COMMENTARY

A connectome is not enough – what is still needed to understand the brain of *Drosophila*?

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ABSTRACT

Understanding the structure and operation of any nervous system has been a subject of research for well over a century. A near-term opportunity in this quest is to understand the brain of a model species, the fruit fly *Drosophila melanogaster*. This is an enticing target given its relatively small size (roughly 200,000 neurons), coupled with the behavioral richness that this brain supports, and the wide variety of techniques now available to study both brain and behavior. It is clear that within a few years we will possess a connectome for *D. melanogaster*: an electron-microscopy-level description of all neurons and their chemical synaptic connections. Given what we will soon have, what we already know and the research that is currently underway, what more do we need to know to enable us to understand the fly's brain? Here, we itemize the data we will need to obtain, collate and organize in order to build an integrated model of the brain of *D. melanogaster*.

KEY WORDS: Neuromodulators, *Drosophila*, Gap junctions, Modeling, Neurotransmitters, Receptors

Introduction

The fruit fly *Drosophila melanogaster* is perhaps the leading candidate in which to study the comprehensive structure and function of the brain, and the mechanistic basis of behavior. The fly supports a wide variety of well-documented behaviors, and yet its brain is small enough (perhaps ∼200,000 neurons; Raji and Potter, 2021) that a functional understanding of its operation seems possible. In addition, a century of work on fly genetics makes manipulation of the brain more practical than in any other animal species.

We are now well on our way to compiling a connectome (a map of all neurons and the chemical synapses between them) of the complete central nervous system (brain plus ventral nerve cord or VNC; see Glossary) of the fruit fly *D. melanogaster*. This is a necessary pre-condition to a functional understanding of how a fly reacts, or even whether and how it might demonstrate cognitive functions beyond reacting to immediate circumstances. However, this anatomical dataset is by no means sufficient to understand how the brain might function in such roles. We need to acquire and integrate many different types of additional knowledge if we wish to understand how the fly's brain works and the richness of its information processing, of which currently we know very little.

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In this Commentary, we attempt to identify and enumerate the missing information, examine current efforts to acquire that information, and identify research paths to collect the data we still lack. Next, we discuss the need for a computational framework that can coherently account for all the concurrent processes that constitute higher brain functions, especially those we consider evidence for cognition. We close with a look forward to the possibility of obtaining a mechanistic description of how the brain of a complex creature might function.

What do we mean by understanding the brain?

Engineers typically say they 'understand' a system when they can predict, without doing an experiment, what will happen if one or more parts of the system are either disabled or activated. Similar experiments can now be performed in animal brains, in which individual neurons (or neuron types) can be activated or deactivated through the use of optogenetic, thermogenetic or chemogenetic tools (see Glossary). Extending this definition to biology, if the results of each such trial can be predicted without undertaking additional experiments, then the system may be said to be understood at a functional, as opposed to an anatomical, level.

The level of detail allowing the claim of mechanistic understanding is somewhat arbitrary. Although each might be described as mechanistic, a control-theory description might not include a circuit representation, a circuit-level description may abstract away detailed biochemistry, and even a biochemical description might skip the details of quantum mechanics. In this Commentary, we search for mechanistic understanding at the level of neurons and their operation. For example, a proprioceptive neuron might report a joint angle. Although the molecular mechanism by which it does so may remain unknown, we could still say we understand its neural operation if we can predict what happens if we disable it or restore its function after it has been disabled.

Some may argue that even if we have a perfect mathematical or simulation model, we cannot be said to understand a system unless we also have a higher level, intuitive description that explains how the system arrives at a result. This charge has been leveled against quantum mechanics (Bunge, 1956) and machine learning (Knight, 2017), among others. We discuss this in more detail in the section 'Computational tools'.

As shown in Table 1, we can identify at least 15 areas in which additional work will be needed to understand the fly's brain following the completion of the connectome. In the sections that follow, we elaborate on the current status and future needs of different research fields that will be needed to understand the *D. melanogaster* brain. These areas are: biochemistry, cell physiology, whole-animal concerns and new tools. Each topic represents the life's work of many groups; thus, we apologize in advance for any missed contributions or overlooked references.

Glossary

Chemogenetic tool
A method that combines cell-specific genetic manipulation with a small molecule that can be supplied externally (typically in the fly's food). This allows the genetic intervention to be selectively activated or deactivated by changing the fly's diet.

Expansion microscopy
A technique in which a sample is embedded in a gel that is then uniformly expanded (typically by a factor of 4–8), in the same manner as a dry sponge expands when exposed to water. This enables structures less than, or closer to, the un-expanded diffraction limit to be resolved.

FISH
Fluorescence *in situ* hybridization (FISH) provides a method to attach, or hybridize, fluorescent molecules to molecules in an intact organism. Multi-FISH is the same technique with multiple binding agents, each tagged with a different chromophore.

Local field potential
The voltage in the extracellular medium surrounding cells. It varies from place to place within the brain in ways that as yet are poorly quantified.

Mushroom body
Named for its characteristic shape, this is a center for learning and memory in the fly and many other arthropods.

Optogenetics
A method that combines genetic manipulation with optically sensitive chemistry. A typical example is using genetic means to selectively produce in certain cells a light-sensitive ion channel. As a result, these cells can be selectively activated or deactivated by exposing them to the appropriate wavelength of light.

Thermogenetic methods
These use genetic means to insert exclusively in select cells temperature-sensitive activators and de-activators of neuronal function. Then, in a cold-blooded terrestrial animal, such as a fly, the function of these cells can be manipulated by changing the ambient environmental temperature to which the animal is exposed.

RNA-seq
RNA sequencing. All cells contain the same DNA, but acquire their individual character by expressing different subsets of all the genes contained in the DNA. To do this, they first transcribe a selected subset into RNA, and from this make the appropriate proteins. By sequencing RNA instead of DNA, we can determine which genes are expressed, or were recently expressed in a particular cell type.

Ventral nerve cord
The fly's equivalent of the mammalian spinal cord, containing complex circuits critical especially for motor behavior.

An understanding of the fly's brain is a cross-disciplinary pursuit
Biochemistry

The molecular underpinnings of neuroscience are extensive and can only be summarized here (e.g. see Südhof, 2017 for a fuller review). We cannot claim to fully understand the brain of the fly until we obtain more detail on multiple aspects of its molecular biology and biochemistry. For example, we need to increase our knowledge of the roles of neurotransmitters and their receptors, neuromodulators and gap junctions. These issues are discussed in more detail below.

Connectomes derived from electron microscopy (EM), at least with current technology, do not incorporate the identity of the neurotransmitters used by synapses, and neither the identity nor the expression sites of the corresponding receptors. This leaves the sign, strength and time constant of connections unknown; it will therefore be important to identify the relevant neurotransmitters – and especially their receptors – if we are to understand fully the function of the brain. Neurotransmitters could be identified through RNA-sequencing (RNA-seq; see Glossary), which could potentially identify all neurotransmitters, or perhaps through some of the many

variants of multiplexed fluorescence *in situ* hybridization (FISH; see Glossary; Frei et al., 2016). Efforts are currently underway to test each *D. melanogaster* cell line for the eight most common neurotransmitters, including acetylcholine, GABA and glutamate. Both RNA-seq and FISH show that a substantial fraction of cells express genes for more than a single neurotransmitter, adding a further layer of combinatorial complexity (Nusbaum et al., 2017), even if not all transcripts may be coextensive in time and space.

Neurotransmitters act at receptors, of which there is a bewildering range and complexity. There can be many different receptors for the same transmitter (Hevers and Lüddens, 1998; Gotti et al., 2007), and – at least in some cases – the location of these is important. The most straightforward technique to identify receptor location would seem to involve some combination of genetic lines, optical labeling and expansion microscopy (see Glossary), or perhaps more challenging immuno-EM methods using highly effective antibodies, which can localize receptor expression to actual synaptic sites.

One advantage of *D. melanogaster* as a model species is that it is likely to exhibit clear cases of receptor localization to specific sites in identified neurons. One potential example is the off-edge motion detector cell, T5, which responds to motion in a particular direction, one that is aligned with the long axis of the T5 dendrite. A different response at the base and the tip of the dendrite is needed to generate the motion sensitivity observed. However, the main cells providing input to T5 all appear to be cholinergic, excluding the most intuitive mechanism that the different responses would be generated by different transmitter systems. Consequently, the most likely method to generate motion selectivity from amongst the different cholinergic inputs appears to be by expressing different receptor types at different locations along the dendrite (Fendl et al., 2020). These must first be identified and then localized to those specific sites if we are to understand fully the function of this system.

Next, many chemical signals act at a distance to influence more than just the adjacent synapse (Marder, 2012). We need to identify these chemical messengers, which are largely the neuropeptides and amines that act as neuromodulators. We must identify where they are released, how they are removed or degraded, where their effective concentration can act on neurons (Bargmann, 2012) and the resulting effects on the cells that receive them. Similarly, we need to understand the response of the nervous system to externally introduced agents such as different foods (Hwangbo et al., 2004), alcohol (Kaun et al., 2011), anesthetics and pheromones (Ottiger et al., 2000). The effects of multiple neuromodulators may not superimpose linearly, so we need to understand the effect of modulator combinations as well. As there are hundreds of known and potential neuromodulators (Bargmann, 2012), there are tens of thousands of potential interactions. Better understanding will be required so each of these cases does not need to be studied individually.

As with receptors, there are many examples of non-local neuronal modulation in *D. melanogaster*. As an example, the operation of dopamine on nearby synapses in the mushroom body (see Glossary) provides fairly strong evidence (Takemura et al., 2017) that this interaction is not strictly local, but instead has a radius of action of roughly 2 µm (approximately 10 cell diameters). Another example is provided by the SIFamide neuron, known to be involved in sleep (Park et al., 2014) and sexual behavior (Terhzaz et al., 2007). Only four neurons are thought to express this peptide (Terhzaz et al., 2007), whereas the receptors are ubiquitous (Sellami and Veenstra, 2015), suggesting that there is volume transmission and a large effective radius of peptide action across the brain for this molecule.

Gap junctions provide direct connections between adjacent neurons, without the intervention of a synapse. At present, the

Table 1. A summary of the status of our understanding of the fly's brain

Issue	Area	Understanding	Status
Neurotransmitter(s) used by each cell type, the receptors for those transmitters and their sites of expression	New biology	Medium	Some known; more in progress
Neuromodulators and hormones – their identities, actions, sources and sinks	New biology	Low	Some work, but no systematic effort
Gap junctions – their location and the operation of their various types	New biology	Medium	Exploration of potential methods for locating sites of expression
Glia – their types, locations and roles	New biology	Low	Several groups actively working
Synaptic plasticity, its time courses and modification rules	New biology	Low	Considerable effort
Mechanical models of all parts of the fly, with integration between the motor outputs and proprioceptors involved	New biology	Medium	Some body parts (wing, neck) studied; no unified efforts
Aerodynamic models of fly flight	New biology	Medium	Considerable effort
Circadian and other biological rhythms	New biology	Medium	Considerable work; effector peptidergic neurons identified
The role of local field potentials	New biology	Low	No current effort
Variation between flies	New biology	Medium	Some work at light microscopy, genetic and behavioral levels
A larger behavioral repertoire	New biology	Medium	Mostly sensory, navigation and courtship so far
An exhaustive library of cell types (including glia) with at least one clean genetic expression line for each	Scaling up	High	EM is generating cell type lists; glia are less well represented
Transcript profiling (RNA-seq) of all cell types, to reveal the genes expressed in each	Scaling up	High	Should follow from clean expression lines
A full connectome of both male and female brains, each including the ventral nerve cord	Scaling up	High	Ongoing
Improved neural simulators that accurately mimic the brain's neural activity and can do so at scale	Computational tools	Medium	Many groups working on scale, few on accuracy
Systems integration – an overall effort to combine knowledge from different subfields and build computational models from the integrated data	Computational tools	Medium	Not started; in prospect
Better theoretical models	Computational tools	Low	Work by theory groups worldwide

Areas are classified as 'new biology', 'scaling up' or 'computational tools'. 'New biology' refers to questions for which we either lack existing methods, or the best way to approach the problem is not yet clear. 'Scaling up' is where methods exist, but do not yet cover the full fly's brain intensively. 'Computational tools' are algorithms, interfaces and databases needed for a more complete understanding.

role of gap junctions in the nervous system is poorly understood and characterized. These junctions, comprising many different types (Dermietzel and Spray, 1993; Shruti et al., 2014), form pathways for communication between neurons, between glia, and between neurons and glia (Rozental et al., 2000; Söhl et al., 2005). In *D. melanogaster*, gap junctions are formed by innexins, a transmembrane protein family that is functionally comparable to connexins in vertebrates (Phelan, 2005). Knowledge of the locations and types of these gap junctions is needed for circuit analysis and simulation, but gap junctions are not well imaged in the EM preparations typically used for connectomics, and we therefore lack reliable knowledge of different types of junctions, and whether these are composed of the same or differing subunits. EM correlates of these innexin subtypes in *D. melanogaster* are thus still lacking. Furthermore, unlike chemical synapses, cell-specific blockers or activators of gap junctions have yet to be reported (Venken et al., 2011).

Given these challenges, how are we to go about identifying the locations of gap junctions within the *D. melanogaster* brain? Cell-type-specific RNA-seq will help, because cell types that fail to produce the requisite innexins can be excluded. Dye-fill techniques can reveal gap junctions but are slow and technically demanding. The use of labeled cell lines combined with labeled gap junctions and expansion microscopy provides an alternative approach (Wassie et al., 2019). In addition, it is possible (though unproven) that a combination of RNA-seq plus dense EM reconstruction could locate gap junctions. RNA-seq will reveal which cell types may express a specific innexin or possibly other gap junction proteins, and dense EM gives a complete list of which cells are physically adjacent. It seems plausible that cells that touch and produce the correct proteins may form gap junctions, perhaps subject to some

surface protein selection rules. This possibility could be tested between cell pairs, such as the adjacent terminals of photoreceptors in the optic lamina (Shaw and Stowe, 1982), which form identified gap junctions.

Finally, armed with knowledge of the exact innexins expressed, and the subtypes that form a particular junction, if we are to understand fully the brain's function we will need a computational model of each type of gap junction, because different gap junctions have different electrical and chemical properties (Dermietzel and Spray, 1993; Rozental et al., 2000; Söhl et al., 2005). With further progress, this combination of requirements may become simpler but currently is technically demanding.

Cell physiology
Nervous systems learn, at least in part, by changing the strength of their connections. Structural changes are also possible, but in the fly's mushroom body, at least, learning appears to happen by changing the strength of existing synapses, as opposed to forming or deleting synapses (Hige et al., 2015). This adjustment, differing in different compartments, appears to be driven by the coincidence of dopamine and Kenyon cell activation (Hige et al., 2015), and will need to be elucidated (or at least modeled) to understand possible changes in behavior brought about through learning (Sutton and Schuman, 2006). Understanding the mechanism of synaptic changes is an ongoing field of research (Gervasi et al., 2010). A logical extension is to circuits in which synapses are generated and disappear. The cellular physiology underlying these changes must be better characterized if we are to properly comprehend the functioning of the *D. melanogaster* brain. Furthermore, we will need to examine more deeply the physiology of all glial and neuronal cell types, as discussed below.

Glial cells were long thought to provide little more than support and insulation for neurons, but are now known to do far more (Deitmer and Steinhauser, 2010). Genetic evidence shows that there are many different types of glial cells in *D. melanogaster*, and these occur in different parts of the brain where many are presumed to play different roles. As an example, the optic lamina alone has six structurally distinct types of glia (Edwards and Meinertzhagen, 2010), approximately half the number of neuron types they surround. One prominent glial function is to serve as a sink for neurotransmitters, neuromodulators and hormones (Eulenburg and Gomeza, 2010). Many chemical synapses exist between neurons and glia, and the role of these synapses is not well understood. The different roles for glia will need to be investigated and understood, and this will likely require the use of genetic lines that uniquely identify the different glial cell types.

Another task yet to be completed is the generation of a complete list of all cell types that constitute the *D. melanogaster* nervous system, both the brain and the VNC, in both male and female flies. Because dense EM reconstruction finds all cells within a given volume (Takemura et al., 2015), once one complete male and one complete female brain have been densely reconstructed, a full library of neurons is assured, at least by morphological criteria and for the particular flies in question. A complete library of glia can perhaps be obtained by related techniques, although this is likely to require different sample preparation methods.

To enable experiments to be undertaken that can explore the function of all identified cell types, it is an enormous advantage to have clean genetic lines – lines that each express exclusively in a single cell type. Lines, such as those obtained using Gal4 or Split-Gal4 methods (Luan et al., 2006; Pfeiffer et al., 2010), are already available for several portions of the fly's brain – the optic lobes, the mushroom body, the central complex and much of the VNC. We will need to continue and expand such efforts to generate clean lines for the remaining unexplored parts of the brain and the VNC. In many cases, this will require us to identify hitherto unknown cell types, which often have very few representatives per type, especially in the central brain.

Knowledge of the molecular expression phenotype of different cell types is an essential next step. RNA-seq (Mortazavi et al., 2008) for each cell type will be a huge help for many subsequent tasks, such as those required to identify gap junctions, neurotransmitters and receptor (sub)types. Given that RNA-seq continues to become cheaper and easier, the limiting factor is therefore likely to be the production of clean genetic lines, and the identification of the exact morphological cell types that these define, as well as the constancy of these in different flies. Another factor, still to be fully recognized, is the transience with which identified genes are expressed, both temporally throughout cell development, and spatially in the fully developed cell (Perez et al., 2021). Multiple time points throughout development, and specific locations in the soma, axons and dendrites, may be required to address these issues.

Finally, unlike most human-designed systems, in the fly, voltages are not measured, nor do they act, with respect to some globally defined ground potential. Instead, the actions of the neurons are defined with respect to the voltage of the surrounding intercellular space, which can vary from place to place – the so-called local field potential (see Glossary). The role of local field potentials is not well understood in *D. melanogaster*, although oscillations have been measured and correlated with sensory input (Paulk et al., 2013). Local field potentials are also thought to contribute to computations in the lamina, where glia form insulating cylinders that isolate adjacent cartridges (Zimmerman, 1978). They are also suspected to be involved in olfactory processing (Prieto-Godino and De Polavieja, 2010), a role played in the locust and the moth *Manduca sexta*.

Whole-animal concerns

The function of any brain is ultimately to generate and control behaviors at the whole-animal level, such as motor behavior, circadian behaviors and the selection of actions from within the behavioral repertoire. All three of these strongly influence the fitness and survival of an animal. Of course, behavior tends to show a high level of variation between individuals, and this must also be taken into account as we work toward an understanding of the fly's brain. These issues are discussed in more detail below.

Modeling motor behavior in the fly requires that we understand how the outputs of the motor neurons are applied to specific muscles to generate the forces that act on the fly's body. Also needed will be models of proprioceptive and other sensory neurons that feed movement information back to the nervous system. This information includes the angles, forces and orientations of the fly's body and body parts (Tytell et al., 2011). There has been some work on mechanical models of *D. melanogaster* movement (Deora et al., 2015; Ríos et al., 2021 preprint), but much remains to be done. Moving and flexible parts include the legs, wings, thorax, head/neck and eyes. In many cases, the mechanical properties of these target structures will need to be measured before they can be modeled. Aerodynamic models of *D. melanogaster* flight are also required. Stabilization and navigation during flight are the job of many of the fly's visual and motor control systems. Understanding the function of these will require knowledge of the details of how muscle action changes the movement of the wings, so that the resulting effect on the state of the animal is determined by aerodynamics, as previously reported (Sane and Dickinson, 2002; Lehmann and Bartussek, 2017).

Both the connections and cell operation of identified neurons are known to change as a function of the fly's circadian rhythm. In the lamina, for example, synapse numbers vary on a daily cycle (Pyza and Meinertzhagen, 1993). Because this change is thought to be mediated by neurotransmitters and/or neuropeptide modulators (Pyza and Meinertzhagen, 1998), such changes may well also happen in other parts of the nervous system. In addition, the internal functions of some neurons also vary as a function of a circadian rhythm. For example, there are neurons that switch between a graded and a spiking operation as a part of a daily cycle (Pimentel et al., 2016). The genome of *D. melanogaster* is known to vary over seasonal time scales (Bergland et al., 2014), a change that may, in turn, induce annual changes in brain operation (Behrman et al., 2018 preprint).

As mentioned above, behavior varies remarkably from one individual to the next: even genetically identical flies do not respond identically to the same stimulus, nor does the same fly react in the exact same way in repeated trials (Anholt and Mackay, 2004). In other words, the fly's circuits (including their internal state) must differ for different trials and different animals, and this diversity must be understood if we wish to possess a predictive model. At the moment, we have no comprehensive idea of how two flies, male or female, might differ in their circuits, though the differences are known to be significant (Cachero et al., 2010). Perhaps more vexing, we also lack significant understanding of how two representatives of either sex might differ from the other. The ideal tool for studying such variation would have high throughput to measure the distributions of small connection variations, and the ability to look at many circuits, which may differ in their variability. No such technique currently exists. Genetic and optical techniques such as t-GRASP (i.e. targeted

GFP reconstitution across synaptic partners; Shearin et al., 2018) and expansion microscopy (Gallagher and Zhao, 2021) can look at a single connection across many animals, with enough throughput for multiple animals and time points. Repeated EM of small circuits (such as a column of the medulla) can show variation of that one circuit across several animals. Comparison of full-scale connectomes shows differences of many circuits, but between very few animals. A combination of these techniques will be needed to form a model of cross-animal variation.

To understand the function of the fly's brain, we also need to study the natural ecology and correspondingly larger behavioral repertoire of unrestrained *D. melanogaster*. By our definition, 'understanding' means being able to predict the fly's behavior without undertaking an experiment. Testing this level of understanding requires comparison with the fly's true behavior. However, fly behavior is known quantitatively in only a few cases, concentrated in a subset of the brain, as shown in Fig. 1B. Behaviors studied, and their brain areas, include motion vision (the optic lobe), olfaction (the mushroom body) and navigation (the central complex). However, even these behaviors are characterized only under a limited set of laboratory conditions, in which flies are tethered or constrained to small arenas. More complex interactions in realistic environments, such as the selection of actions from among feeding, fleeing, fighting and mating, are among the most important functions of the brain, but are currently poorly quantified or totally unknown.

Although there has been great progress in obtaining connectomes for *D. melanogaster*, from the point of view of understanding the brain, the work has only just started. A complete connectome of both male and female brains, at least one of each, will be needed. This is no longer the insuperable obstacle that it would have seemed even a decade ago. Already existing is the full adult fly's brain (FAFB) dataset, one sampled brain obtained with ultramicrotome sections imaged by transmission electron microscopy (TEM). Although more challenging for automated analysis (Huang et al., 2020; Macrina et al., 2021 preprint), a significant number of its neurons have been traced manually (Schlegel et al., 2017), and automated methods are being developed (Li et al., 2019; Buhmann et al., 2021). Newer data, taken using focused ion beam–scanning electron microscopy (FIB-SEM), the analysis of which is easier to automate, contains approximately two-thirds of the center portion of a female brain. These data have been imaged and densely reconstructed, and are currently subject to analysis (Xu et al., 2020 preprint; Scheffer et al., 2020). As of 2021, scientists at the Janelia labs of the Howard Hughes Medical Institute are in the process of imaging the full nervous system of a male fly, including both the brain and the VNC. However, apart from a few circuits compared in two animals (Takemura et al., 2015; Schlegel et al., 2021), we lack duplicate connectomes that could reveal the constancy of the connectome in either sex.

Computational tools

One way to gain an understanding of the fly's brain lies in neuronal simulation of its synaptic circuits. Many such simulation programs are available, such as Neuron (Carnevale and Hines, 2006) or NEST (Gewaltig and Diesmann, 2007), but none exactly provides what is needed to simulate fly behavior accurately. A model of a neuron that assumes the same voltage throughout (a 'single node model') is not sufficient for the longer neurons in the fly's brain (Gouwens and Wilson, 2009; Meier and Borst, 2019). Conversely, modeling the voltage at each point in a neuron is too slow, given the full detail of EM. The latter will require constructing a smaller but still

sufficiently accurate model, a task known as 'model-order reduction'. Generating and validating a reduced model that is fast enough to simulate several seconds of fly behavior, yet accurate enough to reproduce such behavior faithfully, is still a significant research problem (Gornet and Scheffer, 2017 preprint).

If a neural simulator is to replicate fly behavior accurately, it needs to handle both graded (non-spiking) and spiking neurons, and requires a model for the actions of neuromodulators and hormones, including their generation, removal and interactions; each a significant challenge. It also needs to communicate with simulations of mechanical and aerodynamic models of the fly itself. 'Systems integration' is the overall effort to combine the knowledge from several such sub-fields. It is clear that to understand the behavior of a fly, we will need to integrate data from many different sources, acquired by many different techniques. WormBase (Lee et al., 2018) and FlyBase (Gramates et al., 2017) are two examples of such integration in other areas of research, driven by genetics. We will need a similar effort, probably starting with connectomics, to build a machine-readable version of all the diverse data required to understand the fly's brain and its multitudinous functions. The recent FlyBrainLab (Lazar et al., 2021) provides a start in this direction, combining connectomic, genetic and electrophysiology data.

Previous attempts have been made to integrate neural and mechanical simulation. Perhaps the most relevant work is an integrated model of *D. melanogaster* flight (Dickson et al., 2008), albeit this model is quite abstract. The entire nervous system, and the visual sensory system, is replaced by a computational model of the effect of full-field motion, while the mechanical model has three components, a body and two wings, with no representation of muscles or their neural drivers. Two other potential models are AnimatLab and NeuroMechFly. AnimatLab combines a simple neuron model with an arbitrary mechanical model, proprioceptors and muscles (Cofer et al., 2010), but has yet to be used for a moving *D. melanogaster*. NeuroMechFly (Ríos et al., 2021 preprint) combines a detailed mechanical model of *D. melanogaster* with a simple muscle model and an abstract neural model, and has been used to simulate walking flies.

Combining different modes of simulation, as would be necessary to recreate a fly's behavior, is primarily an engineering problem. Fields such as electrical engineering have featured such simulators for decades (Agrawal et al., 1980), normally organized around an event queue that keeps all underlying simulations synchronized. The problems are normally those of software engineering, rather than the available computational power. The simulators that need to be combined are often written in different languages and use different user interfaces, making integration into a unified environment difficult.

A final need is for improved theoretical models. Lord Kelvin famously remarked that when you cannot measure quantitatively, 'your knowledge is of a meagre and unsatisfactory kind'. This criticism has been extended to systems in which we can measure and calculate to any desired degree of accuracy, but for which there is no higher-level, intuitively understandable explanation. Traditional areas in which this applies are quantum mechanics and machine learning. A relevant biological example might be the crustacean stomatogastric ganglion, in which three modes of communication – synapses, gap junctions and neuromodulators – interact. By itself, each interaction seems relatively easy to describe and understand, but the actions of the circuit as a whole have proved notoriously difficult to understand and explain (Marder and Bucher, 2007), even though the circuit has relatively few elements.
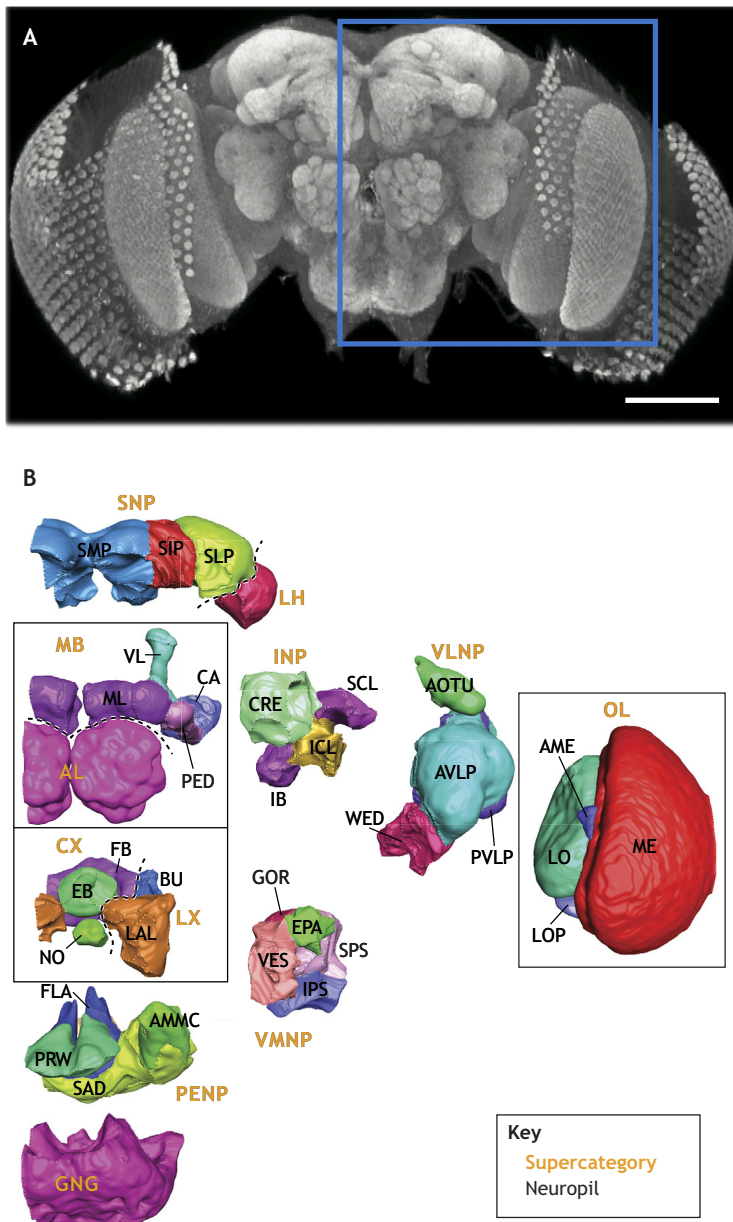
Fig. 1. The tiny brain of *Drosophila melanogaster*, smaller than a poppy seed. (A) The brain labeled with synaptic antibodies. The square indicates the portion of the brain expanded in false color in the adjacent panel. Scale bar: 100 μm. (B) The brain is divided into anatomical compartments which are tightly packed in three dimensions. The optic lobes (OL), central complex (CX) and mushroom body (MB), indicated with boxes, have received significant study; the rest is the brain's 'terra incognita'. AL, antennal lobe; AME, accessory medulla; AMMC, antennal mechanosensory and motor center; AOTU, anterior optic tubercle; AVLP, anterior ventrolateral protocerebrum; BU, bulb; CA, calyx; CRE, crepine; EPA, epaulette; FB, fan-shaped body; FLA, flange; GNG, gnathal ganglia; GOR, gorget; IB, inferior bridge; ICL, inferior clamp; INP, inferior neuropils; IPS, inferior posterior slope; LAL, lateral accessory lobe; LH, lateral horn; LO, lobula; LOP, lobula plate; LX, lateral complex; ME, medulla; ML, medial lobe; NO, noduli; PED, pedunculus; PENP, periesophageal neuropils; PRW, prow; PVLP, posterior ventrolateral protocerebrum; SAD, saddle; SCL, superior clamp; SIP, superior intermediate protocerebrum; SLP, superior lateral protocerebrum; SMP, superior medial protocerebrum; SNP, superior neuropils; SPS, superior posterior slope; VES, vest; VL, vertical lobe; VLNP, ventrolateral neuropils; VMNP, ventromedial neuropils; WED, wedge. Credit: (A) courtesy of I. A. Meinertzhagen; (B) derived with permission from Ito et al. (2014), which defines the regions and their names, and has an interactive 3D model showing more detail.

More successfully, models of network dynamics have provided understandable and interpretable explanations of heading and navigation in the central complex, learning in the mushroom body and motion detection in the optic lobes. These models are both inspired and constrained by connectomic data. Extension of these models to other parts of the brain (such as the pattern generators that are thought to exist in the VNC) will be critical to gain humanly accessible explanations of the brain.

One particular concern is the use of mathematical models suitable for biology. For linear systems of arbitrary size, good mathematical and intuitive tools are available (Chen et al., 2004). Conversely, non-linear systems in their full generality are difficult to describe, exhibiting chaotic behavior with as few as three variables (Wiggins, 2003). Biological systems operate in some middle ground, with strong non-linearities but not extreme sensitivities, insofar as they must operate reliably over a wide range of environmental conditions. However, we have few good mathematical tools for systems in this regime.

Related projects

In addition to the projects already mentioned above, many other existing projects are relevant to the goal of understanding the working of the fly's brain. Examples include extensive work to develop chemical, genetic and optical tools to measure functional properties of nervous systems. Those attempting to understand the *D. melanogaster* brain will be major customers of such efforts, and we will need further improvements in these tools, especially as applied to tiny cells such as those of *D. melanogaster*, to generate data that are currently unavailable.

Another closely related field is the inquiry into how these neural systems are assembled (Clandinin and Zipursky, 2002). Although not strictly required to understand the operation of the adult *D. melanogaster* brain, research in this area shares many of the underlying subfields, such as biochemistry, gene expression in cell types and synapse formation. Indeed, the wiring of some synaptic circuits is molded by patterns of neural activity during development (Kirkby et al., 2013), which mimic the patterns of those synaptic

inputs that will come later. In turn, an understanding of how circuits are built could help other sub-fields, such as connectomics, by helping to define constraints that must be observed by the resulting circuits, and perhaps by deducing the presence of components that are hard to see in EM. For example, if the rules for generating gap junctions were understood, perhaps their presence could be deduced from innexin expression levels in the participating cells, as determined by RNA-seq.

The study of other model organisms may facilitate the interpretation of the *D. melanogaster* connectome, as nervous system operation appears to be largely conserved over a wide range of animals. The seminal *Caenorhabditis elegans* connectome (White et al., 1986) has served this role for decades. The *D. melanogaster* larva connectome is approximately one-tenth the size of the adult connectome, facilitating the analysis of more animals. Although there is extensive remodeling of the nervous system during pupation, the operation of at least some circuits (Eichler et al., 2017) is closely related to the same circuit in the adult, so conclusions may apply to both. Similarly, connectomes of small portions of mammal brains have long been used to study the general principles of olfactory and visual systems, and concerted efforts are underway to further understand the brains of rodents (Abbott et al., 2017; Wool, 2020). Moreover, the completion of the *D. melanogaster* connectome illustrates many of the techniques that will be needed for the much larger brains of mammals or other species (Macrina et al., 2021 preprint), and no less their operating principles.

Conclusions

Most of the sub-fields mentioned here are the subjects of active study. It is reasonable to assume that many of them will make substantial progress in the next decade, just as connectomic studies have done in the last. However, in the absence of a coordinated effort to ensure that all necessary sub-fields are integrated and covered, there will still be gaps in our ability to understand the working of the fly's nervous system. Two sub-disciplines in particular look like limiting factors, with significant gaps in our understanding. Both are hard problems that will only show their value when trying to build an integrated model of the fly's brain; until then they will remain difficult and unrewarding for any individual investigator. These two problems are: (1) the effort required to integrate all the different data sources needed to comprehend the *D. melanogaster* brain, and not least to generate a common language for such disparate data, as we will need to build a combined computational model; and (2) the precise effects of neuromodulators and hormones, and their interactions. Individual investigators study the effects of various long-distance messengers, but so far there has been no effort to compile or study a reasonably comprehensive list. Further, many may interact in unknown ways, which studies of individual neuromodulators will fail to reveal. Progress seems most likely through a combination of fluorescent indicators for neuromodulators (Sabatini and Tian, 2020) and the detection and localization of receptors in neurons (Yano and Matsuzaki, 2009).

Although progress on each of these topics is likely, it will be episodic and uneven, and only a specific project tuned to the overall task is likely to make sufficient progress on all needed fronts. This, in our opinion, would make an excellent umbrella project for any of the world's major biological research institutes. Nevertheless, whether done piecemeal or as a unified effort, success at understanding the operation of the brain of *D. melanogaster* will bring huge scientific benefits. To a great extent, the same

mechanisms must underlie the operation of brains of all sizes, including those of humans. A mechanistic understanding of the brain of a tiny fly would be a critical step on the path to understanding the brains of all creatures great and small, with all the manifold benefits this would bring.

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