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Mapping and Manipulating Neural Circuits in the Fly Brain

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ABSTRACT

Drosophila is a marvelous system to study the underlying principles that govern how neural circuits govern behaviors. The scale of the fly brain (~100,000 neurons) and the complexity of the behaviors the fly can perform make it a tractable experimental model organism. In addition, 100 years and hundreds of labs have contributed to an extensive array of tools and techniques that can be used to dissect the function and organization of the fly nervous system. This review discusses both the conceptual challenges and the specific tools for a neurogenetic approach to circuit mapping in *Drosophila*.

I. INTRODUCTION

Why would you want to map neural circuits? In our quest to understand how the brain controls appropriate responses to environment and experience, we must track which neurons are connected and what jobs they do together. The wiring diagram and associated behavioral functions of neurons are prerequisites for the kind of experiments that will truly parse what the nervous system as an interconnected network does. Research for mapping neural circuits required for specific behaviors has shifted from hunting for the responsible *genes* to the responsible *neurons*. The "lesion approach," where damaged brain regions are correlated with behavioral changes, has been highly effective in vertebrates—humans, too (Damasio *et al.*, 1994)—but the spatial and temporal precision with which we can generate "lesions" in the genetic model organisms is unrivaled. This kind of targeted genetic lesion is a way to make circuit breaking into "a science of control and causality rather than a science of observation and correlation" (Holmes *et al.*, 2007). This is an exciting time to be studying neuroscience, both because of the tools available and because the trend toward multidisciplinary science and freer journal access has pushed previously under connected fields together: information from other scientific disciplines (systems neuroscience, neuroethology) and other organisms (stick insects, bees, locusts) are now informing the experiments we do in *Drosophila*, which has long been a genetic powerhouse for studying development and biochemical signaling pathways.

There are different kinds of information that can be gathered about neural circuits. One could collect anatomical information by labeling individual neurons or fiber tracts and determining neural shape and region-level connectivity at the light level or by electron microscopy. One could record activity in individual neurons or populations with optical reporters or electrodes. One could do careful behavioral assays and deduce what sorts of circuits must underlie particular computations from latency to response to a sensory stimulus,

differences in execution/performance, or types of errors. One could screen for mutations that disrupt neural circuit formation or function. One could do injury or lesion studies to see where structural perturbations disturb behavioral output. And now we are using new technology to make genetically targeted lesions to disrupt function in specific neurons to map neural circuits directly. Figure 3.1 shows a schematic of this approach. In this section, I will discuss briefly what has been learned from these various approaches but I will devote most of the review to discussion of the tools available for generating genetically targeted disruptions in neural activity.

This review attempts to cover four areas—spatial control for targeting small groups of neurons reproducibly, visualization of the activity and connectivity of neurons, temporal control of neural activity, and behavioral assessment of defective flies. I try to give both the original references where tools were developed and examples of circuit dissection where the tools have been used particularly well. I have drawn almost exclusively from the literature on adult flies rather than larva. As a practitioner of this ilk of circuit tracing, I have used many of the reagents discussed and I have tried to inject cautionary notes based on my own experience and those of my colleagues that may not have made it into print since negative results often go undocumented. I have tried to compile best practices, appropriate controls, and areas ripe for improvement and discovery. Construct names are in **bold** for easy spotting and the ***bold italics*** text highlights references for particularly good examples of the use of the tool for circuit bashing.

Some aspects of this chapter have been ably covered in recent reviews and I refer you to them for additional information and different perspectives. Specifically, I suggest reviews of spatial control of gene expression and neuronal targeting (***Luan and White, 2007***); manipulation of neural activity (***Holmes et al., 2007***); fly circuit analysis with emphasis on electrophysiology, functional imaging, and neural computations (***Olsen and Wilson, 2008a***); vertebrate and invertebrate techniques (***Luo et al., 2008***); and genes and behavior (***Baker et al., 2001***; ***Dickson, 2008***; ***Vosshall, 2007***).

A. Genes for behavior

There are genetic mutations that affect behavior. Genes encode the proteins required to specify neural cell type, guide axons to their appropriate targets, drive the membrane potential changes that allow action potentials, and synthesize and release neurotransmitters. Mutagenesis screens have uncovered many of these genes. In some cases, gene expression is restricted to small groups of neurons, which gives a starting point for circuit identification. In other cases, reexpressing the missing gene in restricted subsets of neurons to show that function in these neurons is sufficient to restore normal behavior has identified the circuits underlying a given behavior.

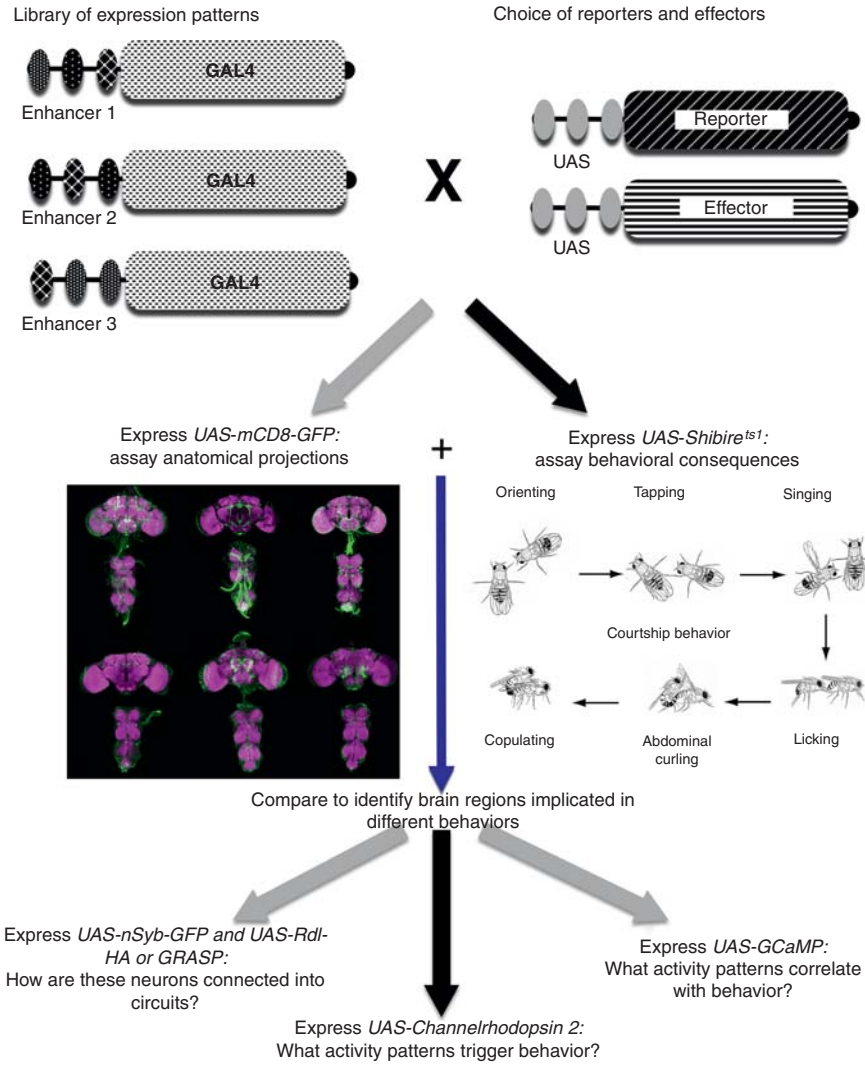

Figure 3.1. *Screening approach to identify circuit components by targeted genetic lesions*. A library of GAL4 lines is crossed to anatomical markers to determine the identity and potential connectivity of neurons. The same lines are crossed to neural activity blockers or activators and behavioral effects assayed. Lines that show similar behavior defects can be compared to look for shared neurons. The complex expression patterns can be further dissected by intersectional strategies described in Fig. 3.2. Functional imaging can also be tried to identify relevant neurons. Examples of use of this approach include Armstrong *et al.* (2006), Baker *et al.* (2007b), Gordon and Scott (2009b), Hughes and Thomas (2007), Katsov and Clandinin (2008), Kitamoto (2002), and Pitman *et al.* (2006).

There is a long history of performing radiation, chemical, or transposon mutagenesis and screening for behavioral defects. Seymour Benzer (1921–2007) was a pioneer of this approach in *Drosophila*. He identified flies defective in fast phototaxis and circadian rhythms, for example (Benzer 1967, 1973). It is tricky to identify mutants that only affect an adult behavioral phenotype since most mutants are pleiotropic, contributing to animal function during development and/or in multiple tissues. The ability to screen vast numbers of flies allowed people to obtain hypomorphic and neomorphic alleles which had more subtle effects on phenotypes (Greenspan, 1997). The Per^{long} and Per^{short} alleles of the circadian rhythm gene *Period* are examples of this (Konopka and Benzer, 1971). David Suzuki searched specifically for conditional alleles, shifting to nonpermissive temperatures in the adult to obtain specific behavioral defects (Homyk *et al.*, 1980; Suzuki *et al.*, 1971). Many of these mutations were eventually mapped to ion channel genes. Sokolowski and colleagues took advantage of a natural behavioral variant to identify the *foraging* gene in which two different alleles, neither of which is a null, affect larval feeding behavior (de Belle *et al.*, 1989). Natural variants have also been identified in population selection screens for increased lifespan and response to gravity (Lin *et al.*, 1998; Song *et al.*, 2002). Screens for the failure of the jump-escape circuit led to the cloning of an invertebrate gap junction component, the ShakingB Neural innexin (Thomas and Wyman, 1984). Screens for grooming behavior and response to ethanol have yielded mutants in adhesion molecules and cell signaling cascades (Moore *et al.*, 1998; Phillis *et al.*, 1993). How these genes contribute to the performance of these behaviors remains mysterious. Localizing which neurons require these proteins has been key for identifying the neural circuits involved.

Sometimes the genes are expressed in restricted patterns that suggest which neurons are critical for the behavior affected by mutant alleles (Hamada *et al.*, 2008; Renn *et al.*, 1999). People have used the behavioral mutants to identify the neurons participating in particular behaviors by restoring functional protein selectively—rescuing in specific cell types or time points. *CamKII* mutants are defective in the memory of bad experiences during courtship, but restoring CamKII in the mushroom bodies rescues normal memory performance (Joiner and Griffith, 1999). Flies mutant for *rutabaga* have visual memory defects that are restored by replacing rutabaga function in different layers of the fan-shaped body—as well as in some other areas of the brain (Liu *et al.*, 2006; Zars *et al.*, 2000). Expressing *taybridge* in the central complex rescues that mutant's locomotor and anatomical defects (Poeck *et al.*, 2008).

B. Neurons for behavior

Attempts to map the parts of the brain that drive behaviors go back to the days of gynandromorphs or sexual mosaics. The parts of the brain that must be genetically male to drive appropriate male courtship behaviors have been known at a

rough level for decades (Hall, 1979; Hotta and Benzer, 1970; Tompkins and Hall, 1983). Laborious histological screens were done to isolate mutants with visible anatomical defects in particular brain regions; behavior analysis lead to the hypothesis that the central complex is critical for coordinated locomotion (Ilius *et al.*, 1994; Strauss and Heisenberg, 1993). Drug ablation of the mushroom bodies implicated them in memory formation and retrieval (de Belle and Heisenberg, 1994). The modern methods for targeting neural activity modifiers to specific groups of neurons and assaying behavioral consequences discussed below are a logical continuation of this tradition for circuit mapping.

In the genetic tradition, a gene is considered *necessary* for a process if null mutants disrupt the process, and *sufficient* if restoration of the gene ameliorates the phenotype. This is usually taken as proof that a given gene is the cause of a phenotype. The circuit mapping analogy is that if blocking neural activity in a group of neurons disrupts a behavior, those neurons are in some way *necessary* for the performance of that behavior. If restoring neural activity—or function of a necessary gene—specifically in a group of neurons rescues the behavior, these neurons are thought to be *sufficient*. If triggering activity in a group of neurons evokes the behavior, those neurons are capable of causing the behavior, whether they normally play this role or not. These standards of proof for implicating neurons in behavioral control are useful, but the circuits that normally drive behavior can be complex and redundant, so care should be taken to interpret the results of necessity and sufficiency experiments. With neurons as well as with genes, the expression levels and extent of rescue are rarely perfectly measured or controlled. Blocking and activating experiments in the style depicted in Fig. 3.1 are useful for identifying the component parts of neural circuits, but the way these neurons work together to drive behavior is a network property; the list of parts is necessary but not sufficient to explain circuit function.

C. Anatomy and stereotypy

Sometimes the anatomy alone gives clues about neural function and connectivity into circuits. For example, the "parts list" for the retina suggests where color comparisons could be made (Fischbach and Dittrich, 1989; Morante and Desplan, 2008). The morphology of the lobular plate tangential cells suggests that they may detect horizontal or visual motion (Joesch *et al.*, 2008; Scott *et al.*, 2002). Although there is no published quantification, there are thought to be on the order of 100,000 neurons in the adult fly nervous system: 30,000 are part of the central brain (includes the subesophogeal ganglia), 15,000 in each optic lobe, and another 15,000 in the ventral nerve cord or thoracic and abdominal ganglia. Approximately 3600 ascending and descending neurons pass through the cervical connective to connect the brain and thoracic ganglia. Neuronal cell bodies are between 2 and 5 μm in diameter, dendritic fields can span 50 μm, and

neurites can extend 100 μm. In *Drosophila*, the cell bodies are located on the outside surface of the brain—the cortical rind—while the neurites project inside to form the synaptic neuropil. This region is divided into compartments by glial sheaths and axon tracts. The fly uses the canonical neurotransmitters (including acetylcholine, glutamate, GABA, histamine, dopamine, and serotonin (Bicker, 1999; Littleton and Ganetzky, 2000)) as well as tyramine, octopamine, and neuropeptides (Nassel and Homberg, 2006; Roeder, 2005; Taghert and Veenstra, 2003). How many types of neurons the fly has is the subject of much debate, but this largely depends on how one defines type: origin or lineage, transmitter type, gene expression profile, morphology, connectivity, or function. The nomenclature and descriptive anatomy of the adult fly brain is still being studied and described—no atlas or comprehensive textbook exists—although there is a serious effort underway to standardize naming conventions and disseminate this information to the research community. There remains a lot of terra incognita: brain regions whose function and connectivity is unknown.

In order for circuit mapping to be meaningful, we must ask if the circuits that drive a behavior in one individual will be similar to those that do so in another. We believe neural identity and connectivity in the fly are relatively stereotyped. The sensory projections and the circuits governing innate behaviors seem to be grossly similar from individual to individual where they have been carefully studied. For review of the olfactory projection neurons as an example, see Cachero and Jefferis (2008). The motor neurons and photoreceptors connect precisely to their targets even in the absence of neural activity (Baines *et al.*, 2001; Broadie and Bate, 1993; Hiesinger *et al.*, 2006). There are examples of morphological plasticity: the olfactory glomeruli responding to carbon dioxide expand if the flies are raised in a high CO_2 environment (Sachse *et al.*, 2007). The mushroom bodies are larger in flies raised in mixed gender groups than in those raised in isolation, and the brain areas associated with walking are larger in lab strains while those associated with flight are larger in more wild ones (Heisenberg *et al.*, 1995; Rein *et al.*, 2002). In the optic neuropils, cell size and shape can change with circadian rhythms (Pyza and Meinertzhagen, 1999). Most of these changes are due to increases in arborization or branching, and potentially increases in synaptic connections, rather than the development of entirely new circuits. Activity within a circuit might or might not be stereotypical. For instance, statistical arguments can be made from recording from many mushroom-body Kenyon cell neurons to show that their odor response profiles vary between individuals (Murthy *et al.*, 2008). Whether this affects the animals' behavioral performance is not known. Extensive work—both theoretical and experimental—in the stomatogastric system has shown that functional central pattern generators can be constructed with neurons with a range of firing properties and configurations (Prinz *et al.*, 2004; Schulz *et al.*, 2006). In the behavior assays performed in flies to date, genetically homogeneous populations tend to

perform similarly. It seems reasonable to suppose that the neural circuits that underlie behavior are sufficiently stereotyped in *Drosophila* that we can learn something useful about their organizing principles.

To summarize, genes that have behavioral consequences have been identified. Unusual alleles of these genes have been more informative than nulls. These genes tend to control the development of neurons or be components of the machinery that makes them function (ion channels, SNARE proteins, enzymes, etc.). Systems for targeting neurons, rather than genes, may be more informative for sorting out principles of neural circuit organization. One can disrupt neural function to show necessity or activate neural function to determine sufficiency. The fly brain seems to be sufficiently hardwired and stereotyped that the circuits that drive a behavior should be similar in different individuals of the same genotype, allowing the deduction of general principles of how circuits organize to drive behavior.

II. SPATIAL TARGETING OF NEURON TYPES

One would like to have reproducible genetic access defined populations of neurons for circuit analysis. One can introduce exogenous genes into *Drosophila* using transposable elements (Rubin and Spradling, 1982) and generate markers for given cells by fusing an enhancer directly to an enzymatic or fluorescent reporter protein (for example, see Couto *et al.*, 2005; Wang *et al.*, 2004b). The binary **UAS-GAL4** system (Brand and Perrimon, 1993; Fischer *et al.*, 1988) uses the GAL4 transcription factor from yeast to drive transgenes of choice under the control of the UAS upstream activating sequence. This two-part system is a powerful technique for expressing different genes in the same cell types. It allows reproducible access to a given cell type to perform different manipulations. For example, one can use *ShakingB-GAL4* to express *UAS-mCD8-GFP*, a membrane-targeted green fluorescent protein, to visualize the trajectory of giant fiber neurons in one set of flies and then use the same GAL4 driver to express *UAS-Shibire*^{ts1}, a temperature-sensitive protein that blocks synaptic vesicle recycling, to disrupt neural activity in the same neurons to assay behavioral consequences in another set of flies (see Fig. 3.1). Given the stereotypy of expression from the GAL4, one can be reasonably confident that both manipulations are being done on the same population of neurons (see below—end of Section III—for a discussion of the limits of this assumption). The GAL4 line, which dictates which neurons are targeted, is referred to as the driver, while the UAS construct is called a reporter or effector. In addition to targeting different operations to the same cells, the UAS-GAL4 system also amplifies the

expression level of the reporter transgene. The GAL4 system and its many uses have been reviewed often (Duffy, 2002; Phelps and Brand, 1998); the various intersectional modifications discussed below are summarized in Fig. 3.2.

It is possible to make GAL4 lines by randomly mobilizing a P-element transposon around the genome. This approach is called **enhancer trapping** and has been done extensively (Han *et al.*, 1996). The enhancer trap GAL4 lines might be expressed in the same cells as the gene whose enhancer they trap, but they might have novel patterns since they can land in the middle of enhancers or capture fragments of DNA that are serendipitously capable of driving expression. Whether the neurons labeled by a given GAL4 line constitute a "cell type" is debatable, but they are a group of cells that have at least some element of gene expression in common. P-elements have insertion site preferences (AT rich regions in the 5′ ends of genes) and at this point the genome has been extensively covered with P-element inserts of GAL4. The labs of Kaiser, Ito, and Heberlein have generated large collections (Hayashi *et al.*, 2002; Manseau *et al.*, 1997; Rodan *et al.*, 2002). There are variations on the enhancer trap: a dual-headed trap can pick up enhancers from genes transcribed on either strand to increase the rate of insertions with expression patterns (Lukacsovich *et al.*, 2001). No one has yet published a large-scale GAL4 enhancer trap hop in one of the alternative transposons (piggyBac, Minos, Mariner) which have different insertion biases; this might generate new GAL4 expression patterns. The protein trap approach could also be adapted to select inserts that actually disrupt genes (Lukacsovich *et al.*, 2008; Morin *et al.*, 2001; Quinones-Coello *et al.*, 2007), which will occur less frequently with the alternative transposons.

It is also possible to design GAL4s to reflect expression of specific genes, either by knocking GAL4 into the genomic locus (Rong and Golic, 2000), as was done to make *Fruitless*^{M}*-GAL4s* (Demir and Dickson, 2005; Manoli *et al.*, 2005) or by using large fractions of the DNA surrounding a gene, as for TH-GAL4 (Friggi-Grelin *et al.*, 2003). The latter approach should become easier with the adoption of the bacterial artificial chromosome (BAC) insertion approach (Venken and Bellen, 2005; Venken *et al.*, 2006). It is also possible to take small pieces of DNA upstream of the coding region of interesting genes and fuse this putative regulatory DNA to GAL4 in a transformable vector (Sharma *et al.*, 2002). This designed enhancer approach has been used in the past (Hiromi *et al.*, 1985; Moses and Rubin, 1991) and a large collection of GAL4s using the regulatory regions of neural genes is being generated now (Pfeiffer *et al.*, 2008). This collection is expected to be very powerful because of the high expression level of its GAL4 vector and because all of the constructs are inserted into the same genomic locus using the PhiC1 integration system (Bischof *et al.*, 2007; Fish *et al.*, 2007; Groth *et al.*, 2004), removing position effect variation. The existing and planned GAL4 reagents come close to allowing genetic access to small intersecting subsets of neurons throughout the fly brain.

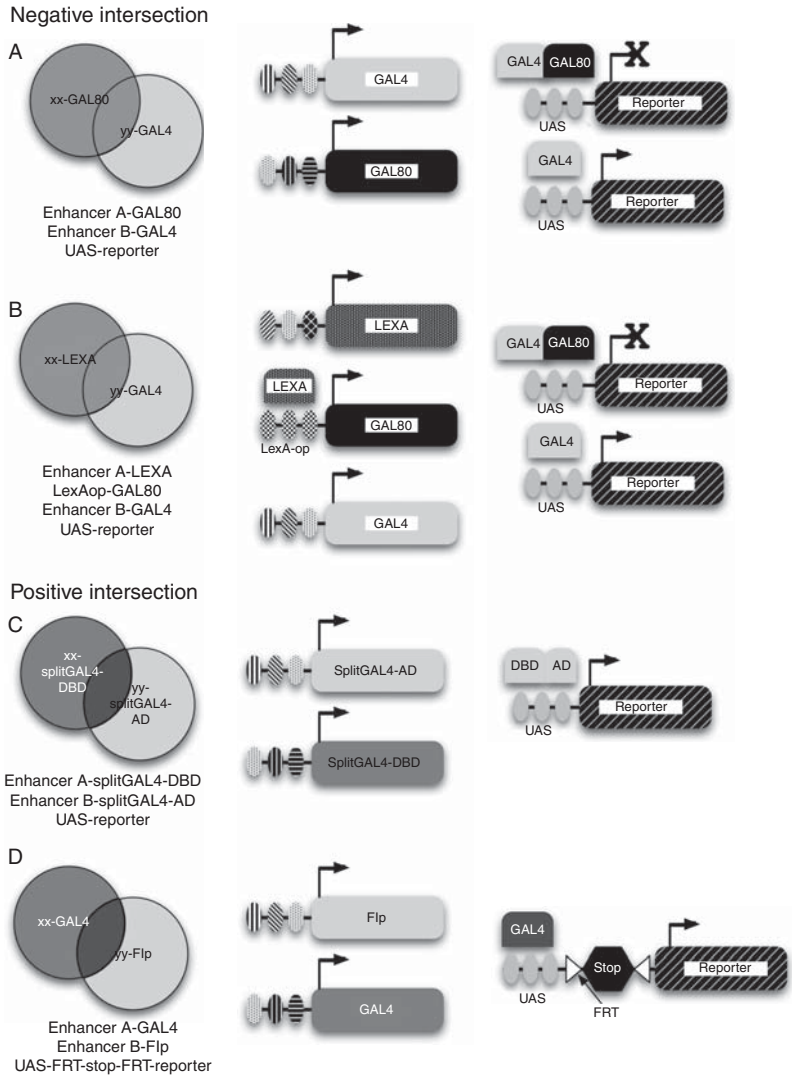

Figure 3.2. *Intersectional strategies to refine spatial expression patterns.* (A) When GAL4 and GAL80 patterns overlap, reporter expression is possible where GAL4 is made and GAL80 is not. (B) GAL80 can be expressed in response to LEXA, which may amplify its expression level. GAL4 function is restricted to the nonoverlapping region. (C) Expressing both halves of split-GAL4 in overlapping patterns restricts functional GAL4 production to the overlap. (D) Expressing a recombinase allows the removal of a stop cassette flanked with target sites. If the reporter is under UAS control as well, it is only made where the recombinase and the GAL4 expression coincide. Other strategies and combinations can be constructed from these basic building blocks.

We have not yet achieved the kind of control where one can design a regulatory sequence of transcription factor and repressor binding sites to dictate the location and level of expression of GAL4. There are promising steps in that direction with a few well-established transcription factor-binding sites dictating wing stripes and embryo segment patterns (Guss *et al.*, 2001; Markstein *et al.*, 2004; Moses and Rubin, 1991). With comparative analysis of the 12 sequenced *Drosophilid* genomes, the transcription factor binding site mapping projects (Gallo *et al.*, 2006), and the antibody generation effort to map the expression pattern of transcription factors (http://www.modencode.org/), it is rational to hope that this kind of designer control element may someday exist.

An additional level of spatial expression control can be added to the UAS-GAL4 system by including **GAL80**. GAL80 is another yeast protein that binds to GAL4 and prevents it from activating transcription (Ma and Ptashne, 1987). More GAL80 may be needed to neutralize a given amount of GAL4. A GAL4 line and a GAL80 line with overlapping expression patterns can be combined (Lee and Luo, 1999). The UAS reporter line will only be expressed in places where the GAL4 is present but not the GAL80, providing a negative intersectional strategy (Suster *et al.*, 2004). It is hard to see where a GAL80 line is expressed: there is no good antibody for immunohistochemistry and the protein is likely to be nuclear or cytoplasmic, making it difficult to extrapolate which neurons express it. It is possible to convert a GAL4 enhancer trap line into a GAL80 line by P-element replacement (Sepp and Auld, 1999) but screening for this can be hard to do visually and PCR screening is sometimes required.

This kind of intersection can also occur in time as well as space. The **TARGET** approach uses ubiquitous expression of a temperature-sensitive version of GAL80 to suppress GAL4 function while the flies are at permissive temperature (McGuire *et al.*, 2003). The flies can be temperature-shifted, which inactivates GAL80, and now the GAL4 is able to activate reporter genes. The ramp up of GAL4 is gradual and the temperature shifting may not be appropriate for all experiments, but this strategy was a major advance for temporal as well as spatial control of gene expression. Another method, **GeneSwitch**, adds temporal control with a drug-sensitive GAL4 (Osterwalder *et al.*, 2001; Roman *et al.*, 2001). Animals are fed RU486, which then binds the modified GAL4 to activate gene expression. This approach requires rebuilding the GAL4 lines of interest and it also has slow kinetics (on the order of 24 h). Both methods suppress GAL4 expression during development and then allow function to be turned on; they are less effective for rapidly turning GAL4 function off. The TARGET and GeneSwitch methods have been reviewed (McGuire *et al.*, 2004). A temperature-sensitive version of GAL4 itself is another alternative (Mondal *et al.*, 2007). The **Tet-on/Tet-off** system requires three transgenes but allows the use of the existing GAL4 collections (Stebbins *et al.*, 2001). It relies on modifying the reporter gene to be drug sensitive and has not been widely adopted. Modifying the reporter to

be produced in a temperature-sensitive fashion produced using **inteins** might also be possible (Zeidler *et al.*, 2004). The slow kinetics of these systems is acceptable if the amount of compensation for the manipulations is minimal and if the behavior under study can be triggered acutely.

Another option for increasing the specificity of a broadly expressing GAL4 is to use it to drive an **RNAi** construct for a transcript that is only present in a subset of cells. This approach was used to identify the *fruitless*-positive median bundle neurons as the critical ones involved in some aspects of courtship behavior (Manoli and Baker, 2004). There are now several collections of RNAi lines for neural genes available (Dietzl *et al.*, 2007; Mathey-Prevot and Perrimon, 2006; Ni *et al.*, 2008; Sepp *et al.*, 2008). Screening genetically targeted RNAi lines has been used to identify the sex peptide receptor and the neurons that express it as critical components of the circuitry for female receptivity behavior (Hasemeyer *et al.*, 2009; Yapici *et al.*, 2008).

Whereas the above methods are negative intersectional strategies, in that they are used to remove part of a GAL4 pattern, positive intersectional strategies have also been developed. These allow the targeting of a reporter to areas only where two expression patterns overlap. The GAL4 protein can be split into two pieces, one of which contains the DNA-binding domain and the other of which activates transcription. (This is the basis for the yeast two-hybrid screening system.) The two pieces can be brought back together again by leucine zipper motifs with high specific affinity and reconstitute a protein that is less effective than the original GAL4 but is still able to activate transcription (Luan *et al.*, 2006b). Each half of the **split GAL4** can be expressed in different patterns, and functional GAL4 is only reconstituted in the overlap zone to drive reporter expression. This approach has been used to identify which neurons within a larger group are really responsible for driving wing expansion (***Luan et al., 2006a***). The split GAL4 technique has great potential utility but requires rebuilding the GAL4 lines of interest. Some examples of astute use of these tools for circuit bashing can be seen in ***Gao et al. (2008)*** and ***Shang et al. (2008)***.

Other positive temporal or spatial intersectional strategies involve the **FLP and Cre recombinases** (reviewed in Bischof and Basler (2008)). During mitosis, they catalyze excision and ligation of double-stranded DNA at defined DNA sequences (FRT or lox sites) (Golic, 1991; Golic and Lindquist, 1989). FLP was initially used for generating chromosomal breaks at the base of each chromosome arm using a heat-shock induced expression of flippase (Basler and Struhl, 1994; Struhl and Basler, 1993; Xu and Rubin, 1993). When the chromosomal break occurs in a dividing cell, it produces a clone of cells that are homozygous mutant in a heterozygous background. Several strategies for making targeted mosaics where specific parts of the fly (usually the eye) expressing the recombinase can become homozygous mutant. This allowed screens for genetic mutations that might have been lethal in the whole animal and was very

successful at identifying components of synaptic function, for example (Blair, 2003; Newsome *et al.*, 2000; St Johnston, 2002; Stowers and Schwarz, 1999). Although recombination events are not reversible, temporal control of the initial recombination event can be achieved with a heat-shock-inducible enhancer (usually from hsp70) or a hormone-inducible motif appended to the recombinase itself (Heidmann and Lehner, 2001). A modification of this approach called mosaic analysis with a repressible cell marker (**MARCM**) combines the use of FLP with GAL4 and GAL80 to mark mutant clones within a given GAL4 pattern (Lee and Luo, 1999, 2001). The MARCM technique allows the intersection of marking based on lineage and marking based on gene expression, which represents an extremely powerful anatomical technique for visualizing cell lineages and single cells (Jefferis *et al.*, 2001).

The recombinases can be used to trigger intrachromosomal recombination events between defined sites as well. Usually this approach involves recombination to remove a stop cassette between UAS and a reporter or effector; it is sometimes called **Flp-out**. The recombination can occur in postmitotic cells and so affects a random set of cells within a given GAL4 pattern. This strategy can be used to equalize expression levels of different reporter constructs, to prolong the expression of a GAL4 that is expressed early in development, or to positively intersect two expression patterns. If the recombination is triggered in a dividing cell, this approach can be used to label neurons related by lineage as is obligatory in MARCM. For example, *TubP-FRT-STOP-FRT-GFP*, *UAS-flippase*, and *PoxN-GAL4* can be combined to cause the expression of GFP to be maintained in all the cells in which the early-expressing GAL4 was active. An enhancer trap GAL4 line could be combined with a line expressing flippase in all the glutamatergic neurons and a *UAS-FRT-STOP-FRT-GFP* to visualize only the glutamatergic neurons within the enhancer trap pattern. The recombinases are reported to work at very high efficiency, especially when catalyzing intragenic—rather than interchromosomal—recombination, and there is a range of matched recombinase binding sites that work in *Drosophila* (Heidmann and Lehner, 2001; Horn and Handler, 2005; Oberstein *et al.*, 2005; Rodin and Georgiev, 2005; Siegal and Hartl, 1996).

To subdivide a GAL4 pattern for imaging, a clonal approach like MARCM or a random approach using a recombinase removes a stop cassette in postmitotic cells are effective alternatives (Chiang *et al.*, 2004; Marin *et al.*, 2002; Wong *et al.*, 2002). These approaches can be used for behavioral analysis, but large numbers of individual animals are needed to get statistical confidence that particular neurons really correlate with a given behavioral defect (Gordon and Scott, 2009a; Kimura *et al.*, 2008; Shang *et al.*, 2008; Yang *et al.*, 2009).

Recently, alternative two-component systems have been transported to the fly. The *yeast* **LexA** transcription factor and the lexOp DNA sequence to which it binds appear to work in flies as well. This allows independent targeting

of different transgenes (Lai and Lee, 2006). For example, one might target *UAS-mCD8-GFP* to the presynaptic side of the neuromuscular junction with *VGlut-GAL4* and *lexOP-CD2-mRFP* to the postsynaptic side with an *MHC-LexA*. LexA and GAL4 can also be combined to expand the repertoire of intersectional strategies. Other transcription factor—binding site systems are under development.

With the library of expression patterns that can be generated by the GAL4-based strategies described above and summarized in Fig. 3.2, we have the tools to image and manipulate neural circuits with unprecedented spatial and temporal precision.

III. IMAGING NEURONS

To visualize the neurons in which GAL4 is expressed, the membrane-targeted green fluorescent protein encoded by **UAS-mCD8-GFP** is most commonly used (Lee and Luo, 1999); alternative anatomical reporters are listed in Table 3.1. The endogenous or intrinsic fluorescence of GFP in live or briefly fixed tissue is usually sufficient to detect the small processes of neurons, but when the tissue can be fixed, the signal is often amplified with primary antibodies against CD8 or GFP itself and bright, photostable, dye-coupled secondary antibodies. For an example protocol, see Wu and Luo (2006). This also allows counterstaining with the mouse nc82 monoclonal antibody to label the whole synaptic neuropil and provides a broad landmark for registering different preparations to a common standard (Jenett *et al.*, 2006; Rein *et al.*, 2002).

While *UAS-mCD8-GFP* provides a good staining of neuronal processes for anatomical analyses, the cytoplasmic **UAS-eGFP** has been reported to be the most innocuous for electrophysiology (Su and O'Dowd, 2003); here the endogenous brightness is essential since GFP here is used to target electrodes in live preparations. GFP has been optimized for brightness, photostability, and pH insensitivity. Most of the GFP in current use is codon optimized for vertebrates, rather than the original jellyfish, and contains the S65T point mutation; thus, it should more precisely be called EGFP (Yang *et al.*, 1996).

The choice of alternative colors of fluorescent proteins is dizzying (Giepmans *et al.*, 2006; Shaner *et al.*, 2005). It is possible to image different neural populations in different colors using direct enhancer fusions or orthogonal expression systems (GAL4 and LexA). We now have photoactivatable and photoswitchable fluorophores, and fluorophores that change color over time (Terskikh *et al.*, 2000) are reviewed (Lippincott-Schwartz and Patterson, 2008). Timer was used to show that the inner fibers of the mushroom bodies are younger than the outer fibers, indicating that unlike tree rings, the

Table 3.1. UAS-Reporters for Visualizing Neurons

Construct name	Localization	Comments	References
Anatomy			
UAS-mCD8-GFP	Membrane	Can also be detected with antibodies to CD8 or GFP	Lee and Luo (1996)
UAS-myr-mRFP	Membrane	Uses endogenous fluorescence of mRFP	H. Chang, flybase
UAS-eGFP	Cytoplasm	Electrophysiologically neutral	Su and O'Dowd (2003)
UAS-nls-GFP	Nucleus	Both GFP and lacZ fusions exist	Robertson *et al.* (2003)
UAS-nSyb-GFP UAS-Syt-GFP UAS-Syt-HA	Synapses	Visualized with antibody to HA	Estes *et al.* (2000), Robinson *et al.* (2002), and Zhang *et al.* (2002)
UAS-DsCam17.1-GFP	Dendrites	May change dendrite morphology	Wang *et al.* (2004a)
UAS-Rdl-HA	Postsynapse	Visualized with antibody to HA	Sanchez-Soriano *et al.* (2005)
UAS-cac-GFP	Active zones	Tested at neuromuscular junction	Kawasaki *et al.* (2004)
UAS-tau-lacZ	Axons	Both GFP and lacZ fusions exist; may affect neuron health	Callahan and Thomas (1994) and Hidalgo *et al.* (1995)
UAS-nod-lacZ	Dendrites	Both GFP and lacZ fusions exist	Anderson *et al.* (2005) andClark *et al.* (1997)
UAS-GAP-GFP	Axons	Tested at neuromuscular junction	Ritzenthaler *et al.* (2000)
UAS-PA-GFP	Cytoplasm	Activated by 710 nm light	Datta *et al.* (2008)
UAS-Timer	Cytoplasm	Switched from red to green over several hours	Verkhusha *et al.* (2001)
Activity			
UAS-GCaMP	Cytoplasmic	Calcium sensor; various improved versions exist	Wang *et al.* (2003a,b)
UAS-Cameleon	Cytoplasmic	FRET calcium sensor; Synapcam is a synaptically targeted variant	Fiala *et al.* (2003), Guerrero *et al.* (2005), and Hendel *et al.* (2008)
UAS-Camgaroo	Cytoplasmic	Calcium sensor	Yu *et al.* (2003)
UAS-TN-XXL	Cytoplasmic	Calcium sensor using troponin C	Mank *et al.* (2008)
UAS-D3cpv	Cytoplasmic	Redesigned M13 peptide	Hendel *et al.* (2008)
UAS-GFP-Aequorin	Cytoplasmic	Bioluminescent Ca^{2+} indicator	Martin *et al.* (2007)
UAS-FlaSh	Membrane; synapse	Voltage sensor; based on a pore-mutated Shaker voltage-gated K+ channel subunit	Siegal and Isacoff (1997)
UAS-hVos	Membrane	Hybrid voltage sensor	Sjulson and Miesenbock (2008)
UAS-SynaptopHluorin	Synaptic vesicles	Vesicle release detector	Miesenbock *et al.* (1998)
UAS-Epac1-camps	Cytoplasmic	cAMP level reporter	Shafer *et al.* (2008)

late-growing axons actually push up through a bundle of established tracts (Verkhusha *et al.*, 2001). Photoactivatable GFP has been used to trace a group of axons with particular odor response profiles (***Datta et al., 2008***).

There are options for targeting reporter proteins to different subcellular compartments. One might use the dendritic and synaptic reporters to deduce input and output zones in a given GAL4 pattern to hypothesize about connectivity or information flow. To visualize neural processes, fusions to the tau motor protein were initially popular and they provide excellent labeling (*UAS-tau-lacZ* and **UAS-tau-GFP**) (Callahan and Thomas, 1994; Hidalgo *et al.*, 1995), but they are deleterious to many neural types (Williams *et al.*, 2000). *UAS-GAP-GFP* (Ritzenthaler *et al.*, 2000) also labels axons. The T-cell membrane-targeting motifs from CD2, CD4, and CD8 (mouse or rat) and myristylation sequences from c-src seem to bring fluorescent proteins to the membrane efficiently in insect cells. To visualize nuclei, nuclear localization signals work well: *UAS-nls-lacZ* and **UAS-nls-GFP** (Hiromi, unpublished Bloomington stock #3955; Robertson *et al.*, 2003). Synaptic targeting can be achieved with fusions to SNARE proteins nSyb and synaptotagmin: **UAS-nSyb-GFP, UAS-Syt-GFP**, *UAS-Syt-HA*, and *UAS-nSyb-mRed* (Estes *et al.*, 2000; Raghu *et al.*, 2007; Robinson *et al.*, 2002; Zhang *et al.*, 2002). There is an active zone marker **UAS-cac-EGFP** that works at the neuromuscular junction in high copy number (Kawasaki *et al.*, 2004). Labeling dendrites or postsynaptic densities is currently the most problematic, but there are reports that it can done with **UAS-dsCam17.1-GFP** (cell adhesion molecule: Wang *et al.*, 2004a) or **UAS-Rdl-HA** (ionotropic GABA receptor: Sanchez-Soriano *et al.*, 2005). *UAS-nod-GFP* (Andersen *et al.*, 2005; Clark *et al.*, 1997), a fusion to another minus-end directed microtubule motor protein, also labels dendrites in some cell types. To move from the possibility of connections suggested by proximity of axons and dendrites to actual connectivity is an important leap that requires further evidence.

The **GRASP** technique for confirming that two neurons are synaptically connected by separately targeting expression of halves of GFP to the pre- and postsynaptic sides of a synapse to reconstitute functional fluorescence (Feinberg *et al.*, 2008), has now been adapted for the fly (Gordon and Scott, 2009a,b). An activity-dependent trans-synaptic tracer that works in many types of neurons would be extremely beneficial for exploratory investigation of neural connectivity, but in spite of hard work in many labs, none is currently available. Electron microscopy can show the presence of synapses and specific neuron classes can be targeted using the GAL4 system to drive *UAS-CD2-HRP* (Larsen *et al.*, 2003); the reaction product of this extracellularly tethered horse radish peroxidase is electron-dense. Synaptic specializations and vesicles may be visible. The number of synaptic contacts and the quantity of docked vesicles might provide some indication of the strength of the connection but the excitatory or inhibitory nature must be deduced by other means.

Please keep in mind some caveats. It is not certain that all of the manipulations we do to visualize neurons are neutral. High levels of GAL4 or reporter proteins may be toxic or alter cell morphology (Kramer and Staveley, 2003). Membrane-targeted proteins may be expressed highly enough to disrupt normal membrane properties. It is possible to have pre- and postsynaptic contacts on the same neurite (Olsen and Wilson, 2008b; Raghu *et al.*, 2007), which makes the analysis of circuitry at the light level more challenging. Confocal microscopy is typically used to visualize these reporters and optimal tissue clearing, laser ramping, and data collection standards are not always achieved. Some serious pitfalls are astutely enumerated in ***Ito et al. (2003)***. The level of the visible reporters may not match the level of effectors expressed, making it difficult to draw firm conclusions about which neurons visualized by *UAS-mCD8-GFP* are the ones responsible for the behavior seen with *UAS-Shibire^{ts1}* expressed by the same GAL4 line. Inserting all reporters and effectors into defined loci with the integrase system may help here by eliminating position effect varigation, and tagging the effectors directly with epitope tags or coexpressing reporters and effectors together with an internal ribosome entry site (IRES) or 2A self-cleaving peptide (Trichas *et al.*, 2008) may go some way toward ameliorating these concerns, but interpretations should be cautious. Detection thresholds for staining and behavior may be very different.

IV. FUNCTIONAL IMAGING: WATCHING NEURONAL ACTIVITY

The promise of functional neuroimaging is to be able to see activity in the processes or compartments of a single identified neuron, or to assay activity in several identified neurons at once, to watch circuit computations in action. Functional neuroimaging can be used to identify relevant neurons or to investigate exactly what previously identified neurons are doing during behavior performance. For neuronal activity, one can monitor membrane voltage or changes in calcium concentration; these reporters have been developed primarily in vertebrate systems and are discussed in greater detail below. The versions of these reporters that are available in the fly are listed in Table 3.1. Reporters for other biological activities exist that have relevance for neural function. There are new reporters for glutamate, usually an excitatory neurotransmitter (Hires *et al.*, 2008b), cAMP levels (Shafer *et al.*, 2008), Creb (Belvin *et al.*, 1999), receptor activation (Barnea *et al.*, 2008), and some kinase activities (Burrone, 2005; Tsien, 2005). **UAS-synaptopHluorin**, pH-sensitive fluorescent protein coupled to neural-synaptobrevin can be used to visualize synaptic vesicle release (Miesenbock *et al.*, 1998; Ng *et al.*, 2002). SynaptopHluorin has also been used to

show that additional neurons become active during the establishment of an olfactory memory (Yu *et al.*, 2004, 2005). Optical reporters represent a powerful, relatively noninvasive, technique for investigating neural circuits.

For neural circuit mapping, reporters that act over a longer timescale may be useful if they help identify brain regions that are active when a behavior is performed repeatedly. In mice, there have been attempts to harness the **immediate early genes** whose transcription is up-regulated by neural activity for this purpose (Barth *et al.*, 2004; Mongeau *et al.*, 2003; Reijmers *et al.*, 2007; Wang *et al.*, 2006). Exactly what these changes in gene expression mean is subject to intense debate. So far, attempts to transport this technique to flies have not been reported.

A. Voltage sensors

Just as it is appealing to be able to activate neurons in a way that mimics action potentials, it would be terrific to see neural activity at the resolution of action potentials. For an example of what can be done with really precise voltage measurements in multiple neurons simultaneously, see the work identifying the neurons that best correlate with the decision to swim rather than crawl in the leech (Briggman *et al.*, 2005). While most of the neurons that drive swimming and crawling are part of a shared network, a few correlate with only one of the two behaviors (Briggman and Kristan, 2006). The fast kinetics from voltage-sensitive dyes are powerful, but the dyes cannot be specifically targeted, penetrate different tissues unevenly, and provide poor spatial resolution. It would be ideal to have a genetically encoded voltage sensor; the current state of the field is well reviewed in Baker *et al.* (2008) and summarized below.

The original voltage-sensing fluorescent proteins were based on ion channels. **FlaSh** tethered GFP to a pore-mutant version of the *Drosophila* Shaker potassium channel close to the membrane so that movement of the voltage-sensing helix affected the fluorescence (Siegel and Isacoff, 1997). This produced a change in fluorescence in *Xenopus* oocytes (5% change with an −80 mV depolarization) but was not able to detect voltage changes in neurons and had kinetics too slow to resolve individual action potentials (on: 100 ms; off: 60 ms). Optimization of the fluorophore improved the response time to ~5 ms but did not make significant improvement in the amount of fluorescence change or the usability in neurons (Guerrero *et al.*, 2002). An alternative to FlaSh, voltage-sensing fluorescent protein 1 (**VSFP1**) was FRET based and used the isolated voltage-sensing S4 domain of the vertebrate potassium channel Kv2.1 (Sakai *et al.*, 2001). Sodium channel protein-based activity reporting construct (**SPARC**) fused GFP between the first and second 6 transmembrane repeat domains of the voltage-sensitive sodium channel rSkM1 (Ataka and Pieribone, 2002). All three of these channel-based voltage-sensors performed poorly in

neurons because they failed to localize well to the plasma membranes, resulting in low signal and high noise levels from the mislocalized fluorescence (Baker *et al.*, 2007a). Endogenous ion channel levels in the plasma membrane are tightly regulated to tune neural activity; perhaps the engineered voltage sensors based on ion channels are subject to the same regulatory mechanisms.

New voltage sensors under development use protein domains from enzymes rather that ion channels (Murata *et al.*, 2005; Ramsey *et al.*, 2006; Sasaki *et al.*, 2006; Tombola *et al.*, 2008). There is some hope that these will ameliorate the plasma localization and protein density limitations that plague the channel-based constructs. This may increase the detectable change in fluorescence. Additional improvements occur all the time (Tsutsui *et al.*, 2008; Villalba-Galea *et al.*, 2009), but whether the sensors will be able to follow the speed of action potentials in neurons *in vivo* is still uncertain.

Since voltage-sensitive chemical dyes can provide the high signal-to-noise ratio and fast kinetics desired for a voltage sensor with action potential resolution, there was some excitement about hybrid systems that couple a dye to a genetically encoded fluorescent donor or acceptor protein (Chanda *et al.*, 2005) which could provide the spatial localization the dyes alone lack. Unfortunately the hybrid voltage sensor (**hVOS**) approach that has been best tested in flies has not performed as well as hoped (Sjulson and Miesenbock, 2008). In a combination of modeling calculations and experiments where flies expressing a membrane-tethered GFP as a FRET donor were exposed to dipicrylamine (DPA, a voltage-sensitive FRET acceptor dye), Sjulson and Miesenbock showed that to see a significant fluorescence change even in a large group of neurons firing synchronously, such a high concentration of dye was required that the quantity of dye intercalating in the membrane changed its capacitance sufficiently to stifle action potentials. Other variants of the dye/genetic hybrid approach are possible but have yet to show positive results for voltage sensing in neurons (Hinner *et al.*, 2006; Lavis *et al.*, 2006).

B. Genetically encoded calcium indicators

More widely used than voltage sensors, calcium sensors act as a proxy to report neuronal activity. Calcium dynamics within neurons are complicated (Yasuda *et al.*, 2004). When a neuron fires an action potential, its membrane depolarizes in a propagating wave moving along the axon toward the synaptic terminal. This depolarization triggers the opening of voltage-gated Ca^{2+} channels (encoded by *cacophony* in *Drosophila*: Kawasaki *et al.*, 2000). The local influx of Ca^{2+} triggers the fusion of vesicles containing neurotransmitter with the plasma membrane, causing the neuron to pass information on to its postsynaptic partners. Repeated action potentials increase the local Ca^{2+} concentration in the neurons and thus Ca^{2+} levels are an indicator of how active the neuron is. Genetically encoded

calcium indicators (GECIs) can also be used to look at the Ca^{2+} dynamics in subcellular compartments such as dendritic branches, where calcium enters through nonselective ion channels, including the ligand-gated ionotropic neurotransmitter receptors such as the glutamate and voltage-gated NMDA receptor. *Drosophila* has NMDA receptors (Xia *et al.*, 2005) but whether their contribution to Ca^{2+} influx can be seen with GECIs has not been explored. Pumping the Ca^{2+} into intracellular stores in the endoplasmic reticulum or out of the cell with Ca^{2+}-ATPase pumps (PMCA) gradually restores the Ca^{2+} levels.

There are highly sensitive chemical indicators of calcium level (Fura dyes and Calcium green; for example use in fly, see Wang *et al.* (2001)), and these can be used with genetic markers of cell identity (Ritter *et al.*, 2001; Yaksi and Friedrich, 2006). There are also a variety of GECIs (reviewed in Hires *et al.* (2008a) and Miyawaki *et al.* (2005)). These are composed of a fluorescent protein (or two) and a peptide that changes conformation upon Ca^{2+} binding (calmodulin or troponin C). **Camgaroo** is a circularly permuted GFP with the calmodulin Ca^{2+}-binding domain at one end and the M13 calmodulin-binding peptide (from myosin light chain kinase) at the other; it undergoes a reversible conformational change upon ion binding that increases the fluorescence of GFP (Baird *et al.*, 1999). Camgaroo was used in *Drosophila* to visualize activity in the mushroom bodies in response to exogenously applied acetylcholine (Yu *et al.*, 2003). **Pericams** (Nagai *et al.*, 2001) and **GCaMPs** (Nakai *et al.*, 2001) use a similar strategy to detect an increase in Ca^{2+}. The GCaMP sensors are currently the most highly developed of these. Although membrane targeting GCaMP2 does not improve its performance (Mao *et al.*, 2008), new variants have the ability to reliably detect short trains of action potentials in some cell types and more improvements are expected soon. GCaMP and its derivatives have been used to map where different types of tastes and odors are processed (Fischler *et al.*, 2007; Marella *et al.*, 2006; Suh *et al.*, 2004; Wang *et al.*, 2003a) and to detect neuronal activity in the mushroom bodies during olfactory conditioning (Yu *et al.*, 2006). It may also be possible to use this type of imaging to identify which neurons within a complicated GAL4 pattern have activity correlated with the behavior under study and thus narrow down complicated expression patterns to spot the relevant neurons (but see caveats below).

The **cameleon** sensors also use calmodulin and the M13 peptide but in this case Ca^{2+} binding brings together two different fluorophores for fluorescence resonance energy transfer (FRET) (Miyawaki *et al.*, 1997, 1999). Ratiometric imaging of this type has been particularly helpful to compensate for movement artifacts (Kerr *et al.*, 2000). Recent variants have optimized the choice of fluorescent donor–acceptor pairs to maximize FRET and reduce interference with endogenous Ca^{2+} sensors (Yellow cameleons and D3cpv: Nagai *et al.* (2004) and Palmer *et al.* (2006)). Fiala *et al.* used Cameleon in *Drosophila* to examine olfactory responses in projection neurons and to demonstrate that

dopaminergic neurons fire strongly in response to electrical shock during olfactory conditioning assays (Fiala *et al.*, 2002; Riemensperger *et al.*, 2005). Cameleon has also been useful specifically for mapping novel neural circuits: Liu *et al.* used the reporter to identify the thermosensing neurons in larva (***Liu et al., 2003***). **Synapcam** is a synaptically targeted version of cameleon (Guerrero *et al.*, 2005) that shows that more distal boutons along a larval neuromuscular junction have higher levels of Ca^{2+} influx, a result that agrees with Ca^{2+} sensitive dye experiments (Lnenicka *et al.*, 2006).

TN-XXL is an alternative FRET-based Ca^{2+} sensor. Instead of calmodulin and the M13 peptide, it exploits a similar domain from troponin C (which is not present in neurons), and so may not interfere with endogenous calmodulin function. It can be activated by the longer wavelengths required for two-photon imaging *in vivo* in flies and mice. It has reasonable fluorescence change signal and may perform better for detecting changes when the overall Ca^{2+} concentration is low. Its performance has been characterized (Mank *et al.*, 2006, 2008).

As an alternative to fluorescence, a few groups have used **GFP-Aequorin** constructs to measure Ca^{2+} changes with bioluminescence (Martin *et al.*, 2007; Rosay *et al.*, 2001). This sensor requires coelenterazine as a cofactor. For very long timescale experiments, this is a possible alternative sensor.

There are problems with all of the GECIs. They tend to have small dynamic range, poor sensitivity, and slow kinetics. Calcium is an indirect proxy for neural activity and the indicators distort the kinetics of the calcium signal. Several recent reviews have compared the available Ca^{2+} indicators (Hendel *et al.*, 2008; Mao *et al.*, 2008; Martin, 2008; Miesenbock and Kevrekidis, 2005; Pologruto *et al.*, 2004; Reiff *et al.*, 2005). The best choice may depend on the exact preparation and expected Ca^{2+} concentration range. In the best cases it may be possible to detect single action potentials with reasonable reliability, but this has not yet been done in the fly. If the action potentials are sparse, the rise time of the Ca^{2+} indicators is sufficient to detect them with high reliability; the decay time is slower, so if the action potentials occur too close together, they cannot be individually resolved, but rate can be estimated by deconvolution (Kerr and Denk, 2008; Wallace *et al.*, 2008). In many neurons multiple spikes are required to generate a visible fluorescence change and the temporal precision of the indicators may make this difficult. The calcium indicators may buffer the Ca^{2+} they detect and may interfere with normal Ca^{2+} binding proteins. They are not able to detect subthreshold or graded changes in membrane potential. The Ca^{2+} signal almost always under-represents the number of active neurons involved because of the high thresholds of activity required to trigger the sensors. In any case, careful interpretation and system-specific validation is needed to determine exactly what the detected change in Ca^{2+} concentration represents—and what it may miss (Hendel *et al.*, 2008; Jayaraman and Laurent, 2007).

GECIs are a powerful way to identify the neurons involved in particular behaviors or circuits, but can sometime yield different results than electrophysiology. Ca^{2+} dynamics measured with GECIs are slower than changes in membrane potential; this allows summation of weak signals but makes it hard to resolve fast spike trains. Several research groups investigated the transformation of information that occurs at different relay points in the olfactory circuit. The results obtained with GCaMP and SynaptopHluorin differed from that obtained with electrophysiological recordings (Ng *et al.*, 2002; Olsen and Wilson, 2008b; Root *et al.*, 2007; Shang *et al.*, 2007; Wang *et al.*, 2003a; Wilson *et al.*, 2004). Since it is now possible—albeit difficult—to record from neurons in the fly brain during sensory experience (Wilson *et al.*, 2004), it is possible to better calibrate the genetic reagents we use to inhibit, activate, and monitor neurons (Jayaraman and Laurent, 2007).

There is a long history of **electrophysiological recording** from neurons, muscles, and sensory structures *Drosophila*. Technical reviews include (Broadie, 2000a,b; Matthies and Broadie, 2003). Electrophysiological methods have been critical for assaying ion channel properties, synaptic vesicle release and recycling machinery, neurotransmitter identity, mechanisms of synaptic plasticity, and sensory information coding. The new genetic tools for manipulating neural activity (discussed below) have been tested by electrophysiology. For circuit analysis in particular, electrophysiological techniques have been instrumental in identifying brain regions involved in specific behaviors, establishing the temporal code of action potentials generated in response to sensory stimuli, and demonstrating connectivity by paired recording or in combination with activation by Channelrhodopsin or imaging with GCaMP. Although electrophysiology in the fly is limited to one—or at most a few—neurons at a time, it provides unparalleled sensitivity and temporal precision for monitoring neural activity. The technical challenges of recording from small, deep brain neurons in a behaving animal should not be underestimated. Table 3.2 lists some of these electrophysiological techniques and example papers where they are used.

V. CONTROL OF NEURAL ACTIVITY

There are many strategies for manipulating neurons once one has a reproducible way to target them. There are cell killers based on toxins or genes that promote programmed cell death; ion channels and proteins that interfere with a neuron's excitability; toxins, and mutations that disrupt the synaptic vesicle cycle; and a slew of enzyme-specific blockers. I refer to these UAS constructs collectively as "effectors" rather than "reporters," which are usually fluorescent ways to visualize cells. All of these effectors have pros and cons associated with them; available reagents are summarized in Table 3.3 and discussed below.

Table 3.2. Electrophysiological Techniques in *Drosophila*

	Technique	References
Culture		
Embryonic	Neuroblasts from gastrulating embryos are isolated, dissociated, and induced to extend neurites in culture; whole cell patch recordings are performed	O'Dowd (1995), O'Dowd and Aldrich (1988), and Seecof *et al.* (1971)
Giant neurons	Neuroblasts are harvested from embryos and then the last cell divisions are blocked to create large multinucleate neurons that can be targeted with electrodes for whole cell patch recording	Saito and Wu (1991)
CNS neurons	Cells are cultivated from embryos and larvae and genetically labeled neurons are targeted for whole cell patch clamp recording to study electrical properties of the neurons	Sicaeros *et al.* (2007) and Wright and Zhong (1995)
Photoreceptors	Adult or pupal ommatidia are cultured for subsequent whole cell patch clamp recording to characterize electrical properties in genetically identified neurons	Hardie (1991)
Neuromuscular junction (NMJ)		
Giant fiber	Flies are immobilized and recordings from motoneuron, muscle and/or the giant fiber axon is preformed. The giant fiber is electrically stimulated through tungsten electrodes placed in the eyes or brain	Elkins and Ganetzky (1990), Engel and Wu (1996), Fayyazuddin *et al.* (2006), Koenig and Ikeda (1983), and Tanouye and Wyman (1980)
Larval NMJ	Larvae are filleted out and intracellular voltage recordings from the muscle can measure both evoked junctional potentials (EJPs) and excitatory junctional currents (EJCs). Two electrode voltage clamp (TEVC) recordings from the muscle have been used to identify membrane currents	Imlach and McCabe (2009), Jan and Jan (1976), Singh and Wu (1989), Wu and Haugland (1985), and Zhong and Wu (1991)
Larval motor nerves	Recording and stimulating from different points along the nerve bundle shows conduction defects and direction of action potential propagation	Wu *et al.* (1978)
Embryonic NMJ	Whole cell patch clamp and perforated patch recordings from developing muscle are possible in dissected young embryos (<17 h AEL). Older embryos require dissection at 16 h AEL and culturing to the appropriate developmental stage	Broadie and Bate (1993)

(*Continues*)

Table 3.2. (*Continued*)

	Technique	References
Embryonic and larval motor neurons	In the filleted animal, whole cell recordings from identified motoneurons, as well as loose patch recordings over synaptic boutons, are also possible	Baines and Bate (1998), Baines *et al.* (2006), Choi *et al.* (2004), and Rohrbough and Broadie (2002)
Sensory periphery		
Photoreceptors	Flies are immobilized and a small hole made in their cornea to allow *in vivo* recordings using sharp glass microelectrodes. This allows study of signal processing and response dynamics of photoreceptors	Juusola and Hardie (2001) and Niven *et al.* (2003)
Large monopolar cells (lamina)	Small corneal openings in an immobilized fly's eye allow sharp glass microelectrode recordings. Information processing at first synapse of the system can be studied. LMCs are identified by their distinctive electrical properties	Zheng *et al.* (2006)
Electroretinograms	Extracellular recording measures light-induced depolarization of photoreceptors and synaptic activation of second order neurons	Alawi and Pak (1971), Hotta *et al.* (1969), and Kelly and Suzuki (1974)
Mechanosensory bristles	Extracellular transepithelial potential recording measures neuronal response to bristle deflection	Dickinson and Paulka (1987) and Kernan *et al.* (1994)
Electroantennograms	Extracellular recordings from the antennal nerve measures gross output of olfactory sensory neurons	Borst (1984) and Venard and Pichon (1981, 1984)
Olfactory receptor neurons	Flies are immobilized for extracellular recordings using low-impedance glass or tungsten electrodes; recordings are made from base of olfactory sensilla in antennae and maxillary palp which allows isolation of activity of single olfactory receptor neurons in response to odors	Clyne *et al.* (1997), de Bruyne *et al.* (1999), Hallem *et al.* (2006), and Kreher *et al.* (2008)
Central nervous system (CNS)		
Mushroom body kenyon cells and circadian pacemaker neurons from isolated whole brain explants	Whole brains are isolated from adult flies and prepared for whole cell patch clamp from genetically labeled neurons using differential interference contrast (DIC) imaging; this permits examination of electrical properties of neurons under different conditions (e.g., during sleep/awake phases in circadian cycle)	Cao and Nitabach (2008), Gu and O'Dowd (2006, 2007), and Sheeba *et al.* (2008)

(*Continues*)

Table 3.2. (*Continued*)

	Technique	References
Antennal lobe projection neurons from isolated whole brain explants	Whole brains and antennae are isolated from adult *Drosophila* and bathed in saline for loose patch recordings from genetically labeled neurons targeted using two-photon imaging; recordings allow detection of action potentials in response to odor	Root *et al.* (2007)
Antennal lobe projection neurons and interneurons	Flies are immobilized and dorsal sections of cuticle, trachea and sheath removed to expose antennal lobes; the brain is bathed in saline keeping antennae in air for *in vivo* whole cell patch clamp and loose patch recordings from genetically labeled neurons in response to odors (performed under visual guidance using DIC optics or two-photon imaging)	Bhandawat *et al.* (2007), Datta *et al.* (2008), Jayaraman and Laurent (2007), Olsen and Wilson (2008a,b), Wilson and Laurent (2005), and Wilson *et al.* (2004)
Mushroom body kenyon cells	Flies are immobilized and posterior sections of cuticle, trachea and sheath are removed to expose mushroom body. The brain is bathed in saline for *in vivo* whole cell patch clamp recordings from genetically labeled neurons in response to air-delivered odors; recordings are performed under visual guidance (DIC)	Murthy *et al.* (2008) and Turner *et al.* (2008)
Lobular plate interneurons	Flies are immobilized and lateral-posterior sections of cuticle, trachea and sheath removed to expose lobula plate. The brain is bathed in saline while eyes remain in air. This allows visually guided whole cell patch clamp recordings of vertical-sensitive neurons of the lobula plate tangential system in response to visual patterns	Joesch *et al.* (2008)

A. Cell killers

One way to genetically ablate neurons is to express toxins that disrupt protein synthesis. Two such toxins are the poetically named "Blue Death" (**UAS-diptheria toxin A** from bacteria (Lin *et al.*, 1995)) and **UAS-ricinA** (from castor beans (Hidalgo and Brand, 1997; Hidalgo *et al.*, 1995)). Cold-sensitive versions of Ricin exist, which adds a measure of temporal control (Allen *et al.*, 2002; Moffat *et al.*, 1992). Expression of proapoptotic genes like **UAS-reaper, grim**, and **hid** can also be used to induce cell death (Zhou *et al.*, 1997).

Table 3.3. UAS-Effector Constructs for Manipulating Neural Activity

	Encodes	Function	Cell type effected	Inducible or reversible?	Comments	References
A. Cell killers						
Diptheria toxin A	Toxic polypeptide from bacteria	Protein synthesis inhibitor	All cells (neurons and nonneurons)	No	Weaker version: DTI—attenuated mutant I; can take hours for cell death to occur	Lin *et al.* (1995)
RicinA	Toxic polypeptide from castor bean	Protein synthesis inhibitor	All cells	Cold-sensitive version is inducible	Temperature-sensitive version; can take hours for cell death to occur	Hidalgo and Brand (1997) and Moffat *et al.* (1992)
Reaper, grim, hid	Proapoptotic genes from *Drosophila*	Induce apoptosis via caspases	All cells	No	Can take hours for cell death to occur; some genes work better in combination depending on cell type	Zhou *et al.* (1997)
B. Inhibitors						
Tetanus toxin (TNT or TeTxLC)	Toxic light chain from bacteria	Cleaves syb/VAMP and blocks vesicle fusion	Neurons (chemical transmission due to small SVs)	No	May not be effective in all neurons (see Thum *et al.* 2006); unclear how effective TNT blocks DCV release	Martin*et al.* (2002) and Sweeney *et al.* (1995)
Shibirets1	Dominant/negative mutant dynamin gene from *Drosophila*	Blocks endocytosis	Neurons (chemical transmission	Temperature-inducible and rapidly reversible	May effect not just endocytosis, but also other vesicle mobilization properties; may also effect nonneuronal cells	Kitamoto (2001)

Kir2.1	Vertebrate inwardly rectifying potassium channel	Open at rest—decreases excitability of cell by hyperpolarization	Neurons (chemical and electrical transmission), muscle	No	Channels can be blocked with barium	Baines *et al.* (2001), Paradis *et al.* (2001), and Wu *et al.* (2008)
dOrk-deltaC	Modified *Drosophila* open rectifier potassium channel	Constitutively open—decreases excitability of cell by hyperpolarization	Neurons (chemical and electrical transmission), muscle	No		Nitaback *et al.* (2002)
EKO	Modified *Drosophila* voltage-sensitive potassium channel	Open at rest—decreases excitability of cell by hyperpolarization	Neurons (chemical and electrical transmission), muscle	No	Channels can be blocked by 4-AP	White *et al.* (2001)
Halorhodopsin (NpHR)	Chloride pump from halobacteria	Opens in response to yellow/green light and hyperpolarizes cell	Neurons (chemical and electrical transmission), muscle	Light inducible, reversible	Requires retinal cofactor	Unpublished
C. Activators						
NaChBac	Bacterial voltage gated sodium channel	Opens at −60 mV—increases excitability of cell by making it easier to depolarize	Neurons (chemical and electrical transmission), muscle	No	Has been seen to deplete neurons of neurohormone, which renders the cell effectively inactive in older animals; also has been shown to decrease firing frequency in some neurons while increasing the AP size	Nitabach *et al.* (2006) and Sheeba *et al.* (2008)

(*Continues*)

Table 3.3. (*Continued*)

	Encodes	Function	Cell type effected	Inducible or reversible?	Comments	References
TrpVR1, TrpA1	Transient receptor potential cation channel	Opens in response to various physical/chemical stimuli	Neurons (chemical and electrical transmission), muscle	Induced by capsaicin, acid, >43 °C heat; reversible	TrpA1/high temperature; TrpM8/cold temperature; TrpV3/warm temperature; TrpVR1 has been used in combination with a caged capsaicin for light gating	Marella *et al.* (2006) and Rosenzweig *et al.* (2008)
Eag-DN, Shaker-DN, Shaw–DN	Dominant/negative voltage-gated K channel	Increases excitability of cell by depolarization of membrane and preventing repolarization after AP	Neurons, muscle (only cells that normally expressing these channels)	No	May only work in cells normally expressing these channels	Broughton and Greenspan (2004), Hodge *et al.* (2005), and Mosca *et al.* (2005)
P2X2	Ionotropic purinoceptor	Opens in response to light, depolarizes cell	Neurons, muscle	Induced by light (caged ATP); reversible	Requires caged ATP as ligand	Lima and Miesenbock (2005)
Channelrhodopsin (ChR2)	Cation channel from algae	Opens in response to light, depolarizes cell	Neurons, muscle	Induced by blue light, with retinal cofactor; reversible	Volvox varient/red-shifted light; in fly, requires retinal cofactor	Hwang *et al.* (2007), Schroll *et al.* (2006), and Suh *et al.* (2007)

These techniques are compared in *Drosophila* Protocols (Sullivan *et al.*, 2000). It should also be possible to laser ablate cells as is done in *Caenorhabditis elegans* or kill them by expressing phototoxic proteins (Bulina *et al.*, 2006), although neither technique is common in flies. Many of the reagents that kill cells work better during development. A killer expressed only in the adult may not kill the cell or may take hours to act; it is a good idea to include a fluorescent reporter with the cell killer so that one can be sure the cell is really gone. If cell death does occur early in development, there is the possibility that the fly will be able to compensate for the cell loss and that cells that normally play a key role in circuits will be missed because alternative circuits are being used. In addition to the cell's function, it may serve as a scaffold for the growth and path finding of other neurons, so there is no guarantee that a phenotype from the loss of a given cell is truly cell-autonomous. These reagents kill all cell types, not just neurons.

As opposed to killing a cell outright, one can block its function in a variety of ways. It is possible to express constitutively active or dominant-negative versions of enzymes, motor proteins, and transcription factors critical for synaptic plasticity or neural function. There are active and inactive versions of CamKII (Griffith *et al.*, 1993; Koh *et al.*, 1999), protein kinase A (PKA) (Kiger *et al.*, 1999; Li *et al.*, 1995), heterotrimeric G proteins (Connolly *et al.*, 1996; Ferris *et al.*, 2006), CREB (Perazzona *et al.*, 2004; Yin *et al.*, 1994, 1995), fos and jun (Eresh *et al.*, 1997), and glued (Allen *et al.*, 1999). These manipulations are also not neuron specific and have slow time courses of activity but have been productively used to identify neurons responsible for particular behaviors. For examples, see Rodan *et al.* (2002), which used *UAS-PKA*^{inh} for the mapping of brain regions involved in ethanol response and see Joiner and Griffith (1999), which used the CamKII inhibitor *UAS-ala* for mapping circuits needed for courtship conditioning. All of these reagents affect many cell types, not just neurons. In some cases it is desirable to restrict the action of the effector to neurons; some options for this are described below.

B. Synaptic vesicle blockers

The *Clostridium* bacteria produce some of the most potent neurotoxins known: tetanus toxin and botulinum toxin. Each toxin is composed of a heavy and a light protein chain. The heavy chain controls their membrane binding and intracellular trafficking while the light chain encodes a protease that cleaves SNARE components of vesicle release machinery (Lalli *et al.*, 2003; Schiavo *et al.*, 2000). **UAS-TNT** (also known as *UAS-TeTxLC*) expresses the light chain of tetanus toxin and cleaves neural synaptobrevin (VAMP), making it a powerful reagent to specifically block vesicle fusion in neurons (***Sweeney et al., 1995***). The cleavage site is not present in cellular brevin, the v-SNARE that facilitates vesicle release in other cell types. TNT is highly effective in small quantities—

any leakiness from the transgene can kill flies—and it may affect neurons below the GFP detection threshold; it lacks temporal control (Martin *et al.*, 2002). Also, there seem to be some cell types in which *UAS-TNT* is not effective (Rister and Heisenberg, 2006; Thum *et al.*, 2006); perhaps cellular brevin can compensate (Bhattacharya *et al.*, 2002). Recovery from TNT is also slow—the cell must synthesize new nSyb protein. But the bigger problem is that perdurance of small amounts of toxin may be sufficient to keep cleaving nSyb. There are several isotypes of botulinum, each of which produces a toxin that cleaves a different site in the SNARE complex; isotypes (B, D, F, and G) also cleave nSyb (Lalli *et al.*, 2003) which would make them neural specific as well, and perhaps these should be explored.

A revolution in the use of the UAS-GAL4 system for neural circuit mapping occurred when Kitamoto adapted a temperature-sensitive dominant negative mutation in *Shibire*, the *Drosophila* homolog of the vesicle recycling protein dynamin, to make a temporally and spatially controlled neural activity blocker **UAS-Shibire^{ts1}** (***Kitamoto, 2001***). Dynamin is a GTPase that forms rings around the necks of vesicles to pinch them off the membrane; the dominant negative version intercalates into the ring of proteins at the neck, but then it blocks the pinching activity (Danino and Hinshaw, 2001; Hinshaw, 2000). Electron microscopy of the *Shibire^{ts1}* mutation at the nonpermissive temperature shows a series of docked vesicles trapped on the membrane. The speed with which phenotypes manifest suggest that the first effects seen are neural, since neurons should be the cells most vulnerable to depletion of vesicles. Different neurons have different thresholds since they have different amounts of vesicle stores and different release demands. Large amounts of the dominant negative may be required in neurons that have large release zones or extensive vesicle reserves. *UAS-Shibire^{ts1}* has some effects—especially in muscles, for example—even at room temperature, and overexpression of wild-type dynamin can have effects. There is a constitutively dominant-negative *UAS-Shibire DN K44A* (Moline *et al.*, 1999) and a wild-type *UAS-Dynamin* (Entchev *et al.*, 2000) that are plausible controls. *UAS-Shibire^{ts1}* has been used successfully to identify neurons involved in courtship and memory retrieval as distinct from acquisition, a distinction that would not have been possible to make without the acute temporal control of neural activity that *UAS-Shibire^{ts1}* provides (***Broughton et al., 2004***; Dubnau *et al.*, 2001; Kitamoto, 2002; Waddell *et al.*, 2000; Wang *et al.*, 2003b).

UAS-TNT and *UAS-Shibire^{ts1}* act on small synaptic vesicle mediated chemical neurotransmission, which likely makes up the bulk of the information traffic in the fly brain, but they do not affect electrical transmission via gap junctions. If one sees an effect with these reagents, the neurons expressing them are implicated in the behavior, but the absence of an effect does not rule out a contribution from these neurons through electrical coupling. The "wireless

network" of neuromodulators and peptides is critical for normal behavior. It is clear from work on the stomatogastric ganglia in crustaceans and on the leech locomotor choice (Briggman and Kristan, 2006; Friesen and Kristan, 2007; Marder and Bucher, 2007), for example, that the same circuit can produce very different outputs depending on the state of the system as set by neuromodulators. It remains to be proven how well reagents that work against fast neurotransmitter-containing synaptic vesicles block the release of dense-core vesicles containing **neuropeptides or neuromodulators** (Kaneko *et al.*, 2000; McNabb *et al.*, 1997).

C. Electrical blockers

Another strategy to inhibit neural function is to take advantage of a neuron's normal resting membrane potential and introduce a new "shunt" current that decreases excitability. There are several electrical shunts based on modified potassium channels. By increasing the permeability of the membrane at rest to potassium, one hyperpolarizes the resting membrane potential toward the equilibrium potential of potassium, thereby increasing the depolarization needed to fire action potentials. These potassium channel effectors will hyperpolarize muscles as well as neurons and are not readily reversible.

UAS-Kir2.1 (Baines *et al.*, 2001; Paradis *et al.*, 2001) is one such effector. It encodes a vertebrate inwardly rectifying potassium channel. Expression causes a hyperpolarized state and prevents neurons from depolarizing sufficiently to fire action potentials. It was tested in oocytes and at the neuromuscular junction, where its effects on membrane potential were directly measured. It has also been used to identify neurons involved in various behaviors in the adult, but the exact nature of the electrophysiological effect in these neurons has not been measured.

Expression of the **UAS-dOrk-deltaC** open rectifier potassium channel from *Drosophila* also reduces neural activity (Nitabach *et al.*, 2002), again by hyperpolarizing the resting membrane potential, making it more difficult to depolarize and generate action potentials. This was confirmed in oocytes. There is a control construct that contains the dOrk channel with a pore mutation that destroys conductance (*UAS-dOrk-NC*) but does not seem to act as a dominant negative.

UAS-EKO, which stands for electrical knock out, is a truncated version of the Shaker voltage-sensitive potassium channel (White *et al.*, 2001). It also reduces neural activity, but in this case by shortening action potentials and repolarizing neurons more rapidly. The mutations that were added to Shaker to make EKO shift the voltage activation threshold so that the channel opens with less membrane depolarization, so it is now closer to the time course of the sodium channel activation. Potassium channels normally cycle into an inactive state after opening; the ball and chain inactivation gate has been removed in EKO,

allowing the channel to remain open. This speeds the repolarization of neurons and reduces their activity (Holmes *et al.*, 2007). Expressing different levels of *UAS-EKO* can have a graded phenotypic effect.

A comparison of these potassium channel-based constructs in the circadian circuit found that *UAS-Kir2.1* is stronger than *UAS-dOrk*, and both are much stronger than *UAS-EKO* (Holmes *et al.*, 2007). It is not clear if this can be generalized to predict their relative strength in other neurons or muscles.

Tethered toxins from spiders and snails that act against neurotransmitter receptors and ion channels have been used in vertebrates (Ibanez-Tallon *et al.*, 2004) and have been adapted for use in flies (Wu *et al.*, 2008). The toxins are expressed in a given cell, secreted, and GPI anchored to act cell autonomously. Spider toxins that block inactivation of the primary sodium channel encoded by *paralytic*, the presynaptic calcium channel (*cacophony*), and a range of potassium channels have been tested for their effects on the PDF neurons that set the circadian clock. They have a variety of complicated effects on the membrane currents in these cells.

D. Neuronal activators

Circuit redundancy may make it difficult to identify what neurons are essential parts of a circuit simply by blocking their activity, especially with constitutively active reagents. Ectopically triggering behavior by activating neurons is an appealing complementary approach for identifying neurons sufficient to drive behavior. There are several options for increasing neural activity. **UAS-NaChBac** (Nitabach *et al.*, 2006) based on a voltage gated bacterial sodium channel, increases activity in some neurons. NaChBac opens at −60 mV and should make cells more excitable. This may be an oversimplification: in some cells, *UAS-NaChBac* changes the cell from firing small but frequent action potentials to one producing bigger but less frequent spikes (Sheeba *et al.*, 2008). NaChBac can also have side effects that look like inhibition: when expressed in bursicon-secreting neurons, it caused peptide depletion and the neurons were rendered inactive by the time bursicon release is required for cuticle tanning and wing expansion (Luan *et al.*, 2006a).

Other neuronal activators are based on the Trp channels, which are nonselective cation channels that can be opened by ligands such as capsaicin, or by changes in temperature. The capsaicin-sensitive modified **VR1 Trp** channel (*UAS-VR1E600K*) has been used in worms (Tobin *et al.*, 2002) and flies (Marella *et al.*, 2006) to activate neurons in response to the capsaicin ligand. Overexpression of the *Drosophila* **TrpA1** channel renders neurons active upon temperature increase (Rosenzweig *et al.*, 2008). Expression of the mammalian Trp channels may also prove effective. Some Trp channels can be activated at temperatures close to the physiological norm for flies and so may be acceptable for long-term behavioral studies.

In contrast to the potassium channel-based inhibitors described above, reducing potassium channel function should increase neural activity. Voltage-gated potassium channels are homotetramers. This makes it possible to transgenically express a faulty subunit that will intercalate into the tetramer and reduce or eliminate its channel function. Several of these dominant negative potassium channel constructs have been built by truncating the proteins between the N-terminal multimerization domain and the pore-forming transmembrane domains: **UAS-Eag-DN** (Broughton *et al.*, 2004), **UAS-Shaker-DN** (Mosca *et al.*, 2005), and **UAS-Shaw-DN** (Hodge *et al.*, 2005). These dominant negatives should constitutively block potassium channel function and should work only in neurons that normally express these channels.

E. Light-based methods

There are many strategies for using light to alter neural activity. Again, the primary development effort has been done in vertebrate systems. The fly-specific reagents are listed in Table 3.3. The phototransduction cascade itself is designed to convert photons into electrical activity and three proteins that make up this cascade in flies were expressed in vertebrate neurons to elicit neural depolarization in an approach called chARGe (Zemelman *et al.*, 2002). Light can be used to uncage a variety of neurotransmitters, second messengers, synthetic or natural ligands, or chemical modifiers. For example, the SPARK system uses an azobenzene chemical switch that can the bind to either an endogenous potassium channel or a modified channel that is transgenically expressed in specific populations of cells, making the channel light-gated (Banghart *et al.*, 2004; Fortin *et al.*, 2008; Zemelman *et al.*, 2003). Glutamate receptor activation can be triggered by light by expressing a modified LiGluR and using a light-activated glutamate agonist (Szobota *et al.*, 2007). When **UAS-P2X2**, encoding a vertebrate ATP-gated channel, is expressed in the fly giant fiber system and caged ATP is injected, the animals produce the characteristic jump-escape behavior in response to light (***Lima and Miesenbock, 2005***). Light can be used to alter cAMP levels with a photoactivatable adenylyl cyclase (Schroder-Lang *et al.*, 2007).

Another method attracting interest in *Drosophila* is Channelrhodopsin2 (**UAS-ChR2**), a monovalent cation channel from algae that is activated by ~470 nm blue light in the presence of all-trans retinal. ChR2 was cloned (Nagel *et al.*, 2003) and then adapted for neuroscience (Boyden *et al.*, 2005; Li *et al.*, 2005; Nagel *et al.*, 2005). The required retinal cofactor is present in mammalian neurons but not in *Drosophila*; it is generally supplied by feeding both during the larval and adult stages. Effective use of ChR2 may also require high transgene expression levels. There is now a red-shifted version of ChR2 from *Volvox* that is activated by light at 590 nm (Zhang *et al.*, 2008a). A new variant of ChR2 shows increased opening in response to a brief light pulse, potentially reducing the

effect of blue light alone on neurons (Berndt *et al.*, 2009). The two big problems here are light penetration and delivery of chemical cofactors. In some cases, the level of light required to activate the effectors also affects behavior or neural function directly. Ongoing development in this area is fast and hopefully reagents that are activated by longer wavelengths (which are less detectable by fly photoreceptors, penetrate farther into tissue, and might be amenable to two-photon excitation) and that do not require cofactors will be forthcoming.

Halorhodopsin (**NpHR**) is a chloride pump activated by ~580 nm yellow light and also requires retinal as a cofactor. Halorhodopsin has also been engineered to express and reach the membrane at higher levels by adding an ER secretion signal (Gradinaru *et al.*, 2008; Zhao *et al.*, 2008). ChR2 and NpHR can be used together to generate specific firing patterns in neurons: they both have millisecond precision and are activated by different wavelengths of light (Zhang *et al.*, 2007). There are frequent reviews on the state of "optogenetics" (see Deisseroth *et al.*, 2006; Miesenbock and Kevrekidis, 2005) and the intense interest in this highly promising field should continue to improve the reagents. For examples of this approach in flies, see Hwang *et al.* (2007), Schroll *et al.* (2006), and Suh *et al.* (2007).

F. Caveats

There are some cautionary notes for the use of all of the reagents that modify neural activity. First, the way these effectors act may be different in different cell types, and some reagents do not work in some cell types at all (Thum *et al.*, 2006). In most cases, the electrophysiological characterization of these reagents was performed in an exogenous system, or at best in one particular cell type, and the validity of generalization is weak. The utility of the existing reagents on electrically coupled cells or peptidergic neurons is unclear. That makes positive results with the *UAS-effectors* more useful than negative results. (It is legitimate to say disrupting activity in these neurons alters behavior, but one should not conclude that expressing a given effector in a cell type and failing to see an effect really means those neurons are not involved.)

It is important to consider exactly what a given manipulation does to a neuron. For example, light on ChR2 flies triggers depolarization via an influx of cations. Blocking a potassium channel also increases neuronal excitability, but it does so by making it easier to fire action potentials triggered by the cells own sodium channels. It does not initiate firing *de novo*—it just amplifies an existing proclivity or lowers a threshold. These two "activators" might have very different effects on the same neural population.

The expression level of blockers can be critical—and the levels required may differ depending on the cell type. Each of the reporter and effector proteins has its own stability and effective concentration. We visualize the neurons in a

GAL4 pattern with one reporter, detect with antibodies, and then manipulate them with an effector such as *UAS-TNT*, which functions in very low doses, or one like *UAS-Shibire^{ts1}*, which seems to require large amounts in many neurons. Do we see all of the neurons with GFP that express enough TNT to be impaired? Do we see many neurons with antibody-amplified GFP that do not make enough Shibire^{ts1} to be blocked? Both false positives and false negatives are possible and worrying.

Timing of expression may also be critical: channel expression levels seem to be tightly controlled (Mee *et al.*, 2004) and homeostatic mechanisms work quickly to restore the ion balance (Turrigiano, 2008), so systems that rapidly trigger effector production or activity have the best chance of evading compensation. No one has systematically looked at the homeostatic responses of neurons expressing channel blockers or activators over time.

None of these cautions make the lines that alter neural activity invalid as tools for mapping which neurons are implicated in particular behaviors, but it does suggest that careful controls and comparison of the results with different blockers is prudent. Initially we can use the deadly effectors and complete blockers to winnow down which neurons are a key for particular behaviors. Then we can use reagents such as channelrhodopsin to send in signals that resemble those the neurons normally carry to more subtly alter the behavioral output and test our predictions about how neural circuits work to drive behavioral responses.

VI. QUANTITATIVE BEHAVIORAL ASSAYS

What can a fly do? What does a fly do? Developing ethologically relevant, quantifiable behavioral assays has been challenging and there are not that many established paradigms in the fly. For recent reviews of some behaviors studied in the fly, see Sharma *et al.* (2005) and Vosshall (2007). Table 3.4 includes a summary of most of the current behavioral paradigms studied in *Drosophila*. There is ample room for merging the behavior analysis techniques developed for other insects—and indeed other animals—with the genetic and molecular tools available in *Drosophila* to learn new things about how neural circuits drive behavior. For example, walking and searching assays from other insects could be adapted for flies (Buschges *et al.*, 2008; Merkle and Wehner, 2008; Watson *et al.*, 2002; Wittlinger *et al.*, 2006). Clever behavioral analysis alone can generate hypotheses about how neural circuits work. As an example, careful observation showed that emergency and voluntary flight initiation sequences are quite different, which suggests that different circuits underlie these behaviors. Further, the latency to jump is different when the emergency takeoff is initiated by a visual or an olfactory stimulus. Again, the circuits that mediate these responses may differ in more than just the sensory input layer

Table 3.4. Behaviors Commonly Assayed in *Drosophila*

Behavior	Description of behavior and usual assays	References
Locomotion		
General locomotion	It is possible to measure the number of lines crossed or to track flies in an open field. Larval crawling can also be tracked	Hughes and Thomas (2007) and Martin (2003)
Response to startle	Flies exhibit increased velocity in response to air puff, vibration, or odor delivery	Wolf *et al.* (2002)
Circadian rhythms	TriKinetics *Drosophila* activity monitoring system is an automatic beam cross detector that counts movement of individual flies in tubes; flies tend to be more active at dawn and dusk	Nitabach and Taghert (2008), Rosato and Kyriacou (2006), and Zordan *et al.* (2007)
Sleep	TriKinetics or ultrasound monitors watch for bouts of stillness lasting more than 5 min	Cirelli and Bushey (2008)
Righting reflex	Flies are knocked over and tested for their ability to get to their feet	Leal *et al.* (2004)
Jump-escape	Visual or olfactory stimuli can trigger an emergency takeoff, scored by hand or filmed with high-speed video	Trimarchi and Schneiderman (1995)
Flight	Flight can be measured by dropping flies into an oil-coated graduated cylinder where the best fliers stick initiate flight early and stick near the top or in flight arenas where tethered flies fly glued to a stick. Flight initiation can be studied with high-speed video	Benzer (1973), Card and Dickinson (2008a,b), Fry *et al.* (2008), Hammond and O'Shea (2007), Lehmann and Dickinson (2001), and Reiser and Dickinson (2008)
Landing response	Flies will extend their legs toward some objects rather than turning to avoid them in the flight arena	Maimon *et al.* (2008)
Gait analysis	A laser carpet measures foot placement and stride length	Strauss (1995)
Postural control	In an inebriometer, a series of baffles or funnels with slanted sides is used to test for lost of balance in response to ethanol or anesthetics. More resistant flies remain in the device longer, while susceptible ones elute more rapidly	Cohan and Hoffman (1986) and Moore *et al.* (1998)

(*Continues*)

Table 3.4. (*Continued*)

Behavior	Description of behavior and usual assays	References
Gap crossing	Flies will step across gaps of manageable size guided by visual cues.	Pick and Strauss (2005)
Leg resistance reflex	A leg can be moved manually and the required force measured	Ready *et al.* (1997)
Bang sensitivity	Flies usually recover rapidly from hard banging or vortex vibration but some mutants paralyze or seize	Pavlidis and Tanouye (1995)
Sensory		
Gravitaxis	Flies will move against gravity. This can be measured in a vertically oriented Y maze with multiple choice points	Baker *et al.* (2007a,b) and Kamicouchi *et al.* (2009)
Olfaction	An olfactory trap assay lures flies into funnels baited with different odorants; olfactory induced jump and T-maze choice tests can also be used to measure response to odorants. Olfactory and visual cues can be measured during flight. Larva crawls toward odor sources and track gradients	Frye and Dickinson (2004), Lilly and Carlson (1990), Louis *et al.* (2008b), McKenna *et al.* (1989), Suh *et al.* (2004), and Woodard *et al.* (1989)
Gustatory	Food consumption can be measured with liquid food in graduated capillary tubes; choice of egg laying sites can be used as a proxy for taste discrimination. Larva spends longer on certain food sources and their consumption can be measured by including dye in the food. They will avoid bitter food unless starved	Bader *et al.* (2007), Ja *et al.* (2007), Mery and Kawecki (2002), Wu *et al.* (2005), and Yang *et al.* (2008)
Phototaxis	The countercurrent device partitions flies based on how quickly they run toward light; more complicated visual motion tests can be performed in the flight arena with a virtual reality display on LED panels	Benzer (1967) and Reiser and Dickinson (2008)
Color vision or spectral preference	UV–visible light choice assays or motion stimuli in equiluminescent displays detect response to color	Gao *et al.* (2008) and Yamaguchi *et al.* (2008)
Optomotor response	Variants on the "fly stampede" measure response to optic flow or motion vision; flies will move against slow or sparse cues and with fast or dense stimuli	Katsov and Clandinin (2008)

(*Continues*)

Table 3.4. (*Continued*)

Behavior	Description of behavior and usual assays	References
Visual discrimination and persistence	Flies remember the location of an object that disappears from view and can recognize novel and familiar shapes in the flight area	Liu *et al.* (1999, 2006), Neuser *et al.* (2008), Peng *et al.* (2007), and Tang *et al.* (2004)
Audition	Playing courtship song to males induces male–male courtship; song induces females to slow down and accept copulation	Clyne and Miesenbock (2008), Crossley *et al.* (1995), Eberl *et al.* (1997), and Tauber and Eberl (2003)
Magnetosensation	A two choice maze with magnets shows flies sense magnetism	Gegear *et al.* (2008)
Thermosensation	Larva and adults avoid noxious heat and spend more time at preferred temperatures with <1 °C precision on a heated agar block	Hamada *et al.* (2008), Rosenzweig *et al.* (2005, 2008), Sayeed and Benzer (1996), Xu *et al.* (2006), and Zars (2001),
Mechanosensation	Larva back up in response to light touch; this can be measured by tapping them with an eyelash	Kernan *et al.* (1994)
Nociception or pain	Larva roll in response to heat or pinch	Tracey *et al.* (2003)
Hygrosensation	Flies can sense water	Hong *et al.* (2006), Inoshita and Tanimura (2006), and Liu *et al.* (2007)
Complex		
Male courtship	Insectavox measures courtship song production; other steps of courtship are usually scored manually from videotaped mating in small chambers	Gorcyzca and Hall (1987), Greenspan (2000), O'Dell (2003), Villella and Hall (2008), and Villella *et al.* (1997)
Female receptivity	Videotaped and scored manually; egg laying can serve as a proxy	Dickson (2008), Hasemeyer *et al.* (2009), Yang *et al.* (2009), and Yapici *et al.* (2008)
Grooming	Flies remove dust in a coordinated series of movements	Corfas and Dudai (1989, 1990) and Phillis *et al.* (1993)
Proboscis extension reflex	This is the motor program triggered by detection of an acceptable food source through tase bristles on the leg or labellum; it is observed and scored by hand	Shiraiwa and Carlson (2007)
Aggression	Male flies will perform a series of stereotyped movements when competing for resources such as food or mates. The behavior is filmed and scored manually	Chen *et al.* (2002), Dierick (2007), Hoyer *et al.* (2008), Mundiyanapurath *et al.* (2007), and Yurkovic *et al.* (2006)

(*Continues*)

Table 3.4. (*Continued*)

Behavior	Description of behavior and usual assays	References
Learning and memory		
Olfactory shock conditioning	Flies can be trained to avoid an odor associated with an electric shock in a T-maze	Berry *et al.* (2008) and Tully and Quinn (1985)
Habituation or sensitization	Flies respond differently to repeated presentation of sensory stimuli such as odor puffs or bristle touches	Asztalos *et al.* (2007), Engel and Wu (2008), and Joiner *et al.* (2007)
Spatial memory	Flies avoid the half of a box that has been associated with noxious heat	Diegelmann *et al.* (2006)
Courtship suppression	Males whose courtship attempts have been rejected are slower to court receptive females	Ejima *et al.* (2007) and Siegel and Hall (1979)
Appetitive conditioning	Larva and adults can also be conditioned to choose an odder associated with a reward	Fiala (2007) and Schroll *et al.* (2006)

(Card and Dickinson, 2008a,b; Hammond and O'Shea, 2007; Trimarchi and Schneiderman, 1995). The kinds of errors an animal makes also suggest the type or manner of computations it must be doing. It was deduced that motion vision in the fly uses local rather than global comparisons because of careful behavioral analysis of the errors the fly makes when the motion cues are distributed distantly across the ommatidia (Buchner, 1976). Careful behavioral analysis could be used to deepen our understanding of neural circuits in *Drosophila*.

Laboratory behavioral assays are designed to identify the neurons and genes that govern these behaviors in more natural environments. The goal is to understand the neural circuits that have evolved under natural selection and are optimized for what the flies actually do normally. We must be careful to consider how well our lab assays mimic the fly's normal circumstances and that we draw appropriate conclusions. The genetic background of the flies used in these assays and the neutrality of the markers we use to identify transgenes must also be considered. For example, there is literature to suggest that using *mini-white* to mark transgenes that are then assayed for courtship defects can lead to results that are difficult to interpret (An *et al.*, 2000; Borycz *et al.*, 2008; Zhang and Odenwald, 1995). Some behaviors, including locomotion speeds, aggression, and response to magnetic fields, also seem to depend on the genetic background of the flies (Gegear *et al.*, 2008; Hoyer *et al.*, 2008; Wolf *et al.*, 2002). The advantage of using isogenic lines or F1 hybrids is also under debate (Sharma *et al.*, 2005). These caveats are manageable as long as appropriate controls are performed.

VII. CONCLUSIONS

A. Example circuits

A few examples are discussed below to highlight the excellent use of the genetic tools available for circuit analysis in *Drosophila* and to describe the circuits about which we know the most. Beautiful work on olfaction has shown organizational logic of the first order sensory representation maps in the antennal lobe (Couto *et al.*, 2005; Laissue *et al.*, 1999; Marella *et al.*, 2006; Marin *et al.*, 2002; Vosshall *et al.*, 2000; Wang *et al.*, 2002, 2003a,b). Excitatory and inhibitory interneurons modify the representation of odors within the antennal lobe (Olsen and Wilson, 2008b; Olsen *et al.*, 2007; Shang *et al.*, 2007), and projection neurons carry this information on to the lateral horn and the Kenyon cells of the mushroom bodies (Jefferis *et al.*, 2007; Murthy *et al.*, 2008; Turner *et al.*, 2008). Olfactory information is integrated with visual cues (Frye *et al.*, 2003), but where and how this occurs is unknown. In the larva, how the sensory input affects the behavioral response has also been studied (Kreher *et al.*, 2008; Louis *et al.*, 2008a). How the odor sensory experience is connected to the motor neurons that dictate behavioral response is also unknown.

Work on the gustatory system has shown that the sensory neurons carrying different taste qualities project to different areas of the subesophogeal ganglia (Marella *et al.*, 2006; Fischler *et al.*, 2007; Wang *et al.*, 2004b). Some of the motor neurons that drive the extension of the proboscis in response to an attractive taste have been identified, but the sensory neurons do not directly connect to the motor neurons, revealing that this apparently simple reflex has additional circuitry that remains to be uncovered (Gordon and Scott, 2009a,b). Part of a circuit governing response to taste has also been established in the larva (Bader *et al.*, 2007; Melcher and Pankratz, 2005).

Extensive work in the visual system has identified neurons involved in detecting motion and color; these are reviewed in Borst (2009). For recent advances here, see Gao *et al.* (2008), Joesch *et al.* (2008), Katsov and Clandinin (2008), Morante and Desplan (2008), Rister *et al.* (2007), and Yamaguchi *et al.* (2008). Very little is known about auditory processing beyond the sensory apparatus in the Johnston's organ (Kernan, 2007). A careful anatomical analysis of projections from the Johnston's organ has been conducted and the function and destination of these projections is under investigation (Kamikouchi *et al.*, 2006, 2009; Yorozu *et al.*, 2009). The sensory systems have been intensively studied and have seen great progress, but in no case can we trace a circuit all the way from a sensory stimulus to a motor output.

There are many examples where parts of a circuit have been mapped. The giant fiber circuit that mediates the jump-escape response has been studied by mutagenesis screens, anatomical dye fills, and electrophysiology. The giant

fibers and the motor neurons that drive the jump are known, but the neurons that activate the giant fibers in the brain are not (Allen *et al.*, 2006). Some of the neurons that govern circadian rhythms and light mediated arousal have been identified (Nitabach and Taghert, 2008; Shang *et al.*, 2008; Sheeba *et al.*, 2008). The neuropeptide-releasing neurons that initiate the wing expansion after eclosion have been determined using an arsenal of intersectional genetic strategies (Luan *et al.*, 2006a). Neurons in and near the mushroom bodies that govern olfactory memory storage and retrieval have also been characterized, reviewed in Keene and Waddell (2007). Perhaps the most complex circuit under intensive study is the network of neurons that underlie male courtship behavior (Clyne and Miesenbock, 2008; Datta *et al.*, 2008; Kimura *et al.*, 2005, 2008; Manoli *et al.*, 2005; Stockinger *et al.*, 2005). The male-specific isoform of *fruitless* is expressed in sensory, motor, and interneurons that participate in courtship. One of the interesting, unanswered questions here is how much of the difference between male and female courtship behavior is reflected in anatomical differences in the circuitry and how much is due to gender differences in the activity or synaptic weights within anatomically similar circuitry. *Fruitless* might be a "master control gene for behavior" (Yamamoto, 2007) in much the same way that there are hypothesized to be "command neurons" (Weiss and Kupfermann, 1978) that trigger entire behavioral programs. *Fruitless* may be unusual in that it is expressed in many types of neurons that participate in courtship, but even here, there are still missing circuit elements.

B. New tools

To highlight some of the newest developments in the arsenal of tools for circuit mapping in flies, the tethered toxins that block specific ion channels and the Trp channels that activate neurons in response to temperature changes seem very promising. The new generation of GECIs for monitoring neural activity and the positive intersectional methods for narrowing gene expression should also contribute to our ability to refine the maps of which neurons participate in given behaviors.

We can learn from the techniques being developed in vertebrate and nematode systems. A variety of strategies for neuronal activation and inactivation exist there: MIST (Karpova *et al.*, 2005), Allatostatin (Tan *et al.*, 2006), Ivermectin (Lerchner *et al.*, 2007), RASSLs and DREADDs (G-protein coupled receptors with synthetic ligands: Armbruster *et al.* (2007) and Conklin *et al.* (2008)), and modified GABA receptors (Wulff *et al.*, 2007) have all been used for circuit mapping. The use of Cre recombinase lines for positive intersectional approaches to transgene expression and the idea that reporters can be recombined out after stable lines have been established are common practice in transgenic mouse work (Dymecki and Kim, 2007). Channelrhodopsin has been

used for anatomical mapping as well as for linking neural activity to behavioral output (Arenkiel *et al.*, 2007; Petreanu *et al.*, 2007; Wang *et al.*, 2007). This mapping was done with electrodes but there is the possibility that it could be done with optical reporters of neural activity (Airan *et al.*, 2007). For visualizing large numbers of neurons at once and tracing their connections, the Brainbow technique (Lichtman *et al.*, 2008; Livet *et al.*, 2007) and the self-amplifying viral trans-synaptic tracers invite envy (Wickersham *et al.*, 2007). Watching neural circuits in action in a behaving animal has been accomplished in several genetic model organisms (Clark *et al.*, 2007; Dombeck *et al.*, 2007; Faumont and Lockery, 2006; Orger *et al.*, 2008; Zhang *et al.*, 2008b). In *C. elegans*, the GRASP synapse marking strategy and the complete electron microscopy wiring diagram suggest what we could do with their equivalents in the fly (Chalasani *et al.*, 2007; Chen *et al.*, 2006; Feinberg *et al.*, 2008; Gray *et al.*, 2005).

C. Full circle

When the field of "neurogenetics" was born, there was debate about whether it would be possible to find single gene mutations that led to understanding behaviors (Vosshall, 2007). Now it is widely accepted that the genetic approach has yielded insights into behavior, especially in the identification of genes involved in neurodevelopment, axon wiring, and neural function. Some of the early stars of neurogenetics were the conditional mutations that led to the cloning of ion channels and synaptic vesicle release machinery; these same mutations allowed the design of tools to manipulate neural activity. To understand neural circuits, we are now using genetic tools to dissect which neurons play roles in specific behaviors, how these neurons are linked, and what jobs they do.

The correlative tools to observe neural activity—electrophysiologically or optically—while the animal is receiving some sensory stimulation or performing some motor behavior can now be combined with the causative genetic manipulations which change activity in defined populations in neurons to see what behavioral changes they evoke. The powerful genetic toolkit that makes *Drosophila* famous can now be applied creatively to address how neural circuits are organized to control appropriate behavioral responses to the environment and experience of the animal. The general principles determined by understanding circuit logic in the specific cases in the fruit fly should be informative for other systems as well.

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