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Single-cell transcriptomics in the Drosophila visual system: Advances and perspectives on cell identity regulation, connectivity, and neuronal diversity evolution

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A B S T R A C T

The Drosophila visual system supports complex behaviors and shares many of its anatomical and molecular features with the vertebrate brain. Yet, it contains a much more manageable number of neurons and neuronal types. In addition to the extensive Drosophila genetic toolbox, this relative simplicity has allowed decades of work to yield a detailed account of its neuronal type diversity, morphology, connectivity and specification mechanisms. In the past three years, numerous studies have applied large scale single-cell transcriptomic approaches to the Drosophila visual system and have provided access to the complete gene expression profile of most neuronal types throughout development. This makes the fly visual system particularly well suited to perform detailed studies of the genetic mechanisms underlying the evolution and development of neuronal systems. Here, we highlight how these transcriptomic resources allow exploring long-standing biological questions under a new light. We first present the efforts made to characterize neuronal diversity in the Drosophila visual system and suggest ways to further improve this description. We then discuss current advances allowed by the single-cell datasets, and envisage how these datasets can be further leveraged to address fundamental questions regarding the regulation of neuronal identity, neuronal circuit development and the evolution of neuronal diversity.

1. Introduction

The human brain, one of the most fascinating organs in terms of complexity and function, consists of 80 billion neurons belonging to thousands of different cell types (Azevedo et al., 2009). Understanding how this structure develops to support its function is one of the holy grails of developmental neuroscience. However, its sheer complexity poses multiple practical challenges, prompting research in simpler, yet similarly organized systems. The Drosophila visual system, and in particular the optic lobe, has emerged over the last 10 years as an ideal model to address such questions, for many reasons. The Drosophila optic lobes have a moderate complexity with a manageable number of cell types (~200) (Kurmangaliyev et al., 2020; Özel et al., 2020) but can still support complex behaviors (Branson et al., 2009; Cande et al., 2018). Their relative simplicity has allowed large high-throughput efforts that have described in detail the morphology of the different cell types (Fischbach and Dittrich, 1989; Nern et al., 2015), their connectivity (Takemura et al., 2013, 2015), and their transcriptomic diversity (Konstantinides et al., 2018a; Davis et al., 2020; Kurmangaliyev et al., 2020; Özel et al., 2020). Importantly, neuronal identity is genetically hardwired in the optic lobes and does not rely on environmental factors (Izutsu et al., 2015), which allows the study of the genetic basis of different neurodevelopmental processes (such as acquisition of neurotransmitter identity, synaptic specificity, etc). Therefore, as discussed below, we already have a good understanding of how neuronal diversity arises during larval development, as well as of the specific factors that are involved in this process (Li et al., 2013; Suzuki et al., 2013; Bertet et al., 2014; Erclik et al., 2017). Importantly, many of the lessons learned from the fly have also been discovered in vertebrates (Elliott et al., 2008; Konstantinides et al., 2015; Mattar et al., 2015; Holguera and Desplan, 2018).

The Drosophila optic lobes receive visual input from the retina, which is formed of ~800 ommatidia (unit eyes), each of which consists of 8 photoreceptors (R1-R8) (Bate and Martinez Arias, 2009). Each optic lobe is divided into four neuropils (ganglia): lamina, medulla, lobula and lobula plate (Fig. 1A) (reviewed in (Nériec et al., 2016)). Each neuropil is divided into ~800 columns, corresponding to the ~800 ommatidia, which process the information from a given point in space. Therefore, the spatial organization of visual signals is maintained in all neuropils, a property called retinotopy (Fischbach and Dittrich, 1989). Unicolumnar neuronal types mostly transmit information between layers and neuropils and are present as 1 neuron per column, while multicolumnar neuronal types integrate visual signals across several columns and usually comprise less than 1 neuron per column (Erclik et al., 2017). The lamina

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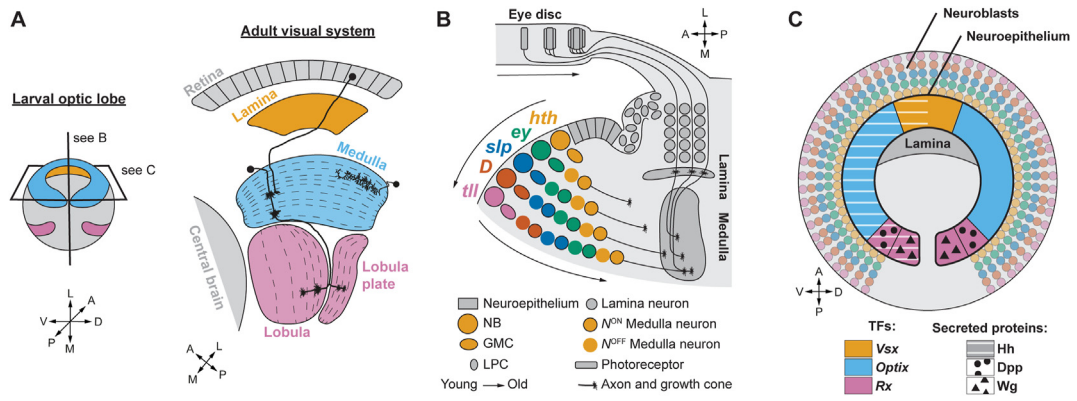


Fig. 1. Anatomy and neuronal type specification mechanisms in the D. melanogaster optic lobe
A – Left: schematic of the larval optic lobe. Blue = part of the outer proliferation center in which medulla neurons are produced. Orange = part of the outer proliferation center in which lamina neurons are produced. Pink = inner proliferation center, in which lobula and lobula plate neurons are produced. Right: schematic of the adult visual system. In black are represented two unicolumnar neurons (left), including one photoreceptor, and one multicolumnar neuron (right).
B – Section of the larval optic lobe, ventral view, illustrating the neuronal type specification mechanisms involving a cascading expression of temporal transcription factors and a Notch-driven binary neuronal fate decision. hth = homothorax, ey = eyeless, slp = sloppy paired, D = Dichaete, tll = tailless, GMC = ganglion mother cell, LPC = lamina precursor cell, NB = neuroblast, N^{ON} = Notch ON, N^{OFF} = Notch OFF.
C – Section of the larval optic lobe, lateral view, illustrating the neuronal type specification mechanisms involving spatial domains of the neuroepithelium. Dpp = Decapentaplegic, Hh = Hedgehog, Rx = Retinal Homeobox, Vsx = Visual system homeobox, Wg = wingless.

receives input from photoreceptors R1-R6, which detect motion (Bausenwein and Fischbach, 1992), while the medulla, which is the largest structure of the optic lobe, receives input from both the lamina neurons and the two color-sensitive photoreceptors R7 and R8 (Fischbach and Dittrich, 1989). The information from the medulla is then forwarded to the lobula that processes color, looming and feature detection amongst others (Wu et al., 2016), and the lobula plate that processes broad field motion (Borst et al., 2010).

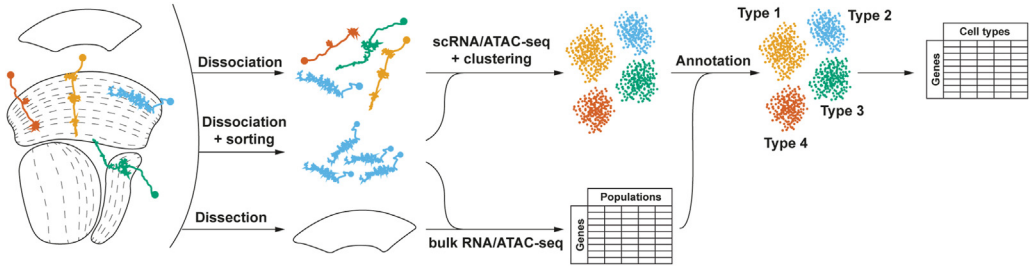
The vast majority of Drosophila optic lobe neurons are generated during the third instar larval stage. Two neuroepithelial structures (Outer Proliferation Center – OPC – and Inner Proliferation Center – IPC) generate neural stem cells (neuroblasts) that divide asymmetrically many times, self-renewing and generating an intermediate progenitor called ganglion mother cell (GMC). The GMC then divides only once to generate two neurons or glia (Hofbauer and Campos-Ortega, 1990). Neuroblasts are capable of generating many different neuronal types using three main mechanisms. i) Temporal patterning (Fig. 1B): in the main part of the OPC, five transcription factors (TFs), homothorax (hth), eyeless (ey), sloppy paired (slp), Dichaete (D), and tailless (tll), are expressed in a temporal sequence in every neuroblast of the medulla, leading to the formation of different neurons at different time windows (Li et al., 2013; Suzuki et al., 2013). A similar mechanism is at play at the tips of the OPC (Bertet et al., 2014), as well as in the IPC (Apitz and Salecker, 2015, 2018; Pinto-Teixeira et al., 2018). ii) Spatial patterning (Fig. 1C): three TFs (Visual system homeobox 1 – Vsx1, Optix, and Retinal homeobox - Rx) and three secreted molecules (hedgehog - hh, wingless - wg, and decapentaplegic - dpp), expressed in sub-regions of the OPC, modify the neuroblast identity without affecting the temporal sequence with the exception of the wg domain, which has a slightly different temporal sequence (Bertet, 2014; Erclik, 2017). The intersection of spatial patterning with temporal specification generates more neuronal types, since neuroblasts of the same age in different subdomains can produce different neurons (Erclik et al., 2017). Similarly, the IPC is spatially compartmentalized by the expression of wg, dpp, and brinker (brk) (Apitz and Salecker, 2015, 2018). iii) Notch-driven binary cell fate decision (Fig. 1B): further neuronal diversity is achieved during the asymmetric division of the GMC by Notch signalling, which is active in only one of the two neurons produced. The combination of spatial and temporal patterning coupled with a Notch-driven binary cell fate decision, as well as cell death (Pinto-Teixeira et al., 2016), are responsible for the generation of most of the observed neuronal diversity.

Here, we review how recent single-cell transcriptomic studies have advanced our understanding of neuronal diversity within the Drosophila visual system from different perspectives. First, we summarize the transcriptomic efforts made to describe the neuronal diversity in the fly visual system (Section 2). Then, we discuss specific applications of single-cell transcriptomics in studying gene regulation and how neuronal characters are regulated (Section 3), neuronal circuitry formation (Section 4), and evolution of neuronal diversity (Section 5). In all cases, we review the current advances with a focus on single-cell sequencing datasets and we present our perspective as to how single-cell sequencing datasets can be used to address the above questions.

2. Transcriptomic diversity of the Drosophila visual system

Several studies took advantage of the modular structure of the Drosophila visual system, in which most neurons are repeated multiple times, to study biological processes at single-cell resolution using two different approaches: a) fluorescence-activated cell sorting (FACS) of a specific cell type followed by bulk RNA- or ATAC-sequencing, and b) single-cell sequencing of the entire tissue and resolution of cell types using clustering approaches (Box 1, Table 1 and Table 2). Two studies focused on the third instar larval eye disc (Ariss et al., 2018; Bravo González-Blas et al., 2020), two studies on the third instar larval brain (Cocanougher et al., 2020; Ravenscroft et al., 2020), and two studies on the third instar larval optic lobe (Konstantinides et al., 2021; Zhu et al., 2021). A group of studies sought to achieve a detailed description of transcriptomic diversity in adults, in the optic lobes (Konstantinides et al., 2018a; Davis et al., 2020; Kurmangaliyev et al., 2020; Özel et al., 2020) or the whole brain (Davie et al., 2018). Moreover, the two most recent optic lobe studies (Kurmangaliyev et al., 2020; Özel et al., 2020) also performed scRNAseq throughout pupal development. Notably, these two studies also achieved a much higher resolution than the earlier ones: they both identified about 200 clusters, as compared to the 52 clusters in (Konstantinides et al., 2018a) and 53 bulk-sequenced isolated populations in (Davis et al., 2020). These datasets represent an unprecedented resource to study all aspects of neuronal development and function, as they contain the transcriptome of more than 170 neuronal clusters throughout their development, with more than 60 of them annotated. Here, we present ways to further take advantage of these datasets, importantly without the need for producing additional sequencing data. It is important to note that, although the available single-cell mRNA

Box 1
Sequencing and data analysis in the Drosophila visual system.



Two main approaches were used to sequence single cell types in the Drosophila visual system. The first one was to perform bulk RNA or ATAC sequencing on a tissue, or on a cell population labeled by the expression of a specific driver and isolated, for instance by fluorescence activated cell sorting. The second was to perform single cell RNA or ATAC sequencing on a dissociated tissue, with or without sorting a specific cell population, via droplet sequencing. The obtained single-cell transcriptomes or accessibility profiles were then grouped informatically (i.e. "clustering") in order to form clusters of profiles similar to each other, which in many cases correspond to cell types. Some studies also corrected for technical variations between data produced in different batches, which is of prime importance to avoid forming clusters based on batches rather than cell types (batch effect correction tools were not always available for earlier studies). The cell types corresponding to the different clusters were then identified by the use of marker genes, or by correlating the average gene expression of the clusters to the one of sorted populations.

sequencing data offer a unique opportunity to study cell type diversity, they do not contain information about chromatin accessibility, gene isoforms, alternative polyadenylation sites, and post-transcriptional regulation (Zhang et al., 2016) that likely also affect neuronal diversity and function (Sen et al., 2019; J. Li et al., 2020; Ha et al., 2021).

2.1. Hidden heterogeneity

Although the above datasets are ready-to-use to explore diverse biological questions, the characterization of cell-type diversity offered by these datasets can be further improved. Many of the clusters contain hidden heterogeneity (Fig. 2): although clusters are group of cells with similar transcriptomes, what "similar" means is highly dependent on the depth of the data (number of cells and of genes per cells sequenced) and the thoroughness of the analysis. It is for instance important to note that, although how the selected clustering parameters are chosen is rarely discussed, they have a considerable influence on the number of clusters obtained for a given dataset: 87–151 (Davie et al., 2018), or 146–229 (Özel et al., 2020). Indeed, the clustering algorithm used to produce the pupal developmental atlases (Kurmangaliyev et al., 2020; Özel et al., 2020), and used in many other studies, has a tendency to either split abundant cell types into artificial groups (for instance based on transcriptome quality) or to group rare cell types (Lancichinetti and Fortunato, 2011). Therefore, many of the smaller neuronal clusters are very likely to contain more than one cell type. This is illustrated in (Davie et al., 2018) in which the authors chose to use the parameters yielding the smallest possible number of clusters (87 out of 151). Independent re-clustering of some of their clusters resulted in subclusters matching to known cell-types, showing that the initial clusters were heterogeneous. In addition to hidden rare cell types, these datasets are also likely to contain hidden subtypes within both rare and abundant cell types. Indeed, the ability of the clustering algorithms to separate cells is dependent on the number of genes that they differentially express. This is why several neuronal subtypes in the optic lobe were distinguishable only at mid pupal stages, during synaptogenesis, when there is an increase in transcriptomic diversity, as described in section 3.3 (Özel et al., 2020). Furthermore, clustering was not able to separate the dorsal and ventral subtypes of two abundant optic lobe neuronal types (Tm4 and cluster 62) that differentially express Wnt4, Wnt10 and a few other genes (Özel et al., 2020). These two populations are however spatially separated on tSNE visualization (without need for reclustering), suggesting that another clustering approach or different clustering parameters might have been able to separate them. However, despite these words of caution (which apply to any scRNAseq dataset), for most applications the developmental datasets can be used "out of the box".

To reveal some of the hidden heterogeneity of the datasets, complementary approaches can nevertheless be applied to the study of one cluster, a group of clusters, or whole datasets. First, merging data from different studies using batch effect removal tools (Luecken and Theis, 2019; Hie et al., 2020) will increase the number of cells that are analyzed, which can in turn increase the ability of the clustering algorithms to isolate rare cell types. However, this could also leave residual batch effects that would need to be carefully controlled for. Second, in the case of developmental atlases, cluster annotation should be made at the stage of maximal transcriptomic diversity and propagated ("transferred") to other developmental stages, instead of propagating a clustering made in adults (Özel et al., 2020). Third, a stepwise approach should be followed in which clustering is first applied to the whole dataset to efficiently identify abundant cell types, and then each cluster is independently re-clustered to verify whether they are heterogeneous. Indeed, clusters could contain either rare or very similar cell types that were not separated in the first clustering step. Fourth, one can use additional bioinformatic approaches, many of them recently reviewed in (Hie et al., 2020). For instance, using algorithms removing multiplets (heterogeneous single cell transcriptomes) or ambient RNA present in the cell suspension media (Fleming et al., 2019; Alvarez et al., 2020; Xi and Li, 2020; Yang et al., 2020; Young and Behjati, 2020) could enhance relative differences between transcriptomes and improve clustering. Several tools have also been developed to improve clustering, for instance by using improved pre-processing (Hafemeister and Satija, 2019; Johnson et al., 2020) or a different clustering algorithm (Traag et al., 2019; Stassen et al., 2020).

In this regard, it is interesting to note that most single-cell algorithms are developed and tested on "ideal datasets" of mammalian cells, with a large number of genes per cell, limited ambient RNA due to the use of cell culture or tissues requiring little to no dissociation (i.e. peripheral blood mononuclear cell), and a small number of cell types which are often of similar abundance. For instance, in one of the most detailed studies of the usual steps of scRNAseq analysis, the authors sought to produce reference datasets by mixing several cell lines, with and without adjunction of RNA to the mixture to model ambient RNA, and used these datasets to test almost 4000 combinations of data analysis methods (Tian et al., 2019).

Table 1
Single-cell RNA- and ATAC-seq datasets produced in the Drosophila visual system. The median number of genes per cell is affected by the version of the technology used, and by sorting or washing the cell suspension before sequencing: this removes cell debris and RNA from burst cells (ambient RNA), but could also increase biases by selecting for less fragile populations or affecting gene expression. The values are indicated after filtering based on quality control when reported in the studies, or before filtering otherwise. The number of clusters is only indicative, as many studies subset and re-cluster part of the dataset. ALH = after larval hatching, APF = after pupal formation, CNS = central nervous system, KC = Kenyon Cells, VNC = ventral nerve chord.

Reference	Material sequenced	Stages sequenced	Sex	Strains sequenced	Number of cells analyzed	Median number of genes/cell	Cell suspension sorting or washing	Sequencing technology	Batch effect correction	Number of clusters
Ariss et al. (2018)	eye disc	3rd instar larva	–	y[1] v[1]; P{y [+t7.7] = CaryP}attP2	11,416	–	–	Drop-Seq	–	15
				Rbf^{120a} mutant	5203					
Davie et al. (2018)	Brain	Adult (0, 1, 3, 6, 9, 15, 30 and 50 days old)	F + M	w^{1118}, DGRP-551	56,902 (157,390 sequenced)	1308 to 810	Washed	10x v.2	–	87
Konstantinides et al. (2018)	Optic lobe	Adult (3 days old)	F	Canton-S	54,974	312	–	Drop-Seq	–	52
(Brunet Avalos et al., 2019)	Brain	1st instar larva	–	Canton-S	4349	1434	Washed	10x v.3	Bioinformatic	29
	Brain (starved)				4347	1962				–
Kurmangaliyev et al. (2019)	Sorted cells (~T4/T5 neurons)	48h APF	F	23G12-Gal4 crossed to UAS-H2A::GFP	3557	1633	FACS	10x v.2	–	8
		24h APF			3071	1447				
Bravo González-Blas et al., 2020)	eye-antennal discs	3rd instar larva	–	hybrid of DGRP-551, DGRP-360, DGRP-907, and DGRP-913	3531	3094	Washed	10x v.2	–	17
					15,387 single-cell ATAC profiles	–	Washed	10x		–
				sens-F2B-GFP	384 single-cell ATAC profiles	–	FACS	Fluidigm C1		–
Cocanougher et al. (2020)	CNS, brain, VNC (including after optogenetic sting or KC overactivation)	1, 24, 48 and 96h ALH	–	–	202,107	–	–	10x v.2	–	70
Hörmann et al. (2020)	Sorted cells (~T4/T5 neurons)	24, 36, 48, 60 and 72h APF	F + M	SS00324-Gal4 recombined with UAS-mCD8:: GFP	~44,000	1627	FACS	10x v.3	Bioinformatic	8
Jain et al. (2020)	Sorted cells (~Lamina neurons)	24, 36, 48, 60, 72, 84 and 96h APF	F + M	w; UAS-H2A-GFP; 9B08-Gal4/Tm6B crossed with DGRP lines	8269	–	FACS	10x v.3	Bioinformatic, and pooled sequencing for part of the data	5
				w; UAS-H2A-GFP; 9B08-Gal4/Tm6B crossed with EcR^{DN}, EcR RNAi, Hr3 RNAi, tdTom or wRNAi	–					
Kurmangaliyev et al. (2020)	Optic lobe	0, 12, 24, 36, 48, 60, 72, 84 and 96h APF	F (M cells removed)	w^{1118}, and w^{1118} crossed with 35 DGRP strains	208,976	1650	FACS	10X v.3	Bioinformatic, and pooled sequencing for part of the data	196 (24–96 APF)
Özel et al. (2020)	Optic lobe	15, 30, 40, 50 and 70h APF, Adult (<3 days old)	F (M cells removed)	Canton-S	275,701	903	–	10X v.2	Bioinformatic	>193
Ravenscroft et al. (2020)	Brain	3rd instar larva	–	Several DGRP strains	5056	–	Washed	10x v.2	Bioinformatic	39
Konstantinides et al. (2021)	Optic lobe	3rd instar larva	F + M	Canton-S	49,893	1343	–	10x v.2	Bioinformatic	–
Michki et al. (2021)	Type-II NBs and some optic lobe cells	3rd instar larva	F + M	;;R9D11-Gal4/UAS-hH2B::2xmNG	6092	–	FACS	10x v.2 and v.3	Bioinformatic	18
(Zhu et al., 2021)	Sorted cells (~Medulla neuroblasts)	3rd instar larva	–	E(spl)mγGFP; SoxNGal4 UAS-RedStinger/TM6B	3074	3682	FACS	10x v.3	Bioinformatic	15

Table 2
Bulk RNA- and ATAC-seq datasets produced in the visual system. APF = after puparium formation.

Reference	Material sequenced	Stages sequenced	Sex	Data produced
Tan et al. (2015)	Isolated populations	40h APF	–	FACSed R7, R8, and L1– L5 neurons.
Zhang et al. (2016)	Isolated populations	24, 35, 40, 45, 53, 65 and 96h APF	–	Sequencing of mRNA associated to ribosomes in photoreceptors. RNA-seq of FACSed photoreceptors at P40 and P53.
Davie et al. (2018)	Brain	Adult	F + M	ATAC-seq, RNA-seq of whole tissue and RNA-seq of isolated nuclei.
Konstantinides et al. (2018)	Isolated populations	Adult (3–5 days old)	F	RNA-seq of 17 FACSed visual system neuronal populations.
Bravo González-Blas et al., 2020)	eye-antennal discs	3rd instar larva	–	Bulk ATAC-seq of sorted sens-F2B-GFP cells, of 29 DGRP lines, and of 14 lines overexpressing a TF.
Davis et al. (2020)	Isolated populations	Adult	F + M	RNA-seq on nuclei of cell populations isolated by INTACT and TAPIN. Visual system populations sequenced: 53 neurons and glia, 6 broader populations (ChAT, Gad1, VGlut, Kdm2, Crz, and NPF), 2 dissected tissues (the lamina and remainder of the optic lobe).
Jain et al. (2020)	Isolated L1 neurons	40, 60 and 72h APF	–	RNA-seq, ATAC-seq.ma
Özel et al. (2020)	Isolated populations	Adult (<3 days old)	F	RNA-seq of FACSed Pm2, T4, elav + cells (neurons) and repo + cells (glia).

However, these datasets contained a mixture of at most 5 human lung adenocarcinoma cell lines, as opposed to the roughly 200 cell types of the optic lobe. The data obtained in the adult optic lobe are much more challenging to analyze: 4 to 6 times fewer genes per cell compared to mammalian cells, higher ambient RNA content due to severing neuronal and glial processes during dissociation, many cell types with a large distribution of abundances, and very similar cell types and subtypes that could not be separated by the algorithms used (Kurmangaliyev et al., 2020; Özel et al., 2020). Therefore, these datasets would be ideal to develop and test new algorithms, especially because the results in adults can be compared to a gold standard of 54 bulk transcriptomes obtained from purified optic lobe neuronal types (Konstantinides et al., 2018a; Davis et al., 2020; Özel et al., 2020).

2.2. Cluster annotation

About two thirds of the neuronal clusters in the pupal and adult atlases remain unannotated, which limits the use of these datasets for several applications. In our experience, it is relatively easy to find the cluster that corresponds to a given cell type, provided the cell type is genetically accessible and can therefore be characterized by specific markers, and is sufficiently abundant to form a cluster (Konstantinides et al., 2018a; Özel et al., 2020). If more than one cluster expresses markers of a given cell type, the scRNAseq data can be used to find marker genes differentiating these clusters and to identify the correct cluster. On the other hand, identifying which cell-type corresponds to a given cluster is more difficult. In theory, since most clusters can be characterized by the differential expression of (semi-)specific genes, sparse labeling of drivers for these genes should allow the identification of the cell-type. A large repertoire of such lines exists or can be made using the Trojan exons (Diao et al., 2015). However, as noticed in the original study, Trojan exons can report the expression of a gene in cells for which the corresponding protein is not detected by antibody staining (Diao et al., 2015). This complicates cluster identification, as in many cases Trojan exons label a higher number of cell types than expected based on mRNA levels, as their expression can be driven by very low mRNA levels of the gene of interest. Therefore, a better way to identify the remaining neuronal clusters would be to first identify driver lines (Trojan exons or other) expressed only in a few neuronal types: the use of sparse labeling will then allow to identify these neurons based on their morphology if the line is specific enough to visualize the morphology of individual neurons. These driver lines should label at least one neuronal type with no known cluster, and their selection can be guided by the scRNAseq data: even if a line labels more neuronal types than expected based on the mRNA levels in the clusters, it can still be sparse enough to provide genetic access to cell types with no known corresponding clusters. Secondly, clusters can be matched to the cell types labeled by a given driver line by identifying markers of these cell types through immunostaining or in-situ hybridization screens. This step can also be guided by the scRNAseq data, by staining in priority for genes differentially expressed between the clusters characterized by high mRNA levels of the driver line.

2.3. Glial clusters

Almost all the studies presented above focused on obtaining and analyzing clusters for neuronal, and not glial, cell types. However, it is crucial to obtain the transcriptome of all glial types in the visual system as they play very important roles in neuronal circuit function and development (Lago-Baldaia et al., 2020). The recent developmental atlases

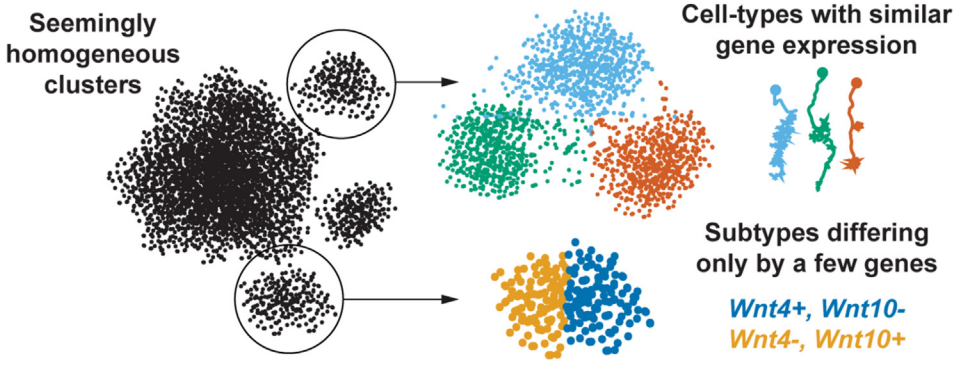
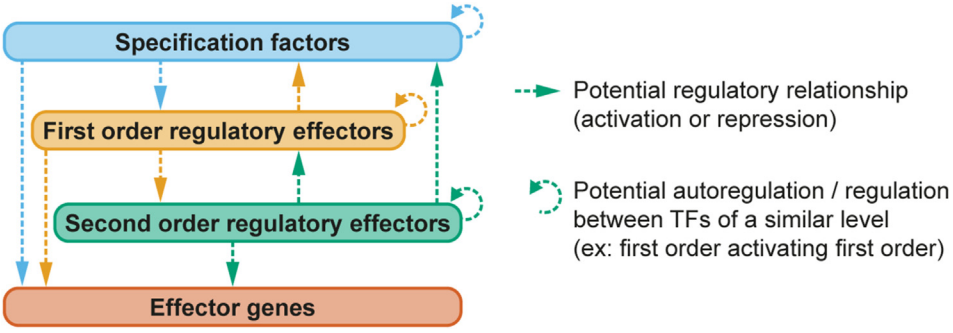


Fig. 2. Potential heterogeneity in seemingly homogeneous clusters. The dots represent single cell transcriptomes, ordered in 2 dimensions by similarity of gene expression. Left: the transcriptomes are organized in 4 clusters of seemingly similar cells. Right: deeper analyses can reveal that these groups comprise either cells from morphologically different neuronal types with closely related transcriptomes, or from morphologically identical neuronal subtypes that differentially express only a few genes.

Box 2
Establishment and maintenance of neuronal identity



Several models have been proposed to explain the establishment and maintenance of neuronal identity, two of the most prominent ones being the concepts of terminal selector (TS (Hobert and Kratsios, 2019),) and core regulatory complex (CoRC (Arendt et al., 2016),). However, these concepts impose strict limitations and, in this review, we chose to use broader terms defined below.

Specification factors: gene expression regulators (TFs, RNA-binding proteins, long-noncoding RNAs, chromatin modifiers …) and secreted molecules whose combination is necessary and sufficient to specify the adult morphological and molecular features of a neuronal type. Specification factors can be expressed at birth of the neuron, or before the neuron is produced and act epigenetically. Specification factors can be expressed or not throughout the life of the neuron, and directly or indirectly regulate the expression of effector genes. The specification factors are therefore the ones selecting the identity of the neurons: all downstream genes are effectors of this selection.

First order regulatory effectors: gene expression regulators (TFs, RNA-binding proteins, long-noncoding RNAs, chromatin modifiers …) directly downstream of the specification factors, and directly or indirectly upstream of effector genes. They include TFs corresponding to the TS and CoRC definitions. However, contrary to TS, they do not have to be expressed throughout development: for instance, some could control axon targeting, therefore the morphology of the neuron, and be expressed only at the beginning of differentiation. Moreover, if their expression is maintained, it does not necessarily need to be through auto-regulation: it can also be maintained by specification factors, other first order regulatory effectors, or by feedback from a second order regulatory effector. Contrary to core regulatory complexes, they also do not have to physically interact and can target either independent or common sets of genes.

Second order regulatory effectors: gene expression regulators (TFs, RNA-binding proteins, long-noncoding RNAs, chromatin modifiers …) downstream of the first order regulatory effectors, and directly or indirectly upstream of effector genes. They do not have to be expressed throughout development, and if they do it is not necessarily through auto-regulation. They do not have to physically interact and can target either independent or common sets of genes, including specification factors, first order regulatory effectors, and other second order regulatory effectors (ex: transcriptional cascades). Notably, TFs can assume different roles through the life of a neuron. For instance, the expression of a specification factor could be turned off early in development and turned back on later, making it a second order regulatory effector.

Effector genes: non-TF genes that are directly or indirectly downstream of the specification factors (therefore they are largely limited to neuronal-type specific genes). They are not required to be expressed in adult, contrary to what is often implied in the concept of "terminal effectors". Indeed, a CSM necessary to target a given layer but not expressed in adults is also an effector of the identity of the cell, as it affects its final morphology.

identified at least 19 glial clusters (Kurmangaliyev et al., 2020; Özel et al., 2020). However, glial clusters seem to be of lower quality than neuronal clusters, i.e. enriched in cells with features of low-quality transcriptomes (low gene count, high mitochondrial gene count), which could be due to a higher sensitivity of glial cells to the dissociation process. This would increase the proportion of glial genes in ambient RNA, and low-quality non-glial transcriptomes would cluster with good quality glial cells. Compared to neuronal clusters, they also contain a more variable proportion of cells originating from different experimental batches (Kurmangaliyev et al., 2020; Özel et al., 2020), indicating that their clustering is partly driven by batch effect. Therefore, rather than directly analyzing glial clusters from these studies, in addition to using dedicated software to remove low-quality transcriptomes (multiplets or ambient RNA (Fleming et al., 2019; Alvarez et al., 2020; Xi and Li, 2020; Yang et al., 2020; Young and Behjati, 2020)) it might be advantageous to separate glial cells from the neuronal cells and cluster them independently. This would allow to reduce the proportion of low-quality transcriptomes in the glial clusters by filtering-out more aggressively the transcriptomes used. Moreover, clustering glial cells in the absence of neurons might increase the relative difference between glia and low-quality non-glial transcriptomes, and therefore allow for a better separation.

3. Use of single-cell approaches to study gene regulation and the link between specification and terminal features

The Drosophila optic lobe consists of ~200 different neuronal types, each of which is specified at the time of neuronal birth. This specification depends on the integration of three mechanisms (Fig. 1): spatial patterning of the neuroepithelium, temporal patterning of the neuroblast, and Notch status of the neuron. Once a neuron is specified, this identity has to be translated into terminal features, such as morphology, connectivity, neurotransmitter identity, etc. Several models have been proposed to explain how cell identity is established and maintained, such as the concepts of terminal selector (Hobert and Kratsios, 2019) or of core regulatory complex (Arendt et al., 2016). However, these impose strong limitations and we chose to use broader terms, as defined in Box 2. While we have a very good understanding of how optic lobe neurons are being specified, we are currently lacking the link between specification and terminal characteristics. Single-cell sequencing offers the opportunity to address this question at different levels.

3.1. Regulation of neuronal characters - investigation of gene regulatory networks

In addition to generic pan-neuronal features, each neuronal type is characterized by a specific set of morphological (neuropil layers targeted, synaptic partners, etc.) and molecular features (cell surface molecules, transporters, neurotransmitter identity, etc.). Understanding the regulation of such features is necessary to understand how neuronal diversity is generated.

The visual system is ideal for this endeavor, as the morphology of each neuronal type is characterized in great detail (Fischbach and Dittrich, 1989; Nern et al., 2015), and we now have access to gene expression during development for more than 170 neuronal clusters (Kurmangaliyev et al., 2020; Özel et al., 2020). One way to study the regulation of a given molecular or morphological feature is to probe for coincident expression between this feature and transcription factors. This can be realized "manually", by comparing the TF expression between a manageable number of cell types, as illustrated by (Kurmangaliyev et al., 2019; Hörmann et al., 2020) who showed that the TF grain instructs both dendritic orientation and axonal targeting of T4/5b and T4/5c neuronal subtypes. However, the large number of neuronal clusters of the transcriptomic atlases also allows the use of machine learning to draw such comparison. For instance, we were able to show that the expression of markers of cholinergic (ChAT), glutamatergic (VGlut), and GABAergic (Gad1) identities is regulated by apterous (ap), traffic jam (tj), and Lim3 transcription factors, respectively (Konstantinides et al., 2018a). This was further validated in vivo: knock-down of apterous, traffic jam, or Lim3 led to reduced expression of ChAT, VGlut, and Gad1 in the optic lobes. Moreover, we showed that the same phenotypes (such as neurotransmitter identity) can be generated by different transcription factors, a phenomenon which we termed phenotypic convergence (Konstantinides et al., 2018a). We found that the expression of 2/3 of all the genes in the optic lobe is better predicted by a combination of TFs than by a single TF. This observation was later generalized beyond the Drosophila optic lobes, in the entire brain (Estacio-Gómez et al., 2020).

In fact, such approaches can be applied to study the regulation of any gene or morphological feature that is expressed in the optic lobes. Numerous gene regulatory inference approaches can be used (Mercatelli et al., 2020) and will be facilitated by the large scale of the newer atlases. Moreover, the use of the developmental atlases can facilitate the inference of the gene regulatory networks that regulate neuronal identity. A first way to do this would be to correlate gene expression not only with the expression of transcription factors, but also with TF accessibility to different regulatory loci. By using new techniques combining RNA- and ATAC-seq in the same individual cells, the accessibility profile of each neuronal cluster can be easily obtained: the mRNA sequenced in each cell of the chromatin accessibility dataset will allow matching it with its corresponding cluster, for instance by using the classifying neural network from (Özel et al., 2020). These accessibility profiles can then be inputted in machine learning models. A second way would be to consider the timing of TF and gene expression when trying to identify gene regulatory interactions, a piece of information that is provided by the developmental atlases. For instance, morphological features of adult neurons are more likely to be correlated with mid-pupal transcriptomes, since genes that are important for proper connectivity and synaptogenesis are enriched around P50 when these processes take place (Kurmangaliyev et al., 2020; Özel et al., 2020). On the other hand, "functional" genes, such as ion channels, are more highly expressed in adulthood.

The establishment and maintenance of neuronal features requires some continuity in gene expression throughout neuronal life, which could be established by a transcriptional cascade (in which different TFs are expressed at different developmental stages), or by maintaining the expression of a "selector" (TF or combination of TFs regulating all specific features of a neuronal type) throughout development, or by a blend of both. For instance, the expression of a first order regulatory effector involved in synaptic specificity might be only maintained from the birth of a neuron until P50. The existence of such a transcriptional cascade is difficult to test, as it requires identifying TFs expressed at different developmental stages that are linked by regulatory relationships. On the other hand, the existence of "selectors" is easier to test. They would be required to be expressed during the whole life of the neuron, and to be necessary and sufficient to establish and maintain all adult features of a neuronal type. The first requirement is fulfilled by numerous TFs, and we have defined for all our neuronal clusters specific combinations of TFs that are maintained throughout differentiation (Özel et al., 2020). The median size of these combinations of TFs is 8, and it is therefore unlikely that in general a unique TF would act as a "selector". There is little doubt that at least some of these TFs are necessary to establish and maintain a subset of the adult features of their neuronal types, but whether they are sufficient will need to be carefully tested through imaging techniques (for the morphology) and transcriptomics (for the molecular phenotype).

3.2. How do temporal and spatial transcription factors regulate neuronal identity?

Spatial and temporal transcription factors (sTFs and tTFs, Fig. 1B and C) are expressed in neuroepithelial cells and neuroblasts, respectively, and regulate the identity of the neuronal progeny (Li et al., 2013; Erclik et al., 2017). In many instances, sTFs and tTFs are not expressed in the neurons. Therefore, it is unclear how these factors act to endow identity to the neurons in the absence of their expression.

Temporal origin: Temporal transcription factors are expressed sequentially in neuroblasts, altering their capacity to generate neuronal cell types. This regulation can happen both through direct effect on effector genes, and through the activation of expression of downstream (first-order) regulatory effectors (Box 2). A question that remains unanswered is whether a neuroblast needs to transition through all previous temporal stages to acquire its competence to generate a specific cell type. Sporadic evidence suggest that this is the case: it was shown recently that for a neuronal type to acquire the proper identity, the neuroblast has to proceed through all the previous temporal windows (Naidu et al., 2020). In this case, the T1 cell type is generated during the Dichaete temporal window. However, T1 expresses three transcription factors that are activated by eyeless, sloppy paired, and Dichaete (oc, Sox102F and Ets65A, respectively), in their respective temporal windows. This means that should the neuroblast skip one of the three temporal windows, T1 will not be generated (Naidu et al., 2020). While this is an intriguing mechanism, which "justifies" the use of temporal patterning as a mechanism to generate diversity (each temporal window adds an extra transcription factor), it is unclear whether it is generalizable. To address this question, one would need to have access to a thorough description of the lineage and the cell types that are generated from each temporal window and use single-cell sequencing data to identify the first-order regulatory effectors that are shared in cell types of the same temporal origin. This would immediately generate candidate regulatory interactions between the tTFs and the first-order regulatory effectors and will provide further insights into the role of the tTFs in activating the first order regulatory effectors, and, hence, regulating neuronal diversity.

However, the identification of the temporal origin of a cell type is not trivial. One could envisage the use of genetic tricks (for example, a combination of MARCM (Lee and Luo, 2001) technologies with memory cassettes) to stably mark only one of the progeny of a dividing neuroblast at a certain temporal window. This, on top of being genetically challenging, may also be subject to maturation delays of Gal4 and could lead to data mis-interpretation. A viable alternative would be to use single-cell sequencing in late larval stages in entire optic lobes that would contain neuroblasts, GMCs, and neurons. This could allow the identification of the temporal origin of neurons based on the expression of temporal factors in their parental GMCs, which should be closely associated with them in the multidimensional space (Fig. 3A). A third, however incomplete, approach would be to use the expression of downstream TFs as a

proxy for temporal origin. For example, we know that Bsh + neurons are born from the hth temporal window, Dfr + neurons arise from ey + neuroblasts, Toy+ and Sox102F + neurons come from the slp and D temporal windows (Li et al., 2013; Naidu et al., 2020), and Ets65A + neurons come from the D temporal window (Naidu et al., 2020). Using these genes as proxy, we should be able to assign putative temporal origin to all sequenced and annotated neuronal types. Finally, a more elaborate way to identify the temporal origin of neurons would be to use recently established cell lineage reconstruction techniques that combine single-cell sequencing and CRISPR-mediated genetic scarring to infer developmental lineages (Alemany et al., 2018; Raj et al., 2018; Spanjaard et al., 2018) that could then be associated with the tTF cascade.

Finally, two recent papers used a combination of single-cell sequencing and trajectory inference (Saelens et al., 2019) to acquire the complete list of tTFs. The neuroblasts in the OPC are progressively generated from medial to lateral; as a result, a wandering third instar larval brain contains neuroblasts at different levels of maturation (Fig. 1B). Therefore, single-cell sequencing of neuroblasts at L3 generated transcriptomes for neuroblasts of different ages. Then, using trajectory inference algorithms, the authors reconstructed the developmental trajectory of the neuroblasts: TFs expressed only in part of this trajectory were also temporarily expressed in-vivo (Konstantinides et al., 2021; Zhu et al., 2021).

Spatial origin: Temporal and spatial factors likely use different mechanisms to regulate neuronal diversity. This is because spatial factors (contrary to the temporal factors) are mostly expressed in the neuroepithelium and not in the neural stem cells (Erclik et al., 2017). In the embryonic ventral nerve cord, neuroblasts differ in their chromatin landscape depending on their spatial identity, which results in the first temporal transcription factor, hunchback, to bind distinct targets in different neuroblasts (Sen et al., 2019). Similar mechanisms to integrate the action of temporally segregated transcription factors have been discovered in other systems. In mouse neuronal stem cells, different proneural genes (Ascl1 and Neurog2) set different chromatin landscapes for the same downstream transcription factors to act (Aydin et al., 2019). In the two neuronal lineages that give rise to the ASE neurons in C. elegans embryos, tbx-37/38 primes the lsy-6 microRNA locus for expression, which is only activated four divisions later by the Che-1 transcription factor (Charest et al., 2020). Thus, it is very likely that the role of sTFs in the optic lobes is also to setup the chromatin landscape for tTF and downstream transcription factors to act. To show whether this is indeed the case, a single-cell ATAC-seq atlas of neurons that come from different spatial domains during the development of the visual system could be matched to the existing single-cell RNA-seq atlas to query for chromatin landscape differences.

To assign a spatial origin to a neuron, one can use memory cassettes during L3 to permanently mark all the cells that are born from a specific spatial domain (e.g. Vsx). Then, these marked cells can be isolated using FACS in the adult and single-cell sequenced. The transcriptomes can then be mapped to the annotated atlases using either integration algorithms such as the one available in Seurat v3 or the neural network that was developed to annotate optic lobe cells (Özel et al., 2020).

Finally, to identify all sTFs, single-cell sequencing of neuroepithelial cells at L3 larval stage could allow to cluster them in groups sharing a common spatial specification factor (the ones already known would serve as positive controls), or alternatively, spatial transcriptomics in L3 optic lobes will provide a direct method to systematically uncover genes expressed in a neuroepithelial subdomain (Sun et al., 2020). The candidate sTFs can then be experimentally validated for their ability to define neural diversity.

Using these data, we could then develop models for the role of the tTFs and sTFs in regulating neuronal features, for instance by comparing gene expression, chromatin accessibility or morphological features in several neurons from the same temporal window or spatial origin. These models can then be tested genetically and lead to general conclusions regarding neurogenesis.

3.3. *Regulation of neuronal characters by extrinsic signals – the role of ecdysone*

Although neuronal identity in the Drosophila optic lobes is almost exclusively cell autonomous and does not rely on extrinsic signals, the role of the environment in the implementation of identity (i.e. differentiation) is undoubted. Transcriptomic studies indicate that neuronal development in the visual system could require the coordinated expression in all neuronal types of both pan-neuronal and neuronal type specific genes. Indeed, it was shown by following 88 neuronal and 6 glial clusters from P24 (i.e. 24% of pupal development) onwards (Kurmangaliyev et al., 2020) that the expression of 200 neuron specific genes was pan-neuronally coordinated during development. Although these genes exhibited various expression dynamics during development, for each given gene these dynamics were the same in almost all neuronal types. This indicates that pan-neuronally coordinated genes may be involved in different steps of neuronal development, and that these steps occur simultaneously in all optic lobe neuronal types. Because these genes are pan-neuronally expressed, they are unlikely to be sufficient for establishing neuronal type specific features, but they may fulfill permissive roles for neuronal development and function. For instance, genes whose expression peaks early could be necessary for axon growth or guidance, while those peaking later might be necessary for synapse formation or cell excitability. Moreover, some of these genes are involved in intercellular communication or encode RNA-binding proteins, indicating potential tissue-wide changes in signal transduction or post-transcriptional

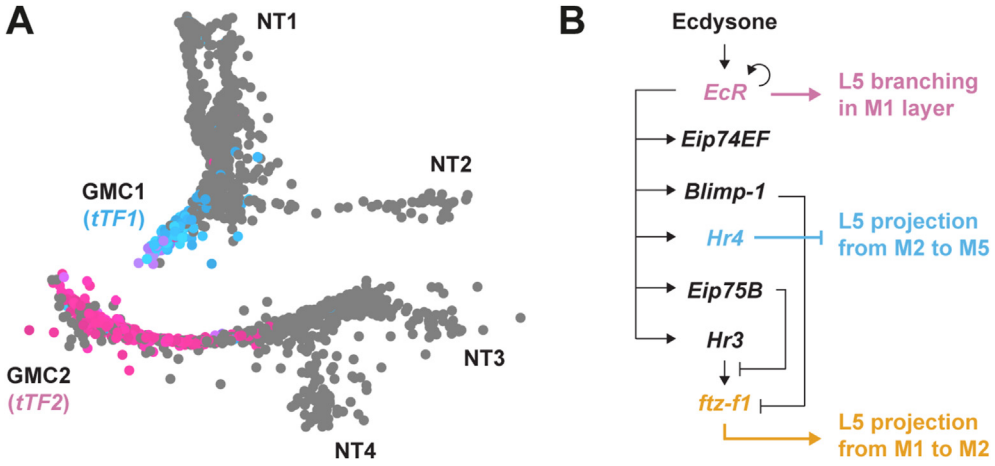


Fig. 3. Neuronal specification
A – UMAP plot of four neuronal types (NT1-4) during larval development. The dots represent single cell transcriptomes, ordered in 2 dimensions by similarity of gene expression. Due to the presence of their parental GMCs in the sequencing, one might be able to assign temporal identity to neuronal types based on their proximity to tTF-expressing GMCs.
B - Members of the ecdysone responsive transcription factor cascade and their involvement in L5 neurons development. Adapted from (Kurmangaliyev et al., 2020).

regulation. The temporal coordination of gene expression during visual system development was also emphasized by analyzing variations in transcriptomic diversity. First, at any pupal stage optic lobe neuronal types comprise individual neurons produced at various timepoints (up to 2 days apart) during larval development (Fig. 1B). It was shown that at early pupal stages the transcriptome of neurons from the same type but of different ages were transcriptionally different, but later converge towards a common state that is reached by P30 (Özel et al., 2020). Second, it was also shown that a peak of transcriptional diversity between all neuronal clusters occurs shortly after, from P40 to P70 (Özel et al., 2020).

These optic-lobe wide variations in transcriptomic diversity and the coordinated expression of some pan-neuronal genes suggests the presence of at least one extrinsic signal that orchestrates this coordination. Several findings suggest that a peak of ecdysone signaling occurring between P30 and P40 (Handler, 1982) could be such a signal, acting via ecdysone responsive TFs (Fig. 3B) (Jain et al., 2020; Kurmangaliyev et al., 2020; Özel et al., 2020). The role of ecdysone signaling on gene regulation was explored in detail in lamina neurons L1-5 by genetic perturbations of ecdysone signaling associated with transcriptomic studies, in which the authors showed that genes with temporal variation of expression are enriched in targets of ecdysone signaling (Jain et al., 2020). Importantly, they also showed that despite ecdysone signaling being pan-neuronal, it could have neuronal type-specific effects. Expression of a dominant negative version of the ecdysone receptor (EcR) often affected a given gene differently in the different lamina neurons, with sometimes opposite variations of expression. Moreover, EcR target genes in the L3 neurons were also enriched in erm targets, indicating that the neuronal-type specificity of EcR disruption could be due to the action of co-factors (erm in the case of L3, other co-factors in other cell types).

The mid-developmental peak of transcriptomic diversity is associated with an upregulation of cell surface molecule (CSM) expression (Özel et al., 2020), which correlates with synapse formation in the optic lobe and occurs just after the peak of ecdysone signaling. This suggests that this peak regulates circuit formation by controlling the expression of hundreds of CSMs during pupal development, which is consistent with work by (Jain et al., 2020). Indeed, the 921 genes they identified downstream of ecdysone signaling in L1-5 are enriched in genes encoding Immunoglobulin Superfamily proteins as well as in GO terms associated with wiring (Jain et al., 2020). In addition, immunostainings in ex-vivo cultures of optic lobes also show that the expression of the CSMs dpr15, dpr17 and dpr6 is dependent on the presence of ecdysone in the medium. Moreover, specific expression of a dominant negative EcR in various cell-types results in mistargeting of R8 photoreceptors, formation of ectopic synapses in the lamina, disorganization of medullar arbors of lamina neurons, and affects the morphology of T4/T5 neuron terminals in the lobula plate. Lastly (Jain et al., 2020), have also shown that different members of the cascade of ecdysone responsive transcription factors can be involved in the step-wise development of neurons (Fig. 3B). They studied L5 neurons, which first send projections to medulla layer 1 around P48, then to medulla layer 2 around P75. The first step is blocked by EcR dominant negative or EcR RNAi, and the second step (but not the first one) by Hr3 and ftz-f1 RNAi (Hr3 and ftz-f1 are themselves EcR targets). Moreover, Hr4 (another EcR target) RNAi causes secondary projections to medulla layer 5. Therefore, different ecdysone responsive transcription factors are involved in different steps of L5 wiring. Consistently, sequencing of L5 cells shows that expression of EcR dominant negative or Hr3 RNAi affects two different sets of CSMs, suggesting that EcR may regulate the CSMs that are responsible for synaptic specificity in layer M1 while Hr3 regulates the ones that control synaptic specificity in layer M2.

These results show that extrinsic signals regulate critical aspects of neuronal identity, such as the timing of the differentiation process both in a pan-neuronal or a cell type-specific manner. Beyond the lamina neurons, the developmental atlases now allow studying the pan-neuronal and specific effects of ecdysone in large populations of neurons, or even the whole optic lobe. Indeed, using data transfer, even a shallow sequencing of the optic lobe after genetic perturbation can produce a "perturbation atlas" with a similar resolution and annotation as the current atlases.

4. Use of single-cell approaches to study neuronal circuit development

In order to form functional circuits, neurons must first reach their target neuropils, then arborize in their target layer, and finally form synapses with their correct synaptic partners. At the time of synapse formation, each growth cone is surrounded by dozens of potential partners yet exhibits synaptic specificity by connecting only to a few stereotyped partners. In the Drosophila visual system as in any other neural system, neuronal targeting and synaptic specificity are mediated by communication between neurons and their environment (surrounding neurons, glia and extracellular matrix) through cell surface and secreted molecules (Plazaola-Sasieta et al., 2017; Sanes and Zipursky, 2020). Despite a general understanding of many mechanisms underlying neural circuit development, the mechanistic detail and the identity of the molecules involved are mostly unknown.

Single-cell transcriptomic atlases, and in particular those that span development, constitute an ideal resource to identify these molecular determinants. Moreover, two other resources considerably facilitate the study of synaptic specificity in Drosophila. First, the detailed knowledge of the connectome of the lamina (Meinertzhagen and O'Neil, 1991; Meinertzhagen and Sorra, 2001; Rivera-Alba et al., 2011), medulla (Takemura et al., 2008; Takemura et al., 2013, 2015), and of part of the lobula and lobula plate (Shinomiya and Horne, 2019; Shinomiya and Huang, 2019). Second, the interactome of 202 CSMs has been characterized (Ozkan et al., 2013; Cosmanescu et al., 2018). Together, one could expect that these resources will allow for important insights into how neuronal circuits are built and especially into how synaptic specificity is achieved, one of the least understood aspects of neuronal circuit development (Sanes and Zipursky, 2020). However, since progress has been limited so far, we will discuss how the transcriptomic datasets advocate for new approaches in the study of neuronal circuits development.

4.1. Neuronal circuit development is unlikely to be explained by a simple molecular code

Numerous known mechanisms are involved in synaptic specificity, including the pruning of synapses between incorrect partners, or the death of improperly connected cells (Sanes and Yamagata, 2009). We will focus on one of the most studied models, initially formulated by Langley and formalized by Sperry (Langley, 1895; Sperry, 1963), in which growth cones carry CSMs and establish synapses only with neuronal types expressing compatible CSMs on their surface. The "molecular labels" allowing the correct matching of the neuronal types have been studied for decades in both vertebrates and invertebrates, and the progresses have been recently reviewed in (Sanes and Zipursky, 2020). However, the details of the mechanisms, such as the number of CSM interactions necessary to establish synapses, are debated. It has been proposed that many of these "molecular labels" come from a few diversified families of cell surface proteins, two of the best studied in Drosophila being the defective proboscis extension response (dpr) and the Dpr-interacting protein (DIP) families (Ozkan et al., 2013; Carrillo et al., 2015; Tan et al., 2015; Cosmanescu et al., 2018). They respectively comprise 21 and 11 members, which can bind to each other either homophilically or heterophilically with varying affinities (Fig. 4A). It has been proposed that the presence of compatible DIPs and Dprs on the projections of two neuronal types in close proximity could instruct the formation of synapses between them. However, although a few examples of such interactions have been described, disrupting DIP or dpr expression most often leads to only partial disruption of connectivity (Courgeon and Desplan, 2019; Sanes and Zipursky, 2020). These observations have

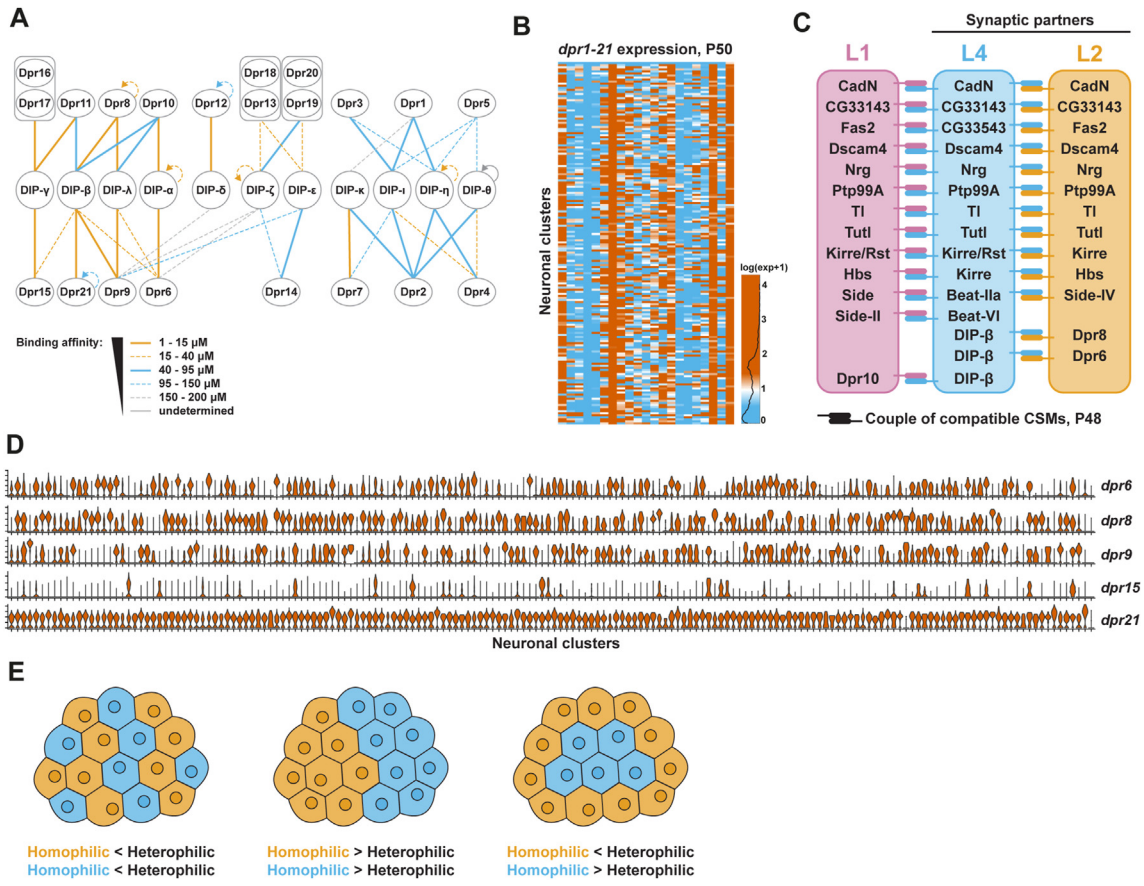


Fig. 4. Molecular mechanisms of neuronal circuit formation

A – Binding affinities of the DIP and Dpr proteins. Adapted from (Cosmanescu et al., 2018).

B – Expression of the 21 dpr coding genes at P50. The black line in the scale represents the density of dpr1-21 expression values. Data from (Özel et al., 2020).

C – Expression of compatible couples of Cell Surface Molecules (CSMs) in lamina neurons L1, L2 and L4 at P48. Adapted from (Kurmangaliyev et al., 2020).

D – Expression of dpr genes with high affinity (<40 µM) to DIP-β, in all neuronal clusters at P50. Data from (Özel et al., 2020).

E – Spatial organization of two populations of cells (orange and blue) as a function of their homophilic and heterophilic membrane adhesivity.

recently led to a more nuanced view of the role of DIP and dpr (and other CSMs), in which a large number of CSMs is required to pair two neuronal types, and these CSMs do not instruct synapse formation in an "all-or-nothing" fashion but rather affect the relative "preference" of a neuronal type towards another (Sanes and Zipursky, 2020).

The developmental transcriptomic atlases very strongly support this more nuanced view of synaptic specificity regulation, as many non-synaptic partners projecting to a common layer express compatible CSMs. In fact, this situation is likely to be the rule rather than the exception due to the widespread expression of many cell surface molecules, as can be seen for the dprs in Fig. 4B or for dozens of CSMs in 13 neurons in (Kurmangaliyev et al., 2020). A transcriptomic study of 5 lamina neurons and two photoreceptors at P40 also showed that hundreds of CSMs are expressed in each cell type, and that between any given pair 49–168 CSMs are differentially expressed (Tan et al., 2015). Moreover, by studying CSM expression in 12 neurons projecting to the lamina (Davis et al., 2020), showed that any pair of these neurons express at least 30 couples of compatible CSMs, and that the number of compatible CSMs expressed is uncorrelated with the number of synapses made by these pairs of neuronal types. Lastly, as an example, virtually all neuronal clusters express one or more dpr with high affinity for DIP-β at P50 (Fig. 4D). Therefore, if the pairing of a DIP/dpr couple were sufficient to establish a synapse, any neuronal type expressing DIP-β could theoretically form synapses with almost all of the neurons in its vicinity. This is obviously not the case, and it has important implications for candidate screens: the expression of a couple of compatible CSMs between two synaptic partners is a weak indication that these CSMs alone play a significant role in the establishment of these synapses.

In addition to non-synaptic partners expressing compatible CSMs, synaptic partners usually express multiple couples of interacting CSMs. Such redundancy has historically been proposed to explain why disrupting the expression of a CSM produces mild or inexistent phenotypes more often than not (Sanes and Zipursky, 2020). This is illustrated in detail (Fig. 4C) by an analysis of the neuronal types L4 and L2, which form synapses in the proximal part of the lamina (Xu et al., 2019; Kurmangaliyev et al., 2020). L4 is the only lamina neuron expressing DIP-β, and L2 expresses several dprs that can interact with DIP-β. If DIP/dpr interactions were necessary and sufficient to establish synaptic specificity, it would be reasonable to expect that DIP-β and its compatible dprs would instruct the establishment of L2-L4 synapses. However, although DIP-β loss of function produces ectopic synapses in the distal lamina, synapses still form in the proximal lamina. This could be explained by the fact that 11 other couples of interacting CSMs are still expressed between L4 and L2 neurons. Interestingly, L1 and L4 similarly also express these 11 couples of interacting CSM but do not from synapses, which further illustrates that non interacting partners can express a large number of compatible CSMs.

In addition to its implications for the regulation of synaptic specificity, the large number of CSMs expressed in each neuronal type leads to a large number of candidates for any neuronal targeting feature. This is for instance shown by two developmental studies of the T4 and T5 neuronal types, which are ideally suited to the study of both neuronal targeting and synaptic specificity (Kurmangaliyev et al., 2019; Hörmann et al., 2020). They each comprise 4 neuronal subtypes (a, b, c and d),

projecting to different lobula plate layers and to the same medulla layer (T4 subtypes) or lobula layer (T5 subtypes). The dendrites of each of the T4/T5 subtypes are orientated in one of 4 cardinal directions. Because each subtype seemingly differs only by one of the lobula plate layers they target and by the orientation of their dendrites in the medulla or lobula, CSMs differentially expressed between them are ideal candidates for the regulation of their morphology. In (Kurmangaliyev et al., 2019), several CSMs were found differentially expressed between neurons of the same type but targeting different lobula plate layer. For instance, just among the top 20 genes differentially expressed between T4a and T4c were the CSM Fas2, Ptp99A, beat-IIIb, mspo, CG15765, robo2, side-IV, Con, and klg. Similarly, (Hörmann et al., 2020) found 62 CSMs differentially expressed between T4/T5 neurons (including Fas2, beat-IIIb, robo2, side-IV, Con, and klg).

Lastly, these results emphasize how difficult it will be to assign a role to the different CSMs involved in establishing neuronal circuits. Indeed, the role of a candidate CSM is usually tested by assays only able to detect strong phenotypes, such as mislocalization of neuronal arbors. However, since most morphological features are likely to be encoded by redundant CSMs, disrupting the expression of a single candidate CSM is likely to have more subtle effects and should therefore be assessed by assays able to detect subtle changes in neuron morphology (for instance using sparse labeling) and in the number of synapses between neuronal types. Another approach could be to favor strong phenotypes by disrupting the expression of multiple CSMs at once, for instance by using an array of CRISPR guides. Coupled with quantitative assays of synaptic specificity (ex: number of synapses lost or of ectopic synapses), the disruption of varied proportions of CSM interactions between two neurons could also help defining general rules for synaptic specificity. Lastly, the developmental transcriptomic atlases should help for the identification of candidate CSMs, by allowing to study together many neuronal types sharing a common feature. Indeed, if most neuronal types targeting a given layer specifically express a shared CSM, this CSM is likely involved in targeting to this layer, although different neuronal types could also reach a common layer by expressing different sets of CSMs.

4.2. What is missing to understand the genetic regulation of neuronal circuit development?

The fact that many neurons sharing compatible CSMs do not form synapses could, in some cases, be explained by CSMs mediating a repulsive rather than an adhesive interaction. However, several results suggest that understanding synaptic specificity will require models taking into account several additional parameters.

Subcellular localization: The subcellular localization of CSM proteins as well as the localization of neuronal projections during development might have an important role in establishing synaptic specificity. Indeed, even if two neuronal types project to a common layer in adults and express compatible CSMs during synapse formation, they would not form common synapses if these CSMs were not in close proximity during synapse formation. Optic lobe layers are progressively constructed, and neuronal types can target them in a stepwise fashion (Plazaola-Sasieta et al., 2017): for instance, as discussed previously, L5 neurons target different medulla layers at different stages of development. Correlating the CSMs that are expressed in a neuronal type at the time of synapse formation with the localization of its arborizations will therefore be necessary to understand synaptic specificity. Although it would be experimentally challenging to obtain such data for all neuronal types, case studies might lead to the identification of general mechanisms. In addition, the restriction of CSMs to specific subcellular locations is probably a common occurrence. For instance, in (Kurmangaliyev et al., 2019), klg is expressed at P48 in subtypes of T4 and T5 that target 4 different layers of the optic lobe (M10, Lo1, LoPc and LoPd). However, immunostaining against klg.GFP only labels strongly 2 of these layers (LoPc and LoPd). Again, obtaining the subcellular localization of all CSMs in all neuronal types during development would be very challenging, but it can easily be approximated by confocal imaging for a manageable number of CSMs and neuronal types.

In vivo binding affinities: The establishment of synapses between two neuronal types might not be encoded by one or a few CSMs, but instead by the combination of numerous CSMs. Importantly, the interaction between each couple (or more complex arrangements) of compatible CSMs is characterized by different affinities. Therefore, to describe the local interaction between patches of membranes from two neuronal types, several parameters must be taken into account: the CSMs present, their concentration, and the affinities of each couple of CSMs. Notably, this interaction is not a linear combination of the parameters: at similar concentration, CSM couples with higher affinity will produce more dimers than couples with lower affinity, in addition to the higher contribution of each of these couples to the adhesive strength between the two membrane patches (Katsamba et al., 2009). Although the interactome of 202 CSMs has been determined in Drosophila, their binding affinities have been much less studied and, when obtained, were obtained in vitro. Therefore, a crucial piece of information might be largely missing to understand synaptic specificity. The expression levels of all CSMs mRNA is now well characterized during development in most optic lobe neurons (Kurmangaliyev et al., 2020; Özel et al., 2020). However, these could differ from the CSM protein levels, which are the real actors of membrane adhesion. Moreover, signaling pathways activated by the binding of compatible CSMs should also be taken into account (Dalva et al., 2007), a factor that remains largely unexplored.

Neuronal activity: intercellular communication might play a role in synapse formation. Indeed, it has been shown in vertebrates that neurotransmission-mediated processes are involved in synaptic specificity by promoting either the reinforcement or the elimination of synapses (Okawa et al., 2014). Such a mechanism is appealing as it would increase the reproducibility of synapse formation and reduce the number of potential synaptic partners, by allowing only synapses between neuronal types that can communicate with each other (i.e. pre- and post-synaptic cells with compatible neurotransmitter release and reception machinery). However, circuit formation in Drosophila is thought to be mostly independent of neuronal activity, as it was shown that disrupting activity in photoreceptors does not affect their axonal targeting, column organization or synapse numbers, and the latter is also unaffected by changing column composition (Hiesinger et al., 2006). Nevertheless, photoreceptors represent only a small fraction of the visual system neuronal diversity, and activity could affect the proportion of synapses they make with their different partners without affecting the total number of synapses per photoreceptor. Therefore, whether intercellular communication affects synapse formation is an open question and is suggested by several experimental observations in the central nervous system, as reviewed in (Akin and Zipursky, 2020), and in the visual system. Indeed, among the pan-neuronally coordinated genes, the expression of most of the ion channels peak during development and not at adulthood, when they are actually required for neuronal communication, and transcripts for genes involved in neurotransmission are detected early during development (Kurmangaliyev et al., 2020). Moreover, it was shown that the optic lobe exhibits a cell type specific, patterned, stimulus-independent neuronal activity (PSINA) that starts in a subset of processes at P50 and expands to the whole optic lobe by P55 (Akin et al., 2019). PSINA is correlated with glutamate release and changes in membrane voltage in neurons. Very importantly, PSINA patterns are cell-type specific, stereotyped, correlated between synaptic partners, and uncorrelated between non-synaptic partners. Expressing the tetanus toxin in the presynaptic cell of a neuronal pair disrupts the correlation between their PSINA activities, while doing so in the post-synaptic cell does not affect the correlation, indicating that the correlation of PSINA activities requires synaptic activity. Disrupting PSINA activity also "leads to cell-type specific changes in synapse counts in the visual system" (Akin and Zipursky, 2020). Moreover, complementary activity is observed in neurons and astrocytes. Astrocytes have been shown to invade neuropils of the central nervous system during

synaptogenesis, and the number of synapses decreases by up to 50% upon astrocyte ablation (Muthukumar et al., 2014). In addition, expression of the pan-neuronally regulated gene ShakB peaks at mid-pupation, and the gap junction protein it encodes is important for the formation of PSINA in the visual system (Kurmangaliyev et al., 2020). Lastly Hr38, a conserved insect neural activity marker gene and a mediator of an ecdysteroid signaling pathway independent of EcR (Baker et al., 2003), is also a pan-neuronally regulated gene and its expression increases after P36 (Kurmangaliyev et al., 2020). Therefore, ecdysone signaling could trigger the expression neuronal activity related genes (ShakB, Hr38) around the time of synapse formation. Importantly, the correlation of PSINA activity between synaptic partners indicates that some synapses are already present. Therefore, activity is unlikely to be sufficient to specify synaptic specificity but could affect the relative number of synapses made between different potential synaptic partners. Consistently, a study has shown that blocking synaptic transmission in mechanosensory chordotonal neurons (in a larval abdominal segment) does not affect the identity of their post-synaptic partners, but does affect the proportion of the total number of synapses with them (Valdes-Aleman et al., 2021).

Synaptic specificity or synaptic selectivity? The formation of synapses between two neurites might not be an "all-or-nothing" phenomenon, in which synapses are formed each time a certain "affinity threshold" is exceeded. Rather, in each region where they project, neurons might "rank" potential partners and only connect to the highest ranked ones based on a competitive process. In this case, it might be more precise to talk about synaptic selectivity rather than synaptic specificity (Sanes and Zipursky, 2020). In addition to being more consistent with the observations made in the transcriptomic datasets, some experimental observations support this model. For instance, upon deletion of DIP-β in L4 neurons, they form ectopic synapses in the distal part of the lamina: although L4 cells had the molecules required to make these synapses all along, they were not preferred when DIP-β was expressed (Xu et al., 2019). A competitive model of synaptic specificity requires mechanisms by which neurites evaluate different potential partners, and form synapses only with the highest-ranking ones. A simple and passive mechanism could be based purely on membrane adhesivity in a manner analogous to the differential adhesion hypothesis. It postulates that a population of cell types with various adhesive affinities to each other, and for which adhesive interactions are dynamic, will adopt a spatial organization that maximizes adhesive stability (Steinberg, 1970). This is often illustrated with populations of two cells with uniform distribution of CSM on their membrane (Fig. 4E) but can be applied to the local interactions between dozens of neurites with different adhesive strengths to each other. For instance, N-cadherin levels are responsible for the spatial organization of photoreceptors, lamina and medulla neurons terminals (Schwabe et al., 2014; Trush et al., 2019). A second passive mechanism can be imagined if neuronal types form a number of synapses intrinsically determined (but not necessarily constant between individuals), and synapses between pairs of neurites with the highest affinity have a competitive advantage (by being either preferentially formed or stabilized). In that case, the proportion (but not the number) of synapses made with each of their partners will be stereotyped. It was for instance shown that R7 neurons form an intrinsically determined number of synapses by the competitive accumulation of a limited resource, the synaptic seeding factors, in only 1–2 filipodia at a time. Notably, here the competitive mechanism does not impact synaptic specificity but the number of synapses produced (Özel et al., 2019). However, such a mechanism could be involved in synaptic specificity if the accumulation of synaptic seeding factors preferentially occurred between filipodia pairs of the highest affinity. Lastly, if synapses between neurites of the highest affinity were to be produced first, synaptic specificity could also be established by the active elimination of all other synapses. In C. elegans for instance, an E3 ubiquitin ligase complex is responsible for the degradation of presynaptic sites in the HSNL neuron, excepted for those containing the synaptic adhesion molecule SYG-1 that inhibits assembly of the complex (Ding et al., 2007). The preferential accumulation of such a "stabilizing factor" in synapses between filipodia with the highest affinity would also confer them a competitive advantage. Notably, all these examples of mechanisms would lead to reproducible connectivity patterns in wild type animals but are very different from a "hard" encoding of synaptic specificity by a fixed molecular code. Indeed, depleting a CSM in a given neuronal type would only result in subtle changes in the ranking of its preferred synaptic partners and not in a complete loss of synaptic specificity, which is consistent with the mild phenotypes usually observed upon such modifications.

The potentially very complex nature of synaptic specificity regulation makes it difficult to study exclusively by traditional approaches, such as screening for candidate genes one neuronal type at a time. Bioinformatic modeling can help produce falsifiable hypotheses, and therefore to identify potential mechanisms regulating synaptic specificity, based on the study of dozens of neuronal types at once. Such models should be required to accurately predict both synaptic and non-synaptic partners based on the developmental transcriptomic atlases and the connectome. Moreover, the effect of all parameters discussed above (such as CSM subcellular localization) on the accuracy of these predictions can be tested in silico and guide subsequent in vivo validations.

5. Use of single-cell approaches to study visual system evolution

Neurons represent the most diverse cell types in the animal body. Understanding how this remarkable diversity has evolved over the hundreds of millions of years of animal evolution is a fascinating, but still elusive, subject. We have good hypotheses for how neurons first appeared and how they assembled their molecular machinery (Achim and Arendt, 2014; Arendt, 2020). Moreover, recent studies have addressed the evolution of neuronal structures in different vertebrates (Tosches et al., 2018; Norimoto et al., 2020). However, we are lacking good examples for how neuronal cell types evolve. To study neuronal type evolution, we need to perform cross-species comparisons; the advent of single-cell sequencing allows us to study neuronal systems in different animals, even in non-genetically accessible ones, at the level of individual neuronal types.

5.1. Neuronal type evolution

Neuronal types are the basic units of a nervous system; however, the definition of a neuronal type is not always straightforward. A neuronal type has been classically defined as a group of neurons with similar morphology and function that are different from those of other neuronal types. Recent transcriptomic studies in the Drosophila brain have shown that morphological neuronal types agree to a great extent with transcriptomic neuronal types (H. Li et al., 2020; Kurmangaliyev et al., 2020; Özel et al., 2020). However, studies both from the visual and olfactory systems have identified subtypes of morphologically identical cell types, i.e. cell types with the same morphology, but different transcriptomes (Li et al., 2017; Özel et al., 2020). Similar observations have also been made in mice, where the transcriptome appears to have a higher resolution than morphology and physiology (Scala et al., 2020). This transcriptomic diversity is driven by the cell type-specific expression of different genes that are regulated by a set of transcription factors, which can be thought of as the identity of each neuronal type.

A definition of neuronal types based only on their morphology and function does not take into account their evolutionary history: two neuronal types with similar morphology and function can be evolutionarily more distant than two neuronal types with divergent morphologies (Fig. 5). This caveat can be mitigated by the use of single-cell sequencing technologies, which now allows us to study the cell type composition of any neuronal tissue of any animal we have access to at the transcriptomic level. Then, we can compare the neuronal type compositions between different species and try to generate cell type trees, in a fashion similar to phylogenetic trees. This comparison could be made either at the level of the entire transcriptome or at the level of the transcription factors

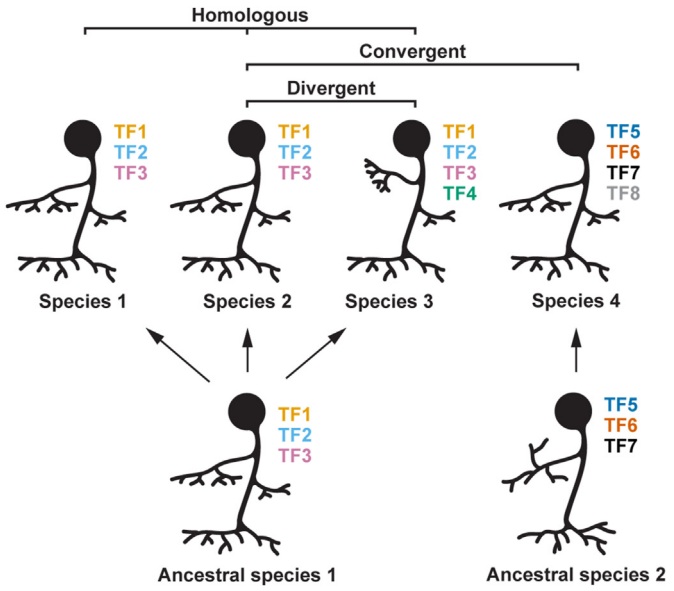


Fig. 5. Neuronal type evolution. Transcription factors can be used to infer evolutionary relationships of homologous cell types. Cell types that share evolutionary history should express a similar set of identity transcription factors. While cases of phenotypic convergence and divergence may blur the morphological comparison of the cell types, comparison of transcription factor signatures should resolve convergent from homologous cell types.

(Konstantinides et al., 2018b) (Fig. 5). In the former scenario, one could use batch-effect correction (data integration) algorithms (Tran et al., 2020) to integrate the single-cell sequencing data from different species, correcting essentially for the batch effect introduced by the independent evolution of cell types upon speciation. Although feasible, this might be blurred by cases of convergence of different cell types (see section 5.2) as well as by divergence of conserved cell types, especially in cases of non-closely related species (Fig. 5). However, two different cell types with separate evolutionary history are unlikely to share common gene regulatory networks, even if they present convergent effector gene expression. Therefore, differentiating between homologous and convergent cell types can benefit from the comparison of transcription factor expression between species (Fig. 5) (Tosches et al., 2018). Ideally, this analysis would be limited to the specification factors and their downstream TFs and would require their identification in each neuronal type before performing such comparisons. Finally, an intermediate approach would be the comparison of entire transcription factor signatures between cell types of different species.

The insect visual system represents an ideal tissue for such studies (Perry et al., 2017). The repetitive nature of the optic lobe neuropils results in a large number of most cell types, which facilitates single-cell sequencing clustering and annotation. Moreover, the recent publication of a detailed and annotated cell atlas in adult Drosophila optic lobes, as well as during development, provides a perfect ground plan for future comparisons and annotation transfers (Kurmangaliyev et al., 2020; Özel et al., 2020). Furthermore, the determination of the developmental origin (spatial and temporal) of all cell types (as described in section 3.3) will facilitate the identification of homologous cell types, as it is expected that homologous cells types come from the same lineage in different animals. Finally, despite being a fairly simple structure (compared to the central brain), the optic lobes are complex enough, composed of ~200 neuronal types that can be compared to other insects and unveil different evolutionary histories.

5.2. Convergent cell types

While these approaches can be successful in relatively closely related species (such as insects), matching cell types across large evolutionary distances might be futile for many reasons. First, the noise introduced by the hundreds of millions of years of independent evolution might prohibit the accurate recovery of the homology signal. Furthermore, there is a smaller number of orthologous cell types in distant animals, and one might instead need to rely on the identification and matching of orthologous cell type families. On the other hand, some of the neuronal functions may have become necessary independently in different animals leading to convergence of non-orthologous neuronal types to the same functions (Fig. 5).

Convergent cell types are of particular interest, as they represent two independent solutions for the same problem. A very interesting question that arises is whether the solution identified in each case is the same, i.e. the same gene regulatory network has been recruited to generate the convergent phenotypes, a phenomenon called deep homology (Shubin, Tabin and Carroll, 1997, 2009), or if they differ between the two species.

Recently, a neuronal population that is reminiscent of the Cajal-Retzius cells of the mammalian cortex (Soriano and Del Río, 2005; Özel et al., 2020) was identified in Drosophila. These neurons are only present during development and only project to the surface of the brain. Cajal–Retzius cells are essential for neuronal migration and other developmental processes and have been implicated in numerous neurological disorder such as lissencephaly, epilepsy, autism, bipolar disorder, and schizophrenia. While it is highly unlikely that these two cell types are orthologous, they could use similar mechanisms to guide neuronal processes to their targets. Other examples of possibly convergent cell types are glial cells (Klämbt, 2009). While vertebrate and invertebrate glial cell types are probably not orthologous, one can find functionally equivalent cell types between the two phyla. Perineurial and subperineurial glia in invertebrates and endothelial cells and astrocytes in vertebrates form septate junctions and provide the blood brain barrier. Similarly, wrapping and ensheathing glia in flies are responsible for wrapping neuronal axons, a function that is undertaken by oligodendrocytes and Schwann cells in vertebrates. Interestingly, despite the differences between vertebrates and invertebrates in gliogenesis, many of the terminal differentiation processes that guide migration and function are evolutionarily conserved (Klämbt, 2009).

5.3. Evolution of neuronal specification mechanisms

Adult cell types are the products of their developmental history and should be studied in that context. Different neuronal types may acquire similar or identical morphological, physiological, or molecular characters using different developmental paths. For this reason, neuronal type evolution cannot be studied except under the light of evolution of neuronal development.

As mentioned earlier, the generation of neuronal diversity in animals relies on two main intrinsic mechanisms: spatial and temporal patterning. While these mechanisms have been and are still being studied in exquisite detail in established genetic models, such as flies and mice, the lack of equivalent information from other representatives of the phylogenetic tree hinders our ability to understand how these mechanisms have evolved.

In the past, researchers had used candidate molecule approaches to study potential conservation of spatial and temporal factors. Given the small number of morphogens that are encoded in the animal genome, it comes as no surprise that the same molecules, such as Dpp/Bmp and Wg/Wnt, are being reused in different animals (Kaphingst and Kunes, 1994; Lee and Jessell, 1999). On the other hand, the main drivers of temporal patterning (i.e. the temporal transcription factors) are not shared between neuronal systems. In fact, even within one species (Drosophila), the ventral nerve cord (Isshiki et al., 2001) and optic lobes (Li et al., 2013) are using different series of tTFs to specify their neuronal progeny. So far, only a few orthologous tTFs have been discovered to play a role in both insects and vertebrates, such as Ikzf1 (the homolog of the Drosophila ventral nerve cord (VNC) first tTF, Hunchback) that specifies

young neural stem cells in the retina (Elliott et al., 2008), and Pou2f1/-Pou2f2 (Javed et al., 2020) and Casz1 (Mattar et al., 2015) (the homologs of the later VNC tTFs, Pdm1/2 and Castor) that specify older retinal progenitors. Finally, FoxG1 (the ortholog of the Drosophila optic lobe temporal factor Slp) is expressed in older cortical progenitors promoting deep-layer neurons (Hanashima et al., 2004; Kumamoto et al., 2013).

Recent transcriptomic studies have performed large-scale single-cell sequencing experiments in the developing spinal cord (Delile et al., 2019) and cortex (Telley et al., 2019) in mice, as well as the developing human brain (Nowakowski et al., 2017; Manno et al., 2020). Common temporal transcription factor codes can be found in neurons that are born sequentially in different mammalian structures, but they are not similar to the fly tTFs: Onecut family transcription factors are expressed in neurons produced in early stages of neurogenesis, followed by the production of neurons that express Pou2f2 and Zfhx3/4 and, finally, neurons that express Nfia/b and Neurod2/6 (Delile et al., 2019; Sagner et al., 2020). This suggests the existence of a temporal series in the progenitors that specifies neuronal identity. With the continued generation of single-cell sequencing datasets from different stages of neuronal development, future meta-analyses will be able to evaluate the presence of temporal series in the neuronal systems of different species and generate hypotheses for the evolution of temporal patterning.

In the meantime, the insect visual system has a unique feature that makes it ideal to study the evolution of temporal patterning. As described earlier, because of the mechanism of generation of neuronal stem cells from the neuroepithelium, the developing optic lobe of Drosophila third instar larva contains neuroblasts of different ages. Single-cell sequencing of these neuroblasts in different insects followed by trajectory inference could give us a full appreciation of the entire temporal progression of the series (see above). Comparison of the temporal series in different insects will allow us to fully comprehend how a complex temporal series that regulates neuronal diversity has evolved. Similarly, an analysis focused on the neuroepithelial cells may allow the identification and comparison of spatial factors in the visual systems of different insects.

Such comparisons of developmental mechanisms between different insects will allow the understanding of what is different between visual systems, but also how these differences evolve by modulating developmental mechanisms.

6. Conclusion

The Drosophila visual system is an exceptionally well-suited model for single-cell studies because of the high repetition of most of its neuronal types. It is also the only one with two high resolution transcriptomic atlases of neuronal development, and for a large part (i.e. lamina and medulla neurons) a detailed view of both its connectome and of the TFs involved in neuronal specification. This presents a unique opportunity to create a complete “toolbox” to study neuronal function and development, by conducting complementary analyses and producing additional datasets. In this review, we have discussed the state-of-the-art as well as potential future projects stemming from our current knowledge. First, completing the characterization of transcriptomic diversity in the visual system by merging and reanalyzing the developmental atlases of the optic lobe, producing comparable atlases of the retina during pupal development and of the whole visual system during larval development, as well as annotating all the unidentified neuronal and glial clusters, would give a complete view of the cell type diversity in the optic lobe. Second, producing a developmental chromatin accessibility dataset of the same resolution as the transcriptomic datasets will allow finer studies of gene expression regulation during development. Interestingly, (Bravo González-Blas et al., 2020) found that, in the eye-antennal disk, the predicted accessibility of 77% of more than 700 enhancer-reporter lines (Jory et al., 2012) is correlated with their activity. Therefore, in addition to gene regulatory inference, accessibility could also be used to gain genetic access to all neuronal types of the visual system. Third, obtaining lineage information for all neuronal clusters, including the temporal window and spatial localization of the neuroblasts producing them, in combination with transcriptomic and chromatin accessibility data for all clusters, would allow to explore the links between specification and terminal identity in several dozens of cell types at once. Fourth, obtaining the missing pieces for the study of synaptic specificity: the interactome, associated affinities and subcellular localization throughout development for all CSMs, would allow us to define general mechanisms of synaptic specificity by bioinformatic modeling and experimental validations. And, finally, comparing cell type composition and specification mechanisms between different animals would help us understand how neuronal specification and neuronal diversity have evolved over time.

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References

Achim, K., Arendt, D., 2014. Structural evolution of cell types by step-wise assembly of cellular modules. Curr. Opin. Genet. Dev. 27, 102–108. https://doi.org/10.1016/j.gde.2014.05.001.

Akin, O., et al., 2019. Cell-type-Specific patterned stimulus-independent neuronal activity in the Drosophila visual system during synapse formation. Neuron 101 (5), 894–904. https://doi.org/10.1016/j.neuron.2019.01.008 e5.

Akin, O., Zipursky, S.L., 2020. Activity regulates brain development in the fly. Curr. Opin. Genet. Dev. 65, 8–13. https://doi.org/10.1016/j.gde.2020.04.005.

Alemany, A., et al., 2018. Whole-organism clone tracing using single-cell sequencing. Nature 556 (7699), 108–112. https://doi.org/10.1038/nature25969.

Alvarez, M., et al., 2020. Enhancing droplet-based single-nucleus RNA-seq resolution using the semi-supervised machine learning classifier DIEM. Sci. Rep. 10 (1), 11019. https://doi.org/10.1038/s41598-020-67513-5.

Apitz, H., Salecker, I., 2015. A region-specific neurogenesis mode requires migratory progenitors in the Drosophila visual system. Nat. Neurosci. 18 (1), 46–55. https://doi.org/10.1038/nn.3896.

Apitz, H., Salecker, I., 2018. Spatio-temporal relays control layer identity of direction-selective neuron subtypes in Drosophila. Nat. Commun. 9 (1), 2295. https://doi.org/10.1038/s41467-018-04592-z.

Arendt, D., et al., 2016. The origin and evolution of cell types. Nat. Rev. Genet. 17 (12), 744–757. https://doi.org/10.1038/nrg.2016.127.

Arendt, D., 2020. The evolutionary assembly of neuronal machinery. Curr. Biol.: CB 30 (10), R603–R616. https://doi.org/10.1016/j.cub.2020.04.008.

Ariss, M.M., et al., 2018. Single cell RNA-sequencing identifies a metabolic aspect of apoptosis in Rbf mutant. Nat. Commun. 9 (1), 5024. https://doi.org/10.1038/s41467-018-07540-z.

Aydin, B., et al., 2019. Proneural factors Ascl1 and Neurog2 contribute to neuronal subtype identities by establishing distinct chromatin landscapes. Nat. Neurosci. 22 (6), 897–908. https://doi.org/10.1038/s41593-019-0399-y.

Azevedo, F.A., et al., 2009. Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. J. Comp. Neurol. 513 (5), 532–541. https://doi.org/10.1002/cne.21974.

Baker, K.D., et al., 2003. The Drosophila orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway. Cell 113 (6), 731–742. https://doi.org/10.1016/S0092-8674(03)00420-3.

Bate, M., Martinez Arias, A., 2009. The Development of Drosophila melanogaster. Cold Spring Harbor Laboratory Press.

Bausenwein, B., Fischbach, K.F., 1992. Activity labeling patterns in the medulla of Drosophila melanogaster caused by motion stimuli. Cell Tissue Res. 270 (1), 25–35.

Bertet, C., et al., 2014. Temporal patterning of neuroblasts controls Notch-mediated cell survival through regulation of Hid or Reaper. Cell 158 (5), 1173–1186. https://doi.org/10.1016/j.cell.2014.07.045.

Borst, A., Haag, J., Reiff, D.F., 2010. Fly motion vision. Annu. Rev. Neurosci. 33 (1), 49–70. https://doi.org/10.1146/annurev-neuro-060909-153155.

Branson, K., et al., 2009. High-throughput ethomics in large groups of Drosophila. Nat. Methods 6 (6), 451–457. https://doi.org/10.1038/nmeth.1328.

Bravo González-Blas, C., et al., 2020. Identification of genomic enhancers through spatial integration of single-cell transcriptomics and epigenomics. Mol. Syst. Biol. 16 (5), e9438. https://doi.org/10.15252/msb.20209438.
Brunet Avalos, C., et al., 2019. Single Cell Transcriptome Atlas of the \textit{Drosophila} Larval Brain, vol. 8. K. VijayRaghavan, eLife, e50354. https://doi.org/10.7554/eLife.50354.
Cande, J., et al., 2018. Optogenetic dissection of descending behavioral control in Drosophila. eLife 7. https://doi.org/10.7554/eLife.34275.
Carrillo, R.A., et al., 2015. Control of synaptic connectivity by a network of Drosophila IgSF cell surface proteins. Cell 163 (7), 1770–1782. https://doi.org/10.1016/j.cell.2015.11.022.
Charest, J., et al., 2020. Combinatorial action of temporally segregated transcription factors. Dev. Cell 55 (4), 483–499. https://doi.org/10.1016/j.devcel.2020.09.002 e7.
Cocanougher, B.T., et al., 2020. Comparative Single-Cell Transcriptomics of Complete Insect Nervous Systems. bioRxiv, p. 785931. https://doi.org/10.1101/785931.
Cosmanescu, F., et al., 2018. Neuron-subtype-specific expression, interaction affinities, and specificity determinants of DIP/dpr cell recognition proteins. Neuron 100 (6), 1385–1400. https://doi.org/10.1016/j.neuron.2018.10.046 e6.
Courgeon, M., Desplan, C., 2019. Coordination between stochastic and deterministic specification in the Drosophila visual system. Science 366 (6463), eaay6727. https://doi.org/10.1126/science.aay6727.
Dalva, M.B., McClelland, A.C., Kayser, M.S., 2007. Cell adhesion molecules: signalling functions at the synapse. Nat. Rev. Neurosci. 8 (3), 206–220. https://doi.org/10.1038/nrn2075.
Davie, K., et al., 2018. A single-cell transcriptome atlas of the aging Drosophila brain. Cell 174 (4), 982–998. https://doi.org/10.1016/j.cell.2018.05.057.
Davis, F.P., et al., 2020. In: Bellen, H.J., VijayRaghavan, K. (Eds.), 'A Genetic, Genomic, and Computational Resource for Exploring Neural Circuit Function', eLife, vol. 9, e50901. https://doi.org/10.7554/eLife.50901.
Delile, J., et al., 2019. Single Cell Transcriptomics Reveals Spatial and Temporal Dynamics of Gene Expression in the Developing Mouse Spinal Cord, vol. 146. ', Development, Cambridge, England. https://doi.org/10.1242/dev.173807, 12.
Diao, F., et al., 2015. Plug-and-play genetic access to drosophila cell types using exchangeable exon cassettes. Cell Rep. 10 (8), 1410–1421. https://doi.org/10.1016/j.celrep.2015.01.059.
Ding, M., et al., 2007. Spatial regulation of an E3 ubiquitin ligase directs selective synapse elimination. Science 317 (5840), 947. https://doi.org/10.1126/science.1145727. LP – 951.
Elliott, J., et al., 2008. Ikaros confers early temporal competence to mouse retinal progenitor cells. Neuron 60, 26–39. https://doi.org/10.1016/j.neuron.2008.08.008.
Erclik, T., et al., 2017. Integration of temporal and spatial patterning generates neural diversity. Nature 541 (7637), 365–370. https://doi.org/10.1038/nature20794.
Estacio-Gómez, A., et al., 2020. Dynamic neurotransmitter specific transcription factor expression profiles during Drosophila development. Biol. Open 9 (5). https://doi.org/10.1242/bio.052928.
Fischbach, K.F., Dittrich, A.P., 1989. The optic lobe of Drosophila melanogaster. I. A Golgi analysis of wild-type structure. Cell Tissue Res. 258, 441–445.
Fleming, S.J., Marioni, J.C., Babadi, M., 2019. CellBender remove-background: a deep generative model for unsupervised removal of background noise from scRNA-seq datasets. bioRxiv. https://doi.org/10.1101/791699.
Ha, K.C.H., et al., 2021. Differential contribution of transcriptomic regulatory layers in the definition of neuronal identity. Nat. Commun. 12 (1), 335. https://doi.org/10.1038/s41467-020-20483-8.
Hafemeister, C., Satija, R., 2019. Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. Genome Biol. 20 (1), 296. https://doi.org/10.1186/s13059-019-1874-1.
Hanashima, C., et al., 2004. Foxg1 suppresses early cortical cell fate. Science (New York, N.Y.) 303 (5654), 56–59. https://doi.org/10.1126/science.1090674.
Handler, A.M., 1982. Ecdysteroid titers during pupal and adult development in Drosophila melanogaster. Dev. Biol. 93, 73–82. https://doi.org/10.1016/0012-1606(82)90240-8.
Hie, B., et al., 2020. Computational methods for single-cell RNA sequencing. Ann. Rev. Biomed. Data Sci. 3 (1), 339–364. https://doi.org/10.1146/annurev-biodatasci-012220-100601.
Hiesinger, P.R., et al., 2006. Activity-independent prespecification of synaptic partners in the visual map of Drosophila. Curr. Biol. 16 (18), 1835–1843. https://doi.org/10.1016/j.cub.2006.07.047.
Hobert, O., Kratsios, P., 2019. Neuronal identity control by terminal selectors in worms, flies, and chordates. Curr. Opin. Neurobiol. 56, 97–105. https://doi.org/10.1016/j.conb.2018.12.006.
Hofbauer, A., Campos-Ortega, J.a., 1990. Proliferation pattern and early differentiation of the optic lobes in Drosophila melanogaster, 198. Roux's Archives of Developmental Biology, pp. 264–274. https://doi.org/10.1007/BF00377393.
Holguera, I., Desplan, C., 2018. Neuronal specification in space and time. Science (New York, N.Y.) 362 (6411), 176–180. https://doi.org/10.1126/science.aas9435.
Hörmann, N., et al., 2020. A Combinatorial Code of Transcription Factors Specifies Subtypes of Visual Motion-Sensing Neurons in Drosophila, vol. 147. Development, Cambridge, England. https://doi.org/10.1242/dev.186296, 9.
Isshiki, T., et al., 2001. Drosophila neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. Cell 106 (4), 511–521.
Izutsu, M., et al., 2015. Dynamics of Dark-Fly Genome under Environmental Selections, vol. 6. G3, Bethesda, Md, pp. 365–376. https://doi.org/10.1534/g3.115.023549, 2.
Jain, S., et al., 2020. A Global Temporal Genetic Program for Neural Circuit Formation. bioRxiv, p. 2020. https://doi.org/10.1101/2020.09.18.304410.
Javed, A., et al., 2020. Pou2f1 and Pou2f2 Cooperate to Control the Timing of Cone Photoreceptor Production in the Developing Mouse Retina, vol. 147. Development, Cambridge, England. https://doi.org/10.1242/dev.188730, 18.
Johnson, E., Kath, W., Mani, M., 2020. A statistical approach to dimensionality reduction reveals scale and structure in scRNA-seq data. bioRxiv. https://doi.org/10.1101/2020.11.18.389031.
Jory, A., et al., 2012. A survey of 6,300 genomic fragments for cis-regulatory activity in the imaginal discs of Drosophila melanogaster. Cell Rep. 2, 1014–1024. https://doi.org/10.1016/j.celrep.2012.09.010.
Kaphingst, K., Kunes, S., 1994. Pattern formation in the visual centers of the Drosophila brain: wingless acts via decapentaplegic to specify the dorsoventral axis. Cell 78, 437–448. https://doi.org/10.1016/0092-8674(94)90422-7.
Katsamba, P., et al., 2009. Linking molecular affinity and cellular specificity in cadherin-mediated adhesion. Proc. Natl. Acad. Sci. U.S.A. 106 (28), 11594–11599. https://doi.org/10.1073/pnas.0905349106.
Klämbt, C., 2009. Modes and regulation of glial migration in vertebrates and invertebrates. Nat. Rev. Neurosci. 10 (11), 769–779. https://doi.org/10.1038/nrn2720.
Konstantinides, N., et al., 2018a. Phenotypic convergence: distinct transcription factors regulate common terminal features. Cell 174 (3), 622–635. https://doi.org/10.1016/j.cell.2018.05.021 e13.
Konstantinides, N., et al., 2021. A Comprehensive Series of Temporal Transcription Factors in the Fly Visual System. bioRxiv, p. 2021. https://doi.org/10.1101/2021.06.13.448242.
Konstantinides, N., Degabriel, S., Desplan, C., 2018b. Neuro-evo-devo in the single cell sequencing era. Curr. Opin. Struct. Biol. 11, 32–40. https://doi.org/10.1016/j.coisb.2018.08.001.
Konstantinides, N., Rossi, A.M., Desplan, C., 2015. Common temporal identity factors regulate neuronal diversity in fly ventral nerve cord and mouse retina. Neuron 85 (3), 447–449. https://doi.org/10.1016/j.neuron.2015.01.016.
Kumamoto, T., et al., 2013. Foxg1 coordinates the switch from nonradially to radially migrating glutamatergic subtypes in the neocortex through spatiotemporal repression. Cell Rep. 3 (3), 931–945. https://doi.org/10.1016/j.celrep.2013.02.023.
Kurmangaliyev, Y.Z., et al., 2019. Modular transcriptional programs separately define axon and dendrite connectivity. eLife 8. https://doi.org/10.7554/eLife.50822.
Kurmangaliyev, Y.Z., et al., 2020. Transcriptional programs of circuit assembly in the Drosophila visual system. Neuron. https://doi.org/10.1016/j.neuron.2020.10.006.
Lago-Baldaia, I., Fernandes, V.M., Ackerman, S.D., 2020. More than mortar: glia as architects of nervous system development and disease. Front. Cell Develop. Biol. 8, 1527. https://doi.org/10.3389/fcell.2020.611269.
Lancichinetti, A., Fortunato, S., 2011. 'Limits of modularity maximization in community detection. Phys. Rev. E, Statistical, Nonlinear, and Soft Matter Phys. 84 (6 Pt 2), 66122. https://doi.org/10.1103/PhysRevE.84.066122.
Langley, J.N., 1895. Note on regeneration of prae-ganglionic fibres of the sympathetic. J. Physiol. 18 (3), 280–284. https://doi.org/10.1113/jphysiol.1895.sp000566.
Lee, K.J., Jessell, T.M., 1999. The specification of dorsal cell fated in the vertebrate central nervous system. Annu. Rev. Neurosci. 22 (1), 261–294. https://doi.org/10.1146/annurev.neuro.22.1.261.
Lee, T., Luo, L., 2001. Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. Trends Neurosci. 24 (5), 251–254.
Li, H., et al., 2017. Classifying Drosophila olfactory projection neuron subtypes by single-cell RNA sequencing. Cell 171 (5), 1206–1220. https://doi.org/10.1016/j.cell.2017.10.019 e22.
Li, H., et al., 2020. Single-cell transcriptomes reveal diverse regulatory strategies for olfactory receptor expression and axon targeting. Curr. Biol.: CB 30 (7), 1189–1198. https://doi.org/10.1016/j.cub.2020.01.049 e5.
Li, J., et al., 2020. Structure of the Teneurin-Latrophilin Complex: Alternative Splicing Controls Synapse Specificity by a Novel Mechanism. bioRxiv, p. 2020. https://doi.org/10.1101/2020.01.13.901090.
Li, X., et al., 2013. Temporal patterning of Drosophila medulla neuroblasts controls neural fates. Nature 498 (7455), 456–462. https://doi.org/10.1038/nature12319.
Luecken, M.D., Theis, F.J., 2019. Current best practices in single-cell RNA-seq analysis: a tutorial. Mol. Syst. Biol. 15 (6), e8746. https://doi.org/10.15252/msb.20188746.
Manno, G. La, et al., 2020. Molecular Architecture of the Developing Mouse Brain. bioRxiv, p. 2020. https://doi.org/10.1101/2020.07.02.184051.
Mattar, P., et al., 2015. A conserved regulatory logic controls temporal identity in mouse neural progenitors. Neuron 85 (3), 497–504. https://doi.org/10.1016/j.neuron.2014.12.052.
Meinertzhagen, I.A., O'Neil, S.D., 1991. Synaptic organization of columnar elements in the lamina of the wild type in Drosophila melanogaster. J. Comp. Neurol. 305 (2), 232–263. https://doi.org/10.1002/cne.903050206.
Meinertzhagen, I.A., Sorra, K.E., 2001. Synaptic organization in the fly's optic lamina: few cells, many synapses and divergent microcircuits. Prog. Brain Res. 131, 53–69.
Mercatelli, D., et al., 2020. Gene regulatory network inference resources: a practical overview. Biochimica et biophysica acta. Gene Regulat. Mech. 1863 (6), 194430. https://doi.org/10.1016/j.bbagrm.2019.194430.
Michki, N.S., et al., 2021. The molecular landscape of neural differentiation in the developing Drosophila brain revealed by targeted scRNA-seq and multi-informatic analysis. Cell Rep. 35 (4), 109039. https://doi.org/10.1016/j.celrep.2021.109039.
Muthukumar, A.K., Stork, T., Freeman, M.R., 2014. Activity-dependent regulation of astrocyte GAT levels during synaptogenesis. Nat. Neurosci. 17 (10), 1340–1350. https://doi.org/10.1038/nn.3791.
Naidu, V.G., et al., 2020. Temporal progression of Drosophila medulla neuroblasts generates the transcription factor combination to control T1 neuron morphogenesis. Dev. Biol. 464 (1), 35–44. https://doi.org/10.1016/j.ydbio.2020.05.005.

Nériec, N., et al., 2016. From the eye to the brain: development of the Drosophila visual system. Curr. Top. Dev. Biol. 116, 247–271. https://doi.org/10.1016/bs.ctdb.2015.11.032.
Nern, A., Pfeiffer, B.D., Rubin, G.M., 2015. Optimized tools for multicolor stochastic labeling reveal diverse stereotyped cell arrangements in the fly visual system. Proc. Natl. Acad. Sci. U. S. A. 112 (22), E2967–E2976. https://doi.org/10.1073/pnas.1506763112.
Norimoto, H., et al., 2020. A claustrum in reptiles and its role in slow-wave sleep. Nature 578 (7795), 413–418. https://doi.org/10.1038/s41586-020-1993-6.
Nowakowski, T.J., et al., 2017. Spatiotemporal gene expression trajectories reveal developmental hierarchies of the human cortex. Science (New York, N.Y.) 358 (6368), 1318–1323. https://doi.org/10.1126/science.aap8809.
Okawa, H., et al., 2014. Illuminating the multifaceted roles of neurotransmission in shaping neuronal circuitry. Neuron 83 (6), 1303–1318. https://doi.org/10.1016/j.neuron.2014.08.029.
Özel, M.N., et al., 2019. Serial synapse formation through filopodial competition for synaptic seeding factors. Dev. Cell 50 (4), 447–461. https://doi.org/10.1016/j.devcel.2019.06.014 e8.
Özel, M.N., et al., 2020. Neuronal diversity and convergence in a visual system developmental atlas. Nature. https://doi.org/10.1038/s41586-020-2879-3.
Ozkan, E., et al., 2013. An extracellular interactome of immunoglobulin and LRR proteins reveals receptor-ligand networks. Cell 154 (1), 228–239. https://doi.org/10.1016/j.cell.2013.06.006.
Perry, M., et al., 2017. Generation and evolution of neural cell types and circuits: insights from the Drosophila visual system. Annu. Rev. Genet. 51, 501–527. https://doi.org/10.1146/annurev-genet-120215-035312.
Pinto-Teixeira, F., et al., 2018. Development of concurrent retinotopic maps in the fly motion detection circuit. Cell 173 (2), 485–498. https://doi.org/10.1016/j.cell.2018.02.053 e11.
Pinto-Teixeira, F., Konstantinides, N., Desplan, C., 2016. Programmed cell death acts at different stages of Drosophila neurodevelopment to shape the central nervous system. FEBS Lett. 590 (15), 2435–2453. https://doi.org/10.1002/1873-3468.12298.
Plazaola-Sasieta, H., et al., 2017. Untangling the wiring of the Drosophila visual system: developmental principles and molecular strategies. J. Neurogenet. 31 (4), 231–249. https://doi.org/10.1080/01677063.2017.1391249.
Raj, B., et al., 2018. Simultaneous single-cell profiling of lineages and cell types in the vertebrate brain. Nat. Biotechnol. 36 (5), 442–450. https://doi.org/10.1038/nbt.4103.
Ravenscroft, T.A., et al., 2020. Drosophila voltage-gated sodium channels are only expressed in active neurons and are localized to distal axonal initial segment-like domains. J. Neurosci.: Off. J. Soc. Neurosci. 40 (42), 7999–8024. https://doi.org/10.1523/JNEUROSCI.0142-20.2020.
Rivera-Alba, M., et al., 2011. Wiring economy and volume exclusion determine neuronal placement in the Drosophila brain. Curr. Biol.: CB 21 (23), 2000–2005. https://doi.org/10.1016/j.cub.2011.10.022.
Saelens, W., et al., 2019. A comparison of single-cell trajectory inference methods. Nat. Biotechnol. 37 (5), 547–554. https://doi.org/10.1038/s41587-019-0071-9.
Sagner, A., et al., 2020. Temporal Patterning of the Central Nervous System by a Shared Transcription Factor Code. bioRxiv, p. 2020. https://doi.org/10.1101/2020.11.10.376491.
Sanes, J.R., Yamagata, M., 2009. Many paths to synaptic specificity. Annu. Rev. Cell Dev. Biol. 25, 161–195. https://doi.org/10.1146/annurev.cellbio.24.110707.175402.
Sanes, J.R., Zipursky, S.L., 2020. Synaptic specificity, recognition molecules, and assembly of neural circuits. Cell 181 (3), 536–556. https://doi.org/10.1016/j.cell.2020.04.008.
Scala, F., et al., 2020. Phenotypic variation of transcriptomic cell types in mouse motor cortex. Nature 1–7. https://doi.org/10.1038/s41586-020-2907-3.
Schwabe, T., et al., 2014. Differential adhesion determines the organization of synaptic fascicles in the Drosophila visual system. Curr. Biol. 24 (12), 1304–1313. https://doi.org/10.1016/j.cub.2014.04.047.
Sen, S.Q., et al., 2019. Neuroblast-specific open chromatin allows the temporal transcription factor, Hunchback, to bind neuroblast-specific loci. eLife 8. https://doi.org/10.7554/eLife.44036.
Shinomiya, K., Huang, G., et al., 2019. Comparisons between the ON- and OFF-edge motion pathways in the Drosophila brain. eLife 8. https://doi.org/10.7554/eLife.40025.
Shinomiya, K., Horne, J.A., et al., 2019. The organization of the second optic chiasm of the Drosophila optic lobe. Front. Neural Circ. 13, 65. https://doi.org/10.3389/fncir.2019.00065.
Shubin, N., Tabin, C., Carroll, S., 1997. Fossils, genes and the evolution of animal limbs. Nature 388 (6643), 639–648. https://doi.org/10.1038/41710.
Shubin, N., Tabin, C., Carroll, S., 2009. Deep homology and the origins of evolutionary novelty. Nature 457, 818–823. https://doi.org/10.1038/nature07891.
Soriano, E., Del Río, J.A., 2005. The cells of cajal-retzius: still a mystery one century after. Neuron 46 (3), 389–394. https://doi.org/10.1016/j.neuron.2005.04.019.
Spanjaard, B., et al., 2018. Simultaneous lineage tracing and cell-type identification using CRISPR-Cas9-induced genetic scars. Nat. Biotechnol. 36 (5), 469–473. https://doi.org/10.1038/nbt.4124.
Sperry, R.W., 1963. Chemoaffinity IN the orderly growth OF nerve fiber patterns and connections. Proc. Natl. Acad. Sci. Unit. States Am. 50 (4), 703. https://doi.org/10.1073/pnas.50.4.703. LP – 710.
Stassen, S.V., et al., 2020. PARC: ultrafast and accurate clustering of phenotypic data of millions of single cells. Bioinformatics 36 (9), 2778–2786. https://doi.org/10.1093/bioinformatics/btaa042.
Steinberg, M.S., 1970. Does differential adhesion govern self-assembly processes in histogenesis? Equilibrium configurations and the emergence of a hierarchy among populations of embryonic cells. J. Exp. Zool. 173 (4), 395–433. https://doi.org/10.1002/jez.1401730406.
Sun, S., Zhu, J., Zhou, X., 2020. Statistical analysis of spatial expression patterns for spatially resolved transcriptomic studies. Nat. Methods 17 (2), 193–200. https://doi.org/10.1038/s41592-019-0701-7.
Suzuki, T., et al., 2013. A temporal mechanism that produces neuronal diversity in the Drosophila visual center. Dev. Biol. 380 (1), 12–24. https://doi.org/10.1016/j.ydbio.2013.05.002.
Takemura, S.Y., et al., 2013. A visual motion detection circuit suggested by Drosophila connectomics. Nature 500 (7461), 175–181. https://doi.org/10.1038/nature12450.
Takemura, S.Y., Lu, Z., Meinertzhagen, I.A., 2008. Synaptic circuits of the Drosophila optic lobe: the input terminals to the medulla. J. Comp. Neurol. 509 (5), 493–513. https://doi.org/10.1002/cne.21757.
Takemura, Shin-ya, et al., 2015. Synaptic circuits and their variations within different columns in the visual system of Drosophila. Proc. Natl. Acad. Sci. U.S.A. 112 (44), 13711–13716. https://doi.org/10.1073/pnas.1509820112.
Tan, L., et al., 2015. Ig superfamily ligand and receptor pairs expressed in synaptic partners in Drosophila. Cell 163 (7), 1756–1769. https://doi.org/10.1016/j.cell.2015.11.021.
Telley, L., et al., 2019. Temporal Patterning of Apical Progenitors and Their Daughter Neurons in the Developing Neocortex. ', Science, New York, N.Y., p. 364. https://doi.org/10.1126/science.aav2522
Tian, L., et al., 2019. Benchmarking single cell RNA-sequencing analysis pipelines using mixture control experiments. Nat. Methods 16 (6), 479–487. https://doi.org/10.1038/s41592-019-0425-8.
Tosches, M.A., et al., 2018. Evolution of pallium, hippocampus, and cortical cell types revealed by single-cell transcriptomics in reptiles. Science 360 (6391), 881–888. https://doi.org/10.1126/science.aar4237.
Traag, V.A., Waltman, L., van Eck, N.J., 2019. From Louvain to Leiden: guaranteeing well-connected communities. Sci. Rep. 9 (1), 5233. https://doi.org/10.1038/s41598-019-41695-z.
Tran, H.T.N., et al., 2020. A benchmark of batch-effect correction methods for single-cell RNA sequencing data. Genome Biol. 21 (1), 12. https://doi.org/10.1186/s13059-019-1850-9.
Trush, O., et al., 2019. N-cadherin orchestrates self-organization of neurons within a columnar unit in the Drosophila medulla. J. Neurosci.: Off. J. Soc. Neurosci. 39 (30), 5861–5880. https://doi.org/10.1523/JNEUROSCI.3107-18.2019.
Valdes-Aleman, J., et al., 2021. Comparative connectomics reveals how partner identity, location, and activity specify synaptic connectivity in Drosophila. Neuron 109 (1), 105–122. https://doi.org/10.1016/j.neuron.2020.10.004 e7.
Wu, M., et al., 2016. Visual projection neurons in the Drosophila lobula link feature detection to distinct behavioral programs. Elife 5. https://doi.org/10.7554/eLife.21022.
Xi, N.M., Li, J.J., 2020. Benchmarking computational doublet-detection methods for single-cell RNA sequencing data. Cell Syst. https://doi.org/10.1016/j.cels.2020.11.008.
Xu, C., et al., 2019. Control of synaptic specificity by establishing a relative preference for synaptic partners. Neuron 103 (5), 865–877. https://doi.org/10.1016/j.neuron.2019.06.006 e7.
Yang, S., et al., 2020. Decontamination of ambient RNA in single-cell RNA-seq with DecontX. Genome Biol. 21 (1), 57. https://doi.org/10.1186/s13059-020-1950-6.
Young, M.D., Behjati, S., 2020. SoupX removes ambient RNA contamination from droplet-based single-cell RNA sequencing data. GigaScience 9 (12). https://doi.org/10.1093/gigascience/giaa151.
Zhang, K.X., et al., 2016. Rapid changes in the translatome during the conversion of growth cones to synaptic terminals. Cell Rep. 14 (5), 1258–1271. https://doi.org/10.1016/j.celrep.2015.12.102.