. (**A**) Schematic of the Drosophila brain. Boxed region corresponds to the optic lobe diagrammed in (**A'**). (**A'**) Schematic of the four main optic lobe neuropils (figure panel modeled after Nériec and Desplan, 2016). R7 (blue) and R8 (purple) photoreceptor neurons innervate distinct layers in the medulla and form synaptic connections with different post-synaptic 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; Chen et al., 2014). R8 photoreceptors assemble approximately twice as many active zones in the medulla (~50) as R7 photoreceptors (~25). Moreover, synaptic organization is distinctly different in these two 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 (purple 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 minutes), but eventually retract. Following retraction, Brp is recruited to the nascent synapse where Liprin-α and Syd-1 reside.

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 active zone 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 (Nériec and Desplan, 2016; Özel et al., 2015, 2019). 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 photoreceptor axon terminals 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 and colleagues (2019) found that one or two long-lived filopodia with characteristic bulbous tips are always present during the period of bulk synaptic addition (Figure 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 accumulation (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 (Astigarraga et al., 2010; Dai et al., 2006; Hakeda-Suzuki et al., 2017; Holbrook et al., 2012; Owald et al., 2010). 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 minutes) synaptogenic bulbous filopodia are present at any given time (Figure 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 active zones in R7 photoreceptor neurons and limits potential targets, suggesting that a temporal model of availability governs synaptic organization. Together with work from Chen and colleagues (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 active zones 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 three-dimensional space.

Neuronal mistargeting affect 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 two steps are separated temporally and while the molecular players that regulate each step can overlap (Hong et al., 2012; Mosca and Luo, 2014; Mosca et al., 2012), 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 post-synaptic partners (Courgeon and Desplan, 2019; Kazama and Wilson, 2008; Takemura et al., 2008, 2013): R7 spans medullary layers M1-6, while R8 spans layers M1-3 (Figure 4A'-B). Considerable work has revealed diverse molecular determinants of layer specificity in the visual system, providing methods to adjust where different neurons project (Akin and Zipursky, 2016; Pecot et al., 2013; Peng et al., 2018; Sanes and Zipursky, 2020; Santiago et al., 2021; Tan et al., 2015; Xu et al., 2019). 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 (Hong et al., 2009; Shishido et al., 1998). 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 (Berger-Müller et al., 2013; Chen et al., 2014; Takemura et al., 2008, 2013): R7 neurons still assemble their characteristic number of active zones (~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, highlighting that synaptic partner matching and synaptic organization are not 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 active zones 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, such as the

extent to which parameters such as how 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; Cosmanescu et al., 2018; Tan et al., 2015; Xu et al., 2019, 2018) in regulating synapse organization and wiring development. Finally, whether molecular codes contribute the bulk of synaptic specificity or if synapse formation and selectivity is 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 active zones 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 post-synaptic 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 active zone 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-hour light/dark cycling (LD). How environmental exposure influences rapid changes in active zone 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 active zones 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 homologue Wingless as a key mediator of visual synaptic plasticity, opening the door to mechanistic studies of synaptic remodeling post-development. Components of the divergent canonical Wnt signaling pathway (Ciani et al., 2004; Miech et al., 2008; Salinas, 2007) are required for activity-dependent AZ remodeling in R8 photoreceptors (Kawamura et al., 2020; Sugie et al., 2015). 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., 2020) 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., 2020). 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 decrease 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.

Dorsocentral mechanosensory neurons

The mechanosensory neurons (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 (Figure 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 three primary axon collaterals that project anteriorly, contralaterally, or posteriorly; each collateral exhibits a characteristic synaptic organization. Visualization of active zones using the genetically encoded labels Brp-Short::GFP, mCherry::Syt1, and Cacophony::GFP showed that each of these axonal compartments of MSNs vary according to local synaptic concentration (Fouquet et al., 2009; Kawasaki et al., 2004; 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

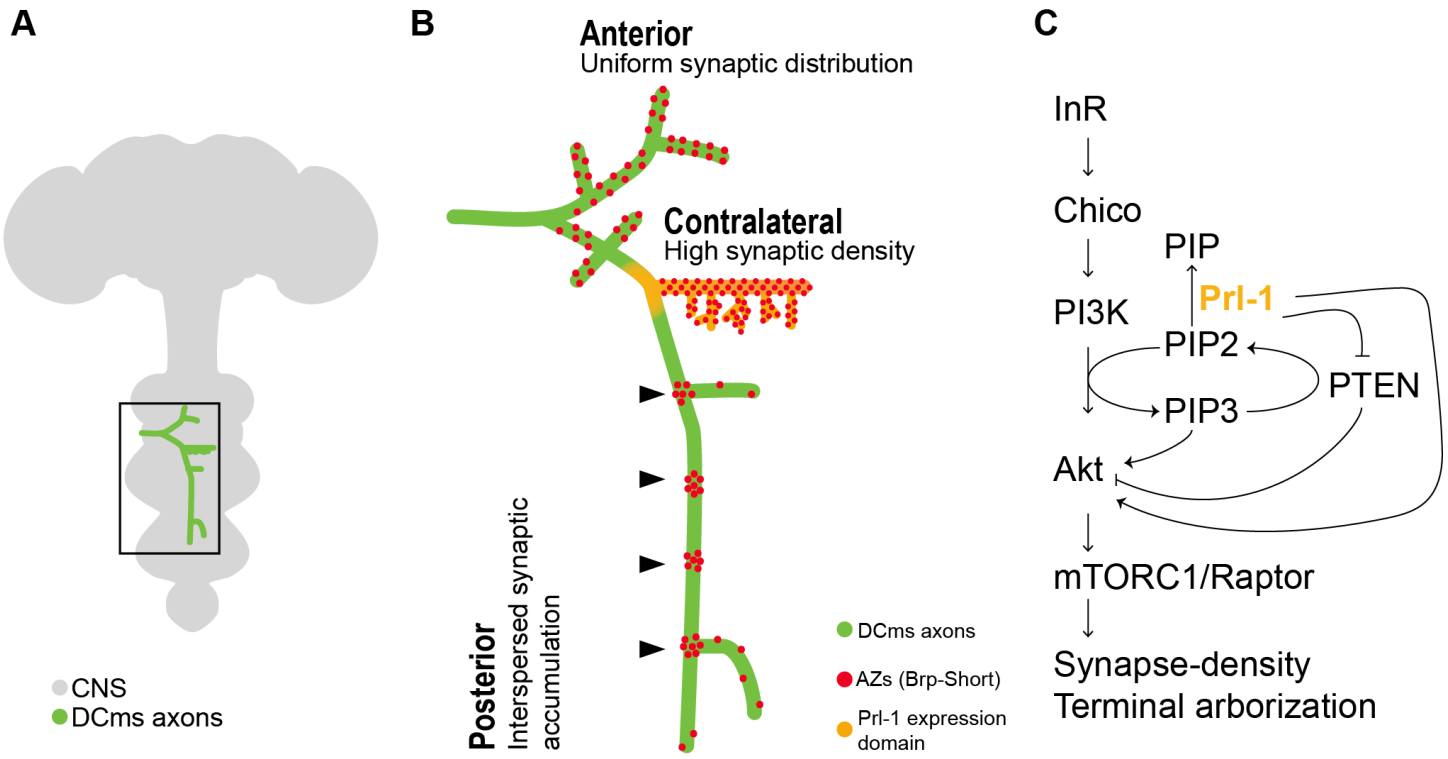

Figure 5. Branch-restricted regulation of synaptic organization in mechanosensory neurons. (**A**) Schematic of the Drosophila adult CNS. A single dorsocentral mechanosensory neuron (MSN; green in box) is diagrammed in the thoracic ganglia (Figure panel modeled after Chen et al., 2006). (**B**) MSNs 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).

synapses along the entirety of its length, the posterior branch is largely devoid of synapses, except at four 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 MSNs and as such, the mechanosensory neurons are ideally suited to investigate mechanisms that control synaptic organization with precise subcellular specificity at the level of individual axonal branches.

Active zone assembly and synaptic development in dorsocentral mechanosensory neurons

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; Li et al., 2014; Owald et al., 2010). 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 active zone 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 pre-synaptic active zones but is dispensable for the actual assembly of active zones. This provides a striking contrast to its role in developing photoreceptor neurons and 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 underscores 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 mechanosensory neuron branches

The contralateral branches of the MSNs have a high density of active zones compared to its other collateral branches (Urwyler et al., 2015) that feature fewer presynapses and distinct synaptic organization (uniform vs sparse; Figure 5B). In an RNAi knock-down screen targeting the fly kinome and phosphatome, Urwyler and colleagues (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 knock-down 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 antennal lobe and mushroom body 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 (Figure 5B-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 active zones. 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 sub-cellular 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 three-dimensional 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. Further, while both central and peripheral synapses rely on some shared core machinery (Featherstone et al., 2001; Kaufmann et al., 2002; Miller et al., 2005; Mosca and Luo, 2014; Mosca et al., 2012; Özel et al., 2019; Pielage et al., 2005, 2006; Urwyler et al., 2015) 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 neuromuscular junction function analogously at different synapses in the central nervous system remains an open and fascinating question for the field to tackle. Beyond this, many key questions remain - we highlight four that stem from the discoveries discussed in this review.

- What factors determine synapse number in a neuron? Distinct classes of ORNs in the antennal lobe 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 three-dimensional 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 Kenyon cell 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). Further, projection neurons in the antennal lobe contain postsynaptic specializations to receive ORN input and dendrodendritic synapses that connect PNs with other PNs, and to LNs (Huang et al., 2010; Liu and Wilson, 2013; Mosca and Luo, 2014; Ramaekers et al., 2005). LNs in the antennal lobe also lack the traditional axon / dendrite structure and instead have neurites containing mixed presynaptic active zones and postsynaptic receptors (Chou et al., 2010; Ng et al., 2002; Wilson, 2013). The physiological function of dendrodendritic presynaptic active zones 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). Further, 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 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 mechanosensory neurons (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 mechanism function at finer scales, for example, to constrain three-dimensional 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 (antennal lobe, Mosca and Luo, 2014; mushroom body calyx, Kremer et al., 2010 and Christiansen et al., 2011; optic lobe, Chen et al., 2014; mechanosensory neuron 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 three-dimensional structure. Small-scale candidate screens in the optic lobe (Kawamura et al., 2020; Sugie et al., 2015) and in mechanosensory neurons (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. Further, 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 three-dimensional 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 be better equipped to interpret the molecular basis for clinically relevant synaptopathies and develop more informed therapeutic strategies to treat these disorders.

ACKNOWLEDGEMENTS

We'd like to thank the members of the Mosca Lab for their camaraderie and their discussions in formulating the ideas of this review. We also thank Michael Aimino, Dr. Kristen Davis, Alison DePew, Jesse Humenik, and S. Zosimus for invaluable comments on the manuscript. Work in the Mosca Lab is supported by grants from the US National Institute of Health grants R01-NS110907 and R00-DC013059, the Alfred P. Sloan Foundation, and the Whitehall Foundation.

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