Preface

STARTED IN 1984 BY RALPH GREENSPAN, LILY JAN, YUH-NUNG JAN, AND PATRICK O'FARRELL, the Neurobiology of *Drosophila* course at Cold Spring Harbor Laboratory has now run every summer for the last 25 years. Those of us that have been fortunate enough to direct the course have been honored to inherit this rich tradition.

Admission to the Neurobiology of *Drosophila* course is very competitive with only 12 students selected each year. This small intimate group allows for a fantastic interactive and hands-on experience as well as for a more personal intellectual interaction between students and with the individual instructors of the course. However, the editors of this book, who themselves were unavoidably responsible for denying the entry of 100s of potential students, recognized that we could temper the guilt by making the course accessible to all through the production of this book, a course companion. Therefore, beyond being a valuable reference for those students lucky enough to have taken the course, the book allows others to "attend the course by proxy," as the format directly follows that of the course. The content includes a brief introduction to the areas of *Drosophila* neurobiology research that are covered each year in addition to detailed protocols for the techniques that are taught in the laboratory.

The course is broken into three week-long blocks focusing on Development, Physiology, and Behavior, respectively. Most of the contributing authors were instructors of the course during the time that the three editors were Directors of the course. Each instructor teaches for one day with the usual format being lectures in the morning and real experiments in the laboratory in the afternoon/evening/night. It is often difficult to convey the complexity of experimental setup within a day (especially true for experiments that ordinarily take several days) and therefore part of the course unavoidably only provides a snapshot of the research. In this book the instructors, as contributing authors, face the additional difficulty of converting their snapshot into a coherent chapter that conveys the flavor of the research that they discussed—all within a fairly strict page limit. This book is not a comprehensive review of the course lectures or *Drosophila* neurogenetics. Each chapter has a short introduction to the relevant "subfield," but the heart of each chapter provides detailed experimental protocols. We applaud and thank the contributors for their invaluable contributions both to the course and to this book. Neither project would have happened without their efforts and the efforts of numerous students and postdocs that accompany them as course aids.

The course exists for the good of the students and we are extremely proud of the achievements of the many students that have graduated. A remarkable number now have their own research programs in *Drosophila* neurobiology and many are very distinguished names in their respective area.

The course would not function without the generous input of a number of large scientific companies and a large number of people at Cold Spring Harbor Laboratory. Some investigators lug their sophisticated gear to Cold Spring Harbor for a day or two of experimentation, but the rest of the large high-tech equipment arrives on loan from a number of companies. Of particular note, Zeiss, Olympus, Nikon, and Leica provided various microscopes, and Axon Instruments (now called Molecular Devices) lent amplifiers and software for electrophysiology. All the loan agreements, ordering, delivery, and handling of participant travel and housing is the realm of the Cold Spring

Harbor Meetings and Courses people. We particularly wish to thank Barbara Zane, Andrea Stephenson, Andrea Newell, and David Stewart for their dedication and energy. David Stewart is also responsible for obtaining and maintaining external funding for the Cold Spring Harbor courses, and without that effort and the financial support generated, this course would not exist.

Lastly, we have been ably assisted in the production of this book by the enthusiastic and professional help of those at Cold Spring Harbor Laboratory Press. We would never have finished the project without them. We are indebted to our Publisher, John Inglis; Acquisition Editor, David Crotty; Developmental Editors, Kaaren Janssen, Catriona Simpson, and Michael Zierler; Project Manager, Mary Cozza; Director of Development, Marketing, and Sales, Jan Argentine; Production Manager, Denise Weiss; Production Editor, Kathleen Bubbeo; and Desktop Editor, Susan Schaefer. We thank them for their enthusiasm, patience, and professionalism throughout the entire process.

— SCOTT WADDELL, BING ZHANG, AND MARC FREEMAN

Some Feedback from the Course

Taking this course had an absolutely enormous impact on my career. Because the course teaches the Latest and Greatest, and the people who take the course are typically in the top labs and the people who teach the course are among the top in the field, the course has a huge impact on research directions.

— NANCY BONINI (1988), now Professor, Howard Hughes Medical Institute and University of Pennsylvania

As a grad student from a relatively small university participating in this course had an enormously positive impact on my subsequent career in science.

— SHELAGH CAMPBELL (1989), now Associate Professor, University of Alberta, Canada

Eleven years after taking the course, I was a course instructor. I am still in the field and have had the pleasure of taking the course, sending my own students to the course, and teaching the course. I think it is an invaluable resource for our community.

— AARON DIANTONIO (1991), now Associate Professor, Washington University

The course was decisive to continue my research as a postdoc in Cambridge. It has inspired me enormously to hear the history of scientists teaching the course thanks to the time spent with each of them.

— ANDREAS PROKOP (1991), now Senior Lecturer, University of Manchester, United Kingdom

I still benefit from the experience in the course, both in terms of the useful contacts as well as with the breadth of techniques that I was exposed to and are still used in the lab.

— PAUL GARRITY (1992), now Associate Professor, Brandeis University

2   Molecular and Cellular Analyses of Larval Brain Neuroblasts in *Drosophila*

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ABSTRACT

Polarized localization of proteins is an evolutionarily conserved mechanism for establishing asymmetry within a cell and producing daughter cells with distinct fates. Neuroblasts (neural stem cells) from *Drosophila melanogaster* are an established paradigm for examining cortical cell polarity and its effects on asymmetric cell divisions. The larval fly brain is ideally suited for studies of asymmetric stem cell division because the larval brain maintains a stable number of about 100 neuroblasts throughout larval development. This chapter describes procedures for the collection and processing of *Drosophila* larval brains for examination by immunolocalization of cell-fate and cell-polarity markers (Protocol 1), 5-ethynyl-2′-deoxyuridine (EdU) labeling of mitotic cells (Protocol 2), and RNA in situ localization (Protocol 3).

OVERVIEW

The *Drosophila* larval brain is a well-established model for investigating the role of stem cells in development. Neuroblasts must be competent to generate many thousands of differentiated neurons through asymmetric divisions during normal development. Given the wide array of genetic and molecular tools available for studying flies, the *Drosophila* larval brain provides a powerful in vivo model system for examining the regulation of neuroblast self-renewal versus differentiation (Wu et al. 2008). Studies in fly neuroblasts have been instrumental in identifying how the establishment and maintenance of cell polarity influence cell fate, and they have produced a wide array of molecular cell-polarity markers. Moreover, neuroblasts and their progeny can be positively identified using a variety of cell-fate markers, which will be discussed in a following section.

In this chapter, we focus on techniques for examining neuroblasts in the larval brain. The larval brain maintains a steady population of approximately 100 neuroblasts, making it possible easily to

identify mutants with atypical expansion or premature loss of neuroblast populations, both of which are indicative of disrupted asymmetric cell division (Rolls et al. 2003; Lee et al. 2006a,b,c). It was recently discovered that a small population of larval brain neuroblasts generate transit-amplifying daughter cells capable of limited rounds of asymmetric divisions. This is a particularly intriguing finding given that transit-amplifying cells are commonly seen during development of vertebrate nervous systems (Morrison and Kimble 2006; Nakagawa et al. 2007; Boone and Doe 2008; Bowman et al. 2008). Genes regulating neuroblast polarity and cell fate are evolutionarily conserved between flies and mammals, thus *Drosophila* provides a powerful model system for identifying molecular mechanisms of asymmetric cell division, potentially advancing therapeutic applications in neurology, stem cell biology, and even cancer biology (Rolls et al. 2003; Lee et al. 2006b; Wu et al. 2008).

MOLECULAR MARKERS OF NEUROBLASTS AND THEIR PROGENY

Neuroblasts in the larval brain can usually be identified by their rounded morphology and relatively large size, and can be unambiguously identified by molecular markers including Deadpan (Dpn), Worniu (Wor), and Miranda (Mira) (Lee et al. 2006b). Recent work has revealed that there are at least two classes of larval brain neuroblasts. More than 90% of larval brain neuroblasts are type I (by far the best-characterized class of fly neuroblasts), which divide asymmetrically to self-renew a daughter neuroblast and produce a ganglion mother cell (GMC) that will divide to generate two differentiated neurons. Type I neuroblasts can be unambiguously identified by coexpression of Dpn and Asense (Ase), and their GMCs can be positively identified by nuclear localization of Prospero (Pros) (Brand et al. 1993; Lee et al. 2006b; Bowman et al. 2008). Differentiating neurons can be detected by expression of the neuronal marker Elav (Embryonic lethal, abnormal vision), which is not detected in neuroblasts or GMCs (Fig. 1; Table 1) (Lee et al. 2006b).

The second class of neuroblasts in the larval brain (type II) have tremendous potential to generate many differentiated neurons via transit-amplifying cells (Boone and Doe 2008; Bowman et al. 2008). There are eight type II neuroblasts mostly located in the dorsomedial region of the larval brain lobe that divide asymmetrically to self-renew and generate. Immature intermediate neural progenitors (INPs) commit to the INP fate through maturation, a differentiation process necessary for specification of the INP identity. INPs express similar molecular markers as Type I neuroblasts,
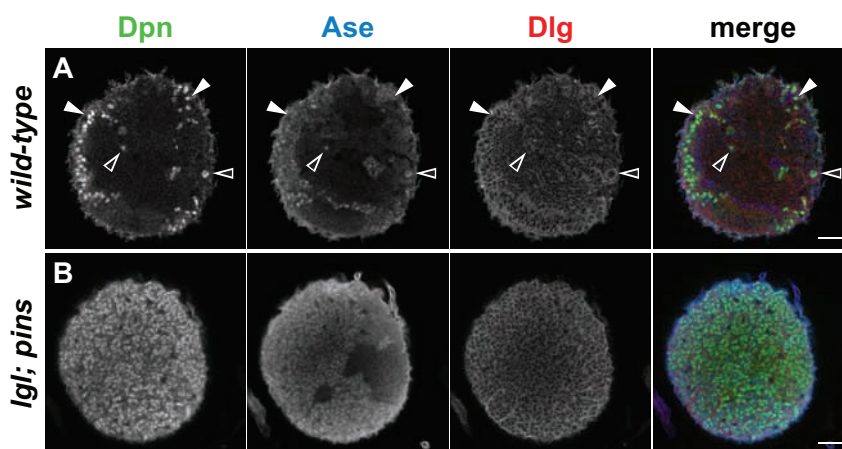
FIGURE 1. Cell-fate markers in larval brain neuroblasts. Third-instar larval brains stained with antibodies against the neuroblast markers Deadpan (Dpn; types I and II; green) and Asense (Ase; type I only; blue), and the cortical marker Discs large (Dlg; red). (*A*) Wild-type brain showing Dpn⁺ Ase⁺ type I neuroblasts (white arrowheads) and Dpn⁺ Ase⁻ type II neuroblasts (black arrowheads). (*B*) An *lgl; pins* double mutant brain showing overproliferation of both type I and type II neuroblasts as determined by coexpression of Dpn and Ase. Anterior is to the *top* in all images. Scale bars, 20 μm. (Images courtesy of J. Haenfler, University of Michigan.)

TABLE 1. Cell-fate markers in neuroblast lineages

Type I	
Neuroblasts	$Dpn^+ Ase^+ Pros^{+cytoplasmic}$
GMCs	$Dpn^- Ase^+ Pros^{+nuc}$
Neurons	$Dpn^- Ase^- Pros^{+nuc}$
Type II	
Neuroblasts	$Dpn^+ Ase^- Pros$
Immature INPs	$Dpn^- Ase^- Pros^-$
INPs	$Dpn^+ Ase^+ Pros^{+cytoplasmic}$
GMCs	$Dpn^- Ase^+ Pros^{+nuc}$
Neurons	$Dpn^- Ase^- Pros^{+nuc}$

GMCs, ganglion mother cells; INPs, intermediate neural progenitors.

including Dpn, Ase, and cytoplasmic Pros, and divide asymmetrically to regenerate and to produce GMCs. Although expression of Dpn is readily detectable in both type II neuroblasts and INPs, these two cell types can be distinguished by size (>10 µm for neuroblasts vs. <6 µm for INPs). Furthermore, Dpn is expressed in all brain neuroblasts, whereas Ase is specific to type I lineages ($Dpn^+ Ase^+$) and thus provides a key reagent to discriminate type I from type II neuroblasts (Fig. 1; Table 1) (Bowman et al. 2008).

MARKERS OF NEUROBLAST CELL POLARITY

In larval brain neuroblasts, cell polarity is established by two protein complexes that localize to the apical cortex: the Par complex (Bazooka–Par6–atypical protein kinase C [aPKC]) and the Partner of Inscuteable (Pins)–G-protein subunit α (Gαi) complex (Rolls et al. 2003; Lee et al. 2006b). Simply put, these protein complexes restrict localization of the neuronal determinants to the basal cortex where they will be inherited by the differentiating GMC. The complexes remain in the apical daughter (the self-renewing neuroblast) during mitosis and provide reliable apical markers of cell polarity (Fig. 2; Table 2). Aurora A kinase (AurA) provides a link between mitosis and asymmetric distribution of fate determinants, initiating cell polarity by phosphorylation of Par6 and activation of aPKC (Lee et al. 2006a; Wirtz-Peitz et al. 2008). The active form of aPKC phosphorylates Lethal giant larvae (Lgl), eliminating it from the apical complex and thereby allowing Bazooka to associate with aPKC and Par6 (Betschinger et al. 2003). The presence of Bazooka in the Par complex facilitates binding to Numb (Nb), promoting phosphorylation of Nb by aPKC and, subsequently, release of Nb from the apical cortex (Smith et al. 2007). Although Nb is required for differentiation of neurons and the end result of this kinase cascade appears to be restriction of Nb to the basal cortex and eventually the GMC, it is not known how Nb might be acting to specify neuronal fate. The most likely mechanism is through modulation of Notch (N) signaling, because Nb is a known repressor of the N receptor (Yoon and Gaiano 2005). Intriguingly, N signaling is active in larval neuroblasts but not GMCs, reinforcing the possibility of this mechanism.

The importance of aPKC in establishing neuroblast cell polarity is apparent when considering aPKC loss-of-function and gain-of-function phenotypes. When aPKC activity is compromised, neuroblasts lose the ability to self-renew and larval brains contain fewer neuroblasts. In contrast, abnormal uniform cortical localization of aPKC in *lgl; pins* double mutants results in the formation of large tumors as both type I and type II neuroblasts execute symmetric divisions to generate ectopic sister neuroblasts (Figs. 1B and 2C).

In addition to regulating distribution of Nb, aPKC is also required for establishing basal localization of additional neuronal determinants Pros and Brain tumor (Brat) to restrict inheritance of these proteins to the GMC during mitosis. The transcription factor Pros is present in neuroblasts, but it remains in the cytoplasm. In GMCs, however, Pros localizes to the nucleus after mitosis where it pre-

8 / *Chapter 2*
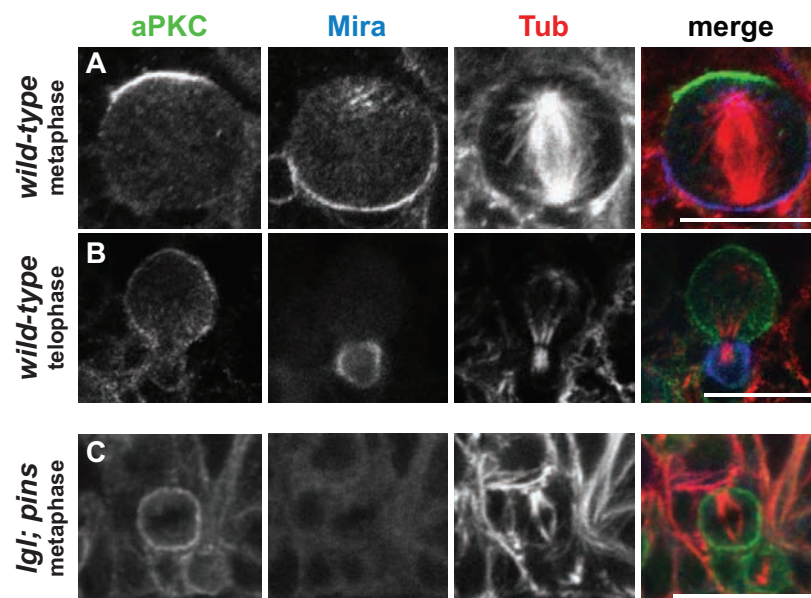
FIGURE 2. Cell-polarity markers in neuroblasts. (*A*) Metaphase wild-type neuroblasts display apical polar localization of aPKC (green) and basal polar localization of Miranda (Mira; blue). The mitotic spindle is visualized with anti-α-tubulin (red). (*B*) During telophase, apical proteins such as aPKC (green) are retained in the neuroblast (apical daughter) and basal proteins such as Mira (blue) are segregated into the ganglion mother cell (GMC; basal daughter). The mitotic spindle is visualized with anti-α-tubulin (red). (*C*) Cell polarity is disrupted in *lgl; pins* double mutants. aPKC (green) is localized uniformly around the cortex, displacing Mira to the cytoplasm (blue). Apical is oriented to the *top*, basal to the *bottom* in all images. Scale bars, 10 μm. (*A,* Images courtesy of C. Gamble, University of Michigan; *C,* images courtesy of J. Haenfler, University of Michigan.)

sumably activates transcription of genes required for neuronal differentiation. Brat is a protein that contains NHL, coiled-coil, and B-box protein–protein interaction domains, and it is predicted to act as a posttranscriptional regulator that likely acts together with Pros to specify neuronal wild-type differentiation, although the exact molecular mechanisms are not known. The cargo-binding protein Mira plays a critical role in localizing Pros and Brat to the basal cortex. Molecular interactions between Mira, Pros, and Brat suggest that Mira acts as a scaffold to retain these neuronal determinants at the basal cortex of the mitotic neuroblast. Mira accumulates on the basal cortex in mitotic neuroblasts, and provides a key marker of basal cortical polarity (Fig. 2; Table 2). The basal localization of Mira is unaffected by loss of Pros or Brat, but loss of Mira causes uniform cytoplasmic localization of Pros and Brat.

Although aPKC is responsible for inactivation of Lgl in the apical cortex, Lgl inhibits aPKC activity at the basal cortex. This mutual inhibition ensures aPKC is restricted to the apical cortex where it will be inherited only by the neuroblast at telophase. Lgl, Scribble (Scrib), and Discs large (Dlg) are all potent tumor suppressors with well-conserved homologs in mammals. Lgl, Scrib, and Dlg are each required for the formation of septate junctions in the basal portion of the cell and for proper segregation of cell-polarity proteins (Albertson and Doe 2003; Humbert et al. 2008). Immunolocalization of Lgl, Scrib, or Dlg provides a useful marker for the cortex and will effectively outline cells in the lar-

TABLE 2. Neuroblast cell-polarity markers

Apical	Basal	Cortical
Atypical protein kinase C (aPKC)	Miranda (Mira)	Scribble (Scrib)
Par6	Brain tumor (Brat)	Discs large (Dgl)
Bazooka (Baz)	Prospero (Pros)	Lethal giant larvae (Lgl)
Inscuteable (Insc)	Numb (Nb)	
Partner of Insc (Pins)		
G-protein subunit α (Gαi)		

val brain, allowing some cells to be identified solely on size or morphology in the absence of specific markers such as Wor or Dpn.

Regulation of symmetric versus asymmetric cell division requires more than proper apical–basal localization of fate determinants: it also requires that cytokinesis occurs in the appropriate plane. Not surprisingly, spindle position is closely tied to cell polarity as Inscuteable (Insc) physically links the Pins–Gαi complex to the Par complex and also connects the mitotic spindle to the apical cortex (Kraut et al. 1996; Wu et al. 2008). Symmetric division of a polarized cell requires that each daughter receive equal amounts of both apical and basal determinants, thus it is easy to see the importance of spindle orientation relative to the apical–basal axis in regulating daughter cell fates. The mitotic spindle is easily visualized by immunolocalization of tubulin, which can be costained with cell polarity markers to assay spindle alignment. Mutations in the Gαi complex cause misalignment of the spindle and ectopic symmetric division of neuroblasts, likely because of improper segregation of fate determinants (Izumi et al. 2004; Nipper et al. 2007). As such, it is easy to see how loss of proper spindle alignment can lead to inappropriate symmetrical division of stem cells and possible tumor formation.

When examining mutants affecting cell fate due to altered asymmetric division patterns, it is important to determine whether cells are mitotically active. Phosphohistone H3 provides a useful immunological M-phase marker for assaying mitotic defects (Lee et al. 2006c). Chemical labeling of newly synthesized DNA (S-phase marker) by incorporation of BrdU (5-bromo-2′-deoxyuridine) can be more informative because this thymidine analog can be used to pulse-label dividing cells and chased to identify the progeny of dividing cells (Lee et al. 2006c). Such pulse-chase experiments can provide additional insight by distinguishing actively dividing cells from those that might be arrested at a mitotic checkpoint. EdU provides a more sensitive and practical alternative to BrdU that can be visualized without the need for harsh DNA denaturation and additional rounds of antibody staining; methodology for EdU labeling is described in Protocol 2 (see also Fig. 3) (Kolb et al. 2001; Rostovtsev et al. 2002; Breinbauer and Köhn 2003; Agard et al. 2004).

EXPERIMENTAL DESIGN NOTES

Equipment and Reagents

Analysis of larval brains does not require much specialized equipment beyond what is needed for standard culturing of flies (Ashburner and Roote 2000). Standard fly food media, egg collection bottles with fruit juice agar caps, and a well-calibrated incubator will be needed in addition to the few specific items described in each protocol below. It is strongly recommended that researchers use a thermometer to monitor carefully the interior temperature of incubators containing samples, particularly when working with temperature-sensitive strains, because the air temperature can vary considerably throughout an incubator.

Genetic Considerations

When designing experiments, it is necessary to be able to distinguish the genotypes of interest during larval stages. It is therefore recommended that researchers use balancer chromosomes carrying GFP (green fluorescent protein) or larval morphological markers such as *tubby*. Also, keep in mind that neuroblasts play a critical role during embryogenesis, and therefore when working with transgenes it is important to avoid causing severe defects (e.g., lethality) too early in development.

Synchronization of Larval Populations

Synchronizing populations of larvae for analysis will ensure that larvae are at the appropriate stage of development for the experiment and that strains are examined at comparable time points. Collection periods as short as 1 h will yield the most tightly synchronized populations, but embryos laid over a span of ~8 h generally provide a good balance between synchronization and having a
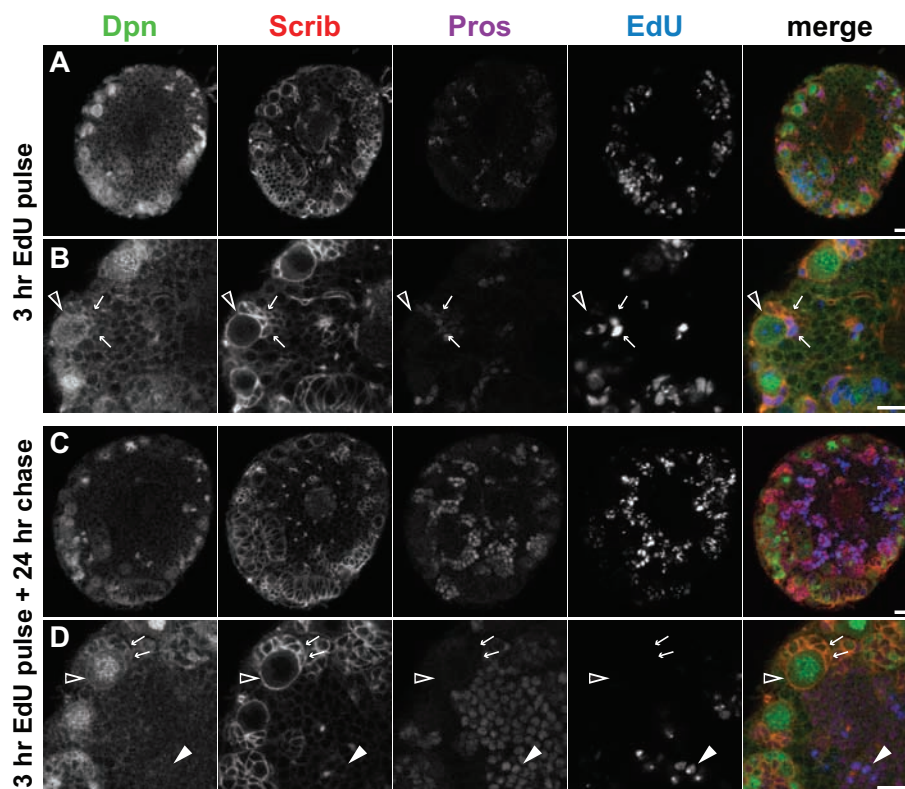
FIGURE 3. EdU pulse-chase in larval brains. (*A*) Wild-type larval brain 72 h after larval hatching (ALH) (25°C) after a 3 h pulse of EdU by feeding. (*B*) High-magnification image of wild-type brain after 3 h pulse. EdU is detected in mitotically active neuroblasts (black arrowheads; Dpn$^+$; green) and GMCs (white arrows), but not differentiating neurons (Pros$^+$; violet). Cells are outlined by the cortical marker Scrib (red). (*C*) Wild-type larval brain 96 h ALH (25°C) after a 3 h pulse of EdU by feeding and a 24 h EdU-free chase. (*D*) High-magnification image of wild-type brain after 3 h pulse and 24 h chase. EdU is no longer detected in neuroblasts (black arrowheads; Dpn$^+$; green) and GMCs (white arrows), but it is now present in differentiating neurons (white arrowheads; Pros$^+$; violet). Cells are outlined by the cortical marker Scrib (red). Scale bars, 10 μm.

large enough population of embryos to work with. Incubate collection plates for ~24 h to allow larvae to hatch before transferring to standard fly food media (meal caps) stored in 60-mm dishes with a moistened tissue to prevent them from drying out. Larvae will generally be in the third-instar stage ~96 h after larval hatching (ALH) at 25°C or 72 h ALH at 30°C.

Protocol 1

Immunofluorescent Antibody Staining of Larval Tissues

This protocol can be used for dissecting, fixing, and staining brains from larvae at any developmental stage. The number of brains processed using this method is limited only by how many brains can be dissected in 20 min, which is the maximum amount of time dissected tissues should remain in buffer before fixation. This protocol can be used for simultaneous costaining of multiple proteins.

MATERIALS

CAUTION: See Appendix for proper handling of materials marked with <!>.
See the end of the chapter for recipes for reagents marked with <R>.

Reagents

Block solution <R>
Fix solution <R>
Glycerol (70%)
Phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) and Triton X-100
    (PBSBT) <R>
PBS containing Triton X-100 (PBST) <R>
PBS stock solution (10x) <R>
ProLong Gold antifade mounting medium (Invitrogen)
      Schneider's insect medium (Sigma-Aldrich)
Triton X-100 <!> (10%)

Equipment

Coverslips (22 x 22-mm [#1 thickness] and 24 x 40-mm)
Dissection dishes
Fine-tipped forceps (two pairs)
Microfuge tubes (0.5-mL)
Microscope slides
Nutator mixer or rocker
Pipettes and sterile tips

METHOD

Dissection of Larvae

1. Fill the wells of dissection dishes with 200–400 µL of cold Schneider's insect medium.

2. Dissect the larvae by rolling them onto their dorsal side so that the denticle belts are facing upward.

3. Using a pair of forceps, gently grasp the larva just posterior of the midpoint. With a second pair of forceps, grasp the anterior end of the larva at the base of the mouth hooks.

4. Carefully tear the cuticle behind the mouth hooks using side-to-side motion while slowly draw-ing the mouthparts out away from the body. The brain will remain attached to the head and be clearly visible among the gut and salivary glands. Remove any excess tissue, but leave the brain attached to the mouth hooks.

> Leaving the brains connected to the mouth hooks will help the brains sink to the bottom of the tube during washing steps below. Moreover, the mouth hooks are dark in color, which makes it easier to see the brains during experimental manipulations.

5. After dissection, place the brains in a 0.5-mL tube containing cold Schneider's insect medium.

> Do not let the tissue sit in Schneider's insect medium for >20 min.

Fixation and Staining

6. Remove the Schneider's insect medium from the samples.

7. Add 500 µL of fix solution and incubate with rocking for 23 min at room temperature.

8. Quickly wash the brains twice in ~500 µL of PBST at room temperature. Wash again in PBST twice for 20 min each at room temperature.

> Once fixed, samples can be held in extended washes to synchronize them before proceeding with further processing.

9. Incubate the samples in ~500 µL of block solution for at least 30 min at room temperature.

10. Incubate in primary antibody diluted in PBSBT for 4 h at room temperature or overnight at 4°C.

> Conditions are dependent on the specific antibody being used. For example, Dpn staining is bet-ter when incubated for 3–4 h at room temperature rather than overnight at 4°C.

11. Quickly wash the brains twice in PBSBT at room temperature. Wash again in PBSBT twice for 30 min each at room temperature.

12. Incubate the samples in secondary antibody for 1.5 h at room temperature or overnight at 4°C. Protect the samples from light after this point.

> Secondary antibodies are typically diluted in PBSBT.

13. Quickly wash the brains twice in PBST at room temperature. Wash again in PBST twice for 30 min each at room temperature.

14. Equilibrate the brains in ProLong Gold at room temperature. Samples can be stored in the dark at room temperature.

Mounting Samples

15. Adhere two 22 × 22-mm coverslips to a microscope slide using a small amount of 70% glycerol, leaving a ~5-mm space between them.

> These coverslips act as spacers to prevent the brains from being deformed by the 24 × 40-mm cov-erslip in Step 19.

16. Transfer the brains to the slide using a pipette with the tip cut off.

17. Using forceps, remove all excess tissue including the optic discs from each brain. Be sure to leave the ventral nerve cord intact, as it will aid in proper orientation of the brain on the slide.

See Troubleshooting.

18. Orient the brains ventral side down.

> If the ventral cord is intact, the brain will sit in the appropriate upright position. Without the ven-tral cord, it is difficult to keep the brain in the proper position and it will tend to end up resting on its anterior or posterior surface.

19. Place a 24 × 40-mm coverslip over the samples and backfill the space between the slide and the coverslip by pipetting a small amount of mounting medium along the edge of the coverslip.
    Backfilling will reduce the formation of air bubbles trapped in the slide.

    See Troubleshooting.

TROUBLESHOOTING

Problem (Step 17): The ventral nerve cord breaks off during dissection.
Solution: Keeping the ventral nerve cord intact requires that you grasp the larva at the right place on its body. Holding the larva at a "sweet spot" near the 4th or 5th abdominal segment will typically allow clean dissection of the brain. Take care to gently break away attached tissues as you tear the head away from the body. The ventral cord is connected to the body by many axons and will likely break off if the head is carelessly pulled from the body.

Problem (Step 19): There is poor signal-to-noise ratio.
Solution: High levels of background staining can result from several steps in this protocol. Ensure that all solutions are at the correct pH because high or low pH levels can negatively affect antibody binding. It is critical to use both primary and secondary antibodies at the appropriate dilution specific for each antibody. The specificity of secondary antibodies should be tested by staining a sample with secondary antibody alone. Thorough washing of samples is also important for reducing background signals, particularly after incubation in primary antibodies. Placing a small, fine pipette tip over a larger 1000-μL tip will help you to remove as much of the wash solutions as possible without losing or damaging the samples. However, note that excessive washing can also lead to weak signal strength. Antibodies can be sensitive to the duration and temperature of incubation. Anti-Dpn, for example, will typically yield cleaner staining when incubated for 3–4 h at room temperature than when incubated overnight at 4°C. Testing different incubation conditions might be necessary to determine the optimal conditions for a particular antibody.

Protocol 2

EdU Labeling of Mitotic Neuroblasts

Like BrdU, EdU is a thymidine analog that is incorporated into newly synthesized DNA during S-phase and it provides an efficient method for identifying mitotic cells. Incorporation of EdU is detected through its reaction with an azide dye that is small enough to penetrate tissues efficiently. This method is highly sensitive and does not require the harsh denaturation of DNA that is necessary for staining with antibodies (Kolb et al. 2001; Rostovtsev et al. 2002; Breinbauer and Köhn 2003; Agard et al. 2004). Visualization of EdU is rapid and does not interfere with subsequent antibody staining. EdU can be used to pulse-label mitotic cells and chased to identify their progeny, just like BrdU. This protocol was modified from BrdU-feeding procedures described by Truman and Bate (1988) and Ito and Hotta (1992). Methods for the detection of EdU are described in Invitrogen product manuals.

MATERIALS

CAUTION: See Appendix for proper handling of materials marked with <!>.
See the end of the chapter for recipes for reagents marked with <R>.
Refer also to the Materials list for Protocol 1.

Reagents

Bromophenol blue <!>
Click-iT EdU imaging kit (Invitrogen)
EdU (Invitrogen)
Kankel–White medium <R>

METHOD

Preparation of Medium

1. Prepare EdU and the detection reagents as instructed by the manufacturer (Invitrogen).
2. Prepare Kankel–White medium and heat to dissolve all components. Add a few granules of bromophenol blue to the medium.
3. Allow the medium to cool to 50°C–60°C. Add EdU to give a final concentration of 0.2 mM.
4. Pour the mixture into plates and allow it to solidify.

Feeding EdU to *Drosophila* Larvae

5. Allow the larvae to feed on EdU-containing medium for 3–4 h.

    The presence of bromophenol blue in the medium will make food in the gut visible. If the larvae are eating, they should be taking up EdU.

6. If no EdU-free chase is required, proceed directly to Steps 7–16 below. If an EdU-free chase is required, transfer the larvae to standard fly food and allow them to recover for the desired amount of time under appropriate experimental conditions.

    If using bromophenol blue in the medium, select larvae with blue food visible in their guts.

Dissection and Staining

7. Dissect the larvae in Schneider's insect medium and remove the brains following the procedure described in Protocol 1, Steps 1–6.

8. Add 500 µL of fix solution to the brains and incubate with rocking for 23 min at room temperature.

9. Quickly wash the brains twice in ~500 µL of PBST at room temperature. Wash again in PBST twice for 20 min each at room temperature.

10. Incubate the samples in ~500 µL of block solution for at least 30 min at room temperature.

11. Quickly wash the brains in PBST at room temperature.

12. Prepare the Click-iT reaction mix as instructed by the manufacturer. To prevent photobleaching, protect the samples from light after this point.

13. Add 500 µL of Click-iT reaction mix and incubate with rocking for 30 min at room temperature.

14. Quickly wash the brains twice in PBST at room temperature.

15. Quickly wash the brains in PBSBT at room temperature.

16. Mount a few brains and scan them to check the efficiency of EdU labeling. Process the remaining brains for antibody staining as described in Protocol 1.

    See Troubleshooting.

TROUBLESHOOTING

Problem (Step 16): EdU signal is too bright, thereby saturating detection.
Solution: Detection of EdU is more sensitive than detecting BrdU. The concentration of EdU in the medium should be lower than is necessary in experiments with BrdU. Also, larvae can be fed for shorter periods of time to reduce the pulse of EdU labeling in cells.

Protocol 3

Multicolor Fluorescence In Situ Hybridization

RNA in situ hybridization is a useful method for determining the transcriptional expression pattern of a gene when antibodies are not available. Using this technique, it is possible to assay the expression of multiple RNA species using distinct labels on RNA probes, or simultaneously examine RNA and protein localization within larval tissues (Fig. 4). This protocol utilizes a fluorophore-conjugated tyramide that is easily made in the laboratory for a fraction of the cost of the commercially produced product. The method was adapted from B. Pearson (University of Utah; pers. comm..) with modifications by H. Komori and A. Daul (University of Michigan). Additional modifications were derived from D. Kosman (http://superfly.ucsd.edu/~davek/) (Kosman et al. 2004).

MATERIALS

CAUTION: See Appendix for proper handling of materials marked with <!>.
See the end of the chapter for recipes for reagents marked with <R>.
Refer also to the Materials list for Protocol 1.

Reagents

Anti-digoxigenin (DIG)-POD or other antibody for detection of riboprobe
Carbonate buffer (2x) <R>
DIG RNA-labeling kit (Roche)
DNA template for riboprobe
Ethanol <!> (70% and 100%)
Horse serum (heat-inactivated)
Hybridization buffer <R>
Hydrogen peroxide <!> (30%, v/v)
Hydrolysis stop buffer <R>
Imidazole <!>
LiCl <!> (4 M)
MABT <R>
PBST <R>
RNase-free water
SSC stock solution (20x) <R>
Tyramide (fluorescently labeled) <R>

Equipment

Heat block (set at 90°C)
Ice bucket
Incubator (set at 37°C)
Microfuge tubes (0.5-mL; RNase-free)
Nutator mixer or rocker
Pipettes and tips (RNase-free)
Water bath (set at 55°C or 65°C)
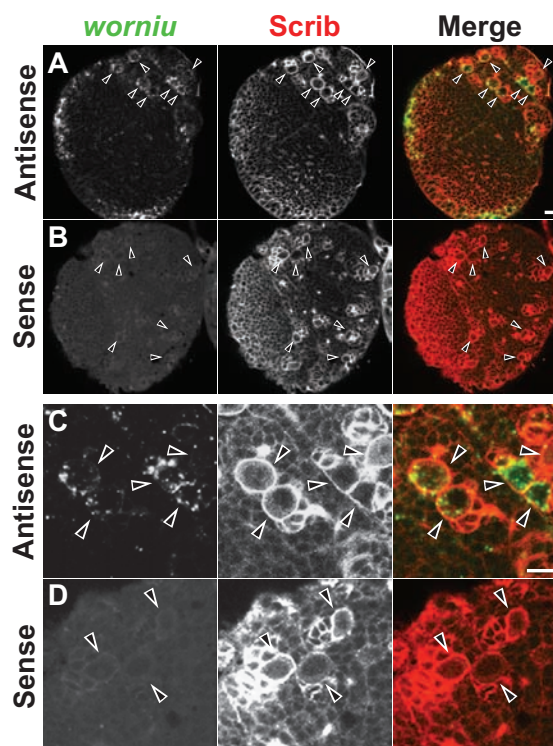
FIGURE 4. Fluorescent in situ localization in larval brains. Wild-type third-instar larval brains hybridized with anti-*worniu* riboprobe and costained with anti-Scrib antibodies. (*A*) *worniu* antisense probe (green) is detected in central brain neuroblasts (arrows). Neuroblasts can be identified by morphology as outlined by the cortical marker Scrib (red). (*B*) No signal is detected when using a *worniu* sense probe. (*C,D*) High-magnification images of *A* and *B*, respectively. (Images courtesy of H. Komori, University of Michigan.)

METHODS

Riboprobe Synthesis

Producing riboprobes requires template DNA flanked by distinct RNA polymerase promoter sites, typically T3, T7, or Sp6. Templates can be easily produced by inserting cDNA or a genomic subclone with minimal intronic sequence into pBluescript or other cloning vector with suitable promoter sites. Having distinct promoters on either end of the template allows the template to be used for transcription of sense and antisense probes. Use a restriction enzyme to cut the plasmid at the opposite end of the template so that transcription of the probe will stop at the cut and not proceed into the vector sequence. Alternatively, template DNA can be made by polymerase chain reaction (PCR) amplification. In this case, the amplified fragment must contain the desired RNA polymerase promoter site at each end in order to transcribe the probe. Regardless of the preparation method, template DNA should cleaned up following the enzymatic reactions and resuspended in RNase-free water. Probes can be labeled with a variety of haptens, including digoxigenin (DIG), dinitrophenol (DNP), biotin (BIO), and fluorescein isothiocyanate (FITC). DIG, BIO, and FITC RNA-labeling kits are available from Roche. DNP-11-UTP is available from PerkinElmer and can be mixed with unlabeled ribonucleotides for use in a similar transcription reaction. Antibodies for the detection of hapten-labeled riboprobes are available from a variety of commercial sources.

1. Combine the following in an RNase-free microfuge tube:

   ~1.5 mg of DNA template
   2 µL of 10x dNTP labeling mix*
   2 µL of 10x transcription buffer*
   1 µL of RNase inhibitor*
   2 µL of RNA polymerase*
   RNase-free water to give a total volume of 20 µL

   > * These components are included in the Roche DIG RNA-labeling kit. The amounts required
   > could vary when not using this kit.

2. Incubate for 2 h at 37°C.

   > At this point, a small amount of probe can be run on an agarose gel to evaluate the efficiency of
   > the transcription reaction.

3. Add 5 µL of RNase-free water and 25 µL of 2x carbonate buffer. Incubate for 45 min at 65°C.

4. Add 50 µL of hydrolysis stop buffer.

5. Add 10 µL of 4 M LiCl and 330 µL of 100% ethanol. Precipitate at –70°C for at least 30 min.
   Spin at top speed for 15 min. Quickly wash the pellet in 70% ethanol. Resuspend the pellet in
   100 µL of hybridization buffer. Store at –80°C. Avoid repeated freezing and thawing.

RNA In Situ Hybridization with Antibody Costaining

6. Dissect the larvae in Schneider's insect medium and remove the brains following the procedure
   described in Protocol 1.

7. Add 500 µL of fix solution and incubate with rocking for 23 min at room temperature.

8. Quickly wash the brains twice in ~500 µL of PBST at room temperature. Wash again in PBST
   twice for 20 min each at room temperature.

9. Incubate in PBST containing 3% hydrogen peroxide for 1 h at room temperature. Seal the tubes
   tightly with parafilm to prevent the caps from blowing open during this reaction!

   > This step eliminates endogenous peroxidase activity.

10. Quickly wash the brains twice in ~500 µL of PBST at room temperature. Wash again in PBST
    twice for 20 min each at room temperature.

11. Incubate the brains in 400 µL of a 1:1 mixture of PBST and hybridization buffer for 30 min at
    room temperature.

12. Wash three times for 10 min each in hybridization buffer at room temperature.

13. Incubate the brains in 400 µL of hybridization buffer for 2 h at 55°C. At 1.5 h after the start of
    this incubation, denature 400 ng (~4 µL) of riboprobe in 400 µL of hybridization buffer for 5
    (step 14)min at 90°C. Cool on ice for 5 min, and then place at 55°C.

14. Incubate the brains in 150–200 µL of the probe/hybridization buffer mixture for at least 16 h
    at 55°C.

15. Remove the probe/hybridization buffer mixture.

    > The mixture can be stored at –20°C and reused.

16. Wash the brains twice in a prewarmed 1:1 mixture of hybridization buffer and 2x SSC for 30
    min at 55°C.

17. Wash twice in prewarmed 2x SSC for 30 min at 55°C.

18. Wash twice in prewarmed 0.2x SSC for 30 min at 55°C.

19. Quickly wash the brains twice in MABT at room temperature.

20. Block in MABT containing 10% heat-inactivated horse serum for 1 h at room temperature.

    BSA has been reported to reduce the intensity of the tyramide reaction.

21. Incubate the brains in anti-DIG-POD (diluted 1:1000 in MABT containing 10% heat-inactivated horse serum) overnight at 4°C.

    If performing in situ hybridization of multiple RNA species using tyramide reactions, each probe must be detected and developed in sequence to avoid cross-reactivity.

22. Wash the brains six times in MABT for 20 min at room temperature.

23. Incubate the brains in PBST containing 10 mM imidazole for 30 min at room temperature.

24. Incubate the brains in the dark in FITC-tyramide solution diluted 1:1000 in PBST containing 10 mM imidazole (1:500 for Cy3-tyramide) for 30 min at room temperature. Protect the samples from light after this point.

25. Develop the signal by adding hydrogen peroxide to a final concentration of 0.002%–0.01%. Incubate for 45 min at room temperature.

    Signal strength can be enhanced by repeating Steps 24–25 up to twice with fresh fluorescently labeled tyramide solution.

26. Quickly wash the brains twice in ~500 µL of PBST at room temperature. Wash again in PBST twice for 30–60 min each at room temperature.

27. If the samples are to be costained with antibodies for protein localization, proceed as described in Step 5 of Protocol 1.

    If the samples are to be processed for the detection of a second riboprobe, quench the residual peroxidase activity by incubating in PBST containing 3% hydrogen peroxide for 1 h at room temperature. Quickly wash the brains twice in ~500 µL of PBST at room temperature. Wash again in PBST twice for 20 min each at room temperature. Proceed by incubating with the second riboprobe and repeat Steps 11–25 above.

RECIPES

CAUTION: See Appendix for proper handling of materials marked with <!>.
Recipes for reagents marked with <R> are included in this list.

Block Solution

Reagent	Quantity (for 5 mL)	Final concentration
PBSBT <R>	5 mL	1x
Normal goat serum (NGS)*	5 µL	0.1% (v/v)
Glycine (1 M in PBS containing 2% sodium azide <!>)	50 µL	1% (w/v)

Prepare fresh and keep cold. *Omit if using antigoat antibodies.

Carbonate Buffer (2x)

Reagent	Quantity (for 100 mL)	Final concentration
Na_2CO_3 <!>	0.636 g	60 mM
$NaHCO_3$	0.336 g	40 mM

Adjust the pH to 10.2. Store at room temperature.

20 / *Chapter 2*

Fix Solution

Reagent	Quantity (for 10 mL)	Final concentration
Formaldehyde <!> (37%, v/v)	1.1 mL	4% (v/v)
PIPES (1 M, pH 6.9)	1.0 mL	0.1 M
Triton X-100 <!> (10%, v/v)	0.3 mL	0.3% (v/v)
EGTA (0.1 M, pH 8.0)	0.2 mL	20 mM
$MgSO_4$ <!> (1 M)	10 µL	1 mM
dH_2O	7.4 mL	

Prepare fresh every time.

Hybridization Buffer

Reagent	Quantity (for 50 mL)	Final concentration
Deionized formamide <!>	25 mL	50%
SSC (20x) <R>	10 mL	4x
Tween-20 (10%, v/v)	0.5 mL	0.1% (v/v)
Heparin <!> (50 mg/mL)	50 µL	0.05 mg/mL
RNase-free dH_2O	14.5 mL	

Hydrolysis Stop Buffer

Reagent	Quantity (for 10 mL)	Final concentration
Sodium acetate	0.166 g	200 mM
Acetic acid <!>	100 µL	1% (v/v)

Adjust the pH to 6.0 with acetic acid. Store at –20°C

Kankel–White Medium

Reagent	Quantity (for 10 mL)
Agar	80 mg
Sucrose	500 mg
Yeast extract	500 mg
Dried yeast	200 mg
dH_2O	10 mL

Heat to dissolve. Do not boil excessively.

MABT

Reagent	Quantity (for 1 L)	Final concentration
Maleic acid <!>	11.6 g	100 mM
NaCl	8.8 g	150 mM
Tween-20 (10%, v/v)	1 mL	0.1% (v/v)

Dissolve the components and adjust the pH to 7.5 with concentrated NaOH <!>.
Adjust the volume to 1 L with dH_2O and sterilize. Store at room temperature.

PBS Stock Solution (10x)

Reagent	Quantity (for 1 L)	Final concentration
NaCl	80 g	1.37 M
KCl <!>	2 g	27 mM
Na_2HPO_4	14.4 g	100 mM
KH_2PO_4	2.4 g	20 mM
dH_2O	to 1 L	

Dissolve the components in 400 mL of dH_2O and adjust the pH to 7.4 with concentrated HCl <!>. Adjust the volume to 1 L with dH_2O and sterilize. Store at room temperature.

PBSBT

Reagent	Quantity (for 25 mL)	Final concentration
PBST <R>	25 mL	1x
Bovine serum albumin (BSA)	0.25 g	1% (w/v)

Make fresh and keep cold. Can be stored short-term at 4°C.

PBST

Reagent	Quantity (for 500 mL)	Final concentration
PBS stock solution (10x) <R>	50 mL	1x
Triton X-100 <!> (10%, v/v)	15 mL	0.3% (v/v)
dH_2O	435 mL	

Store at room temperature.

SSC Stock Solution (20x)

Reagent	Quantity (for 1 L)	Final concentration
NaCl	175.3 g	3.0 M
Sodium citrate <!>	88.2 g	0.3 M
H_2O	800 mL	

Adjust the pH to 7.0 with a few drops of 14 N HCl <!>. Adjust the volume to 1 L with H_2O.

Tyramide (Fluorescently Labeled)

Work should be performed in the hood. For best results, use fresh reagents. *N*-hydroxysuccinimide (NHS) esters are unstable and the coupling reaction should be kept anhydrous.

1. Dissolve 40 mg of fluorescently labeled NHS ester in 4 mL of dimethylformamide (DMF) <!>.

2. Add 10 µL of triethylamine (TEA) <!> to 1 mL of DMF.

3. Dissolve 10 mg of tyramide in 1 mL of TEA–DMF solution.

4. Mix 4 mL of fluorescently labeled NHS ester in DMF with 1.37 mL of tyramide solution. Incubate in the dark for 2 h at room temperature.

5. Add 4.6 mL of ethanol.

Keep protected from light. Store at 4°C or –20°C. The solution is stable for at least 8 mo at 4°C.

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13

Electrophysiological Recordings from the *Drosophila* Giant Fiber System

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ABSTRACT

The giant fiber system (GFS) of *Drosophila* is a well-characterized neuronal circuit that mediates the escape response in the fly. It is one of the few adult neural circuits from which electrophysiological recordings can be made routinely. This chapter describes a simple procedure for stimulating the giant fiber neurons directly in the brain of the adult fly and obtaining recordings from the output muscles of the GFS.

INTRODUCTION

The GFS mediates a fast escape behavior in adult flies (Allen et al. 2006). Behaviorally, it is characterized by an initial extension of the mesothoracic leg, to propel the flies off the substrate, followed by a wing downbeat to initiate flight. The efferent (output) pathways of the GFS have been well defined (Fig. 1) for the most part by work from Wyman and others in the 1980s using a combination of dye injection, electron microscopy, and electrophysiological techniques (Ikeda et al. 1980; King and Wyman 1980; Koto et al. 1981). The two largest interneurons in the fly, the aptly named giant fibers (GFs), relay the signal from the brain to the mesothoracic neuromere where each makes two identified synapses. The first is to a large motorneuron (TTMn) that drives the tergotrochanteral "jump" muscle (TTM), which is also referred to in the literature as the tergal depressor of trochanter or TDT. This GF–TTMn synapse, which is the largest central synapse in the fly, is a mixed synapse with the electrical gap-junction component encoded by the *shaking-B* (*shakB*) gene and the chemical component using acetylcholine its neurotransmitter (Blagburn et al. 1999; Allen and Murphey 2007; Phelan et al. 2008). The second identified synapse of the GF is to another interneuron, the peripherally synapsing interneuron (PSI), which exits the ganglion via the posterior dorsal medial nerve (PDMN) and synapses with dorsal longitudinal motorneurons (DLMns) within the PDMN. The DLMns drive the large indirect flight muscles (DLMs). Electrophysiological recordings can be made from the GFS in a simple noninvasive manner to determine the function of the central synapses within the circuit. Using combinations of adult viable mutants and/or GAL4 lines that express in its neurons, the GFS has provided a useful model circuit to investigate the role of several molecules in the formation of
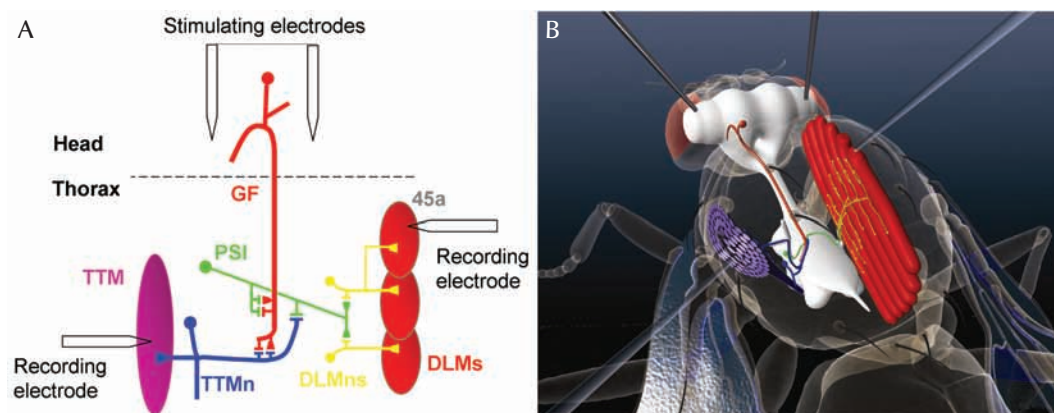
FIGURE 1. The giant fiber system (GFS): neurons and muscles. (*A*) Schematic indicating the neurons and connections of the GFS. For clarity, only one-half of the bilateral circuit is shown. The giant fiber (GF; red) relays information from the brain to the thoracic ganglia where it makes an electrochemical synapse to the tergotrochanteral motor neuron (TTMn; blue), which innervates the tergotrochanteral muscle (TTM). It also makes an electrochemical synapse to the peripherally synapsing interneuron (PSI; green), which, in turn, makes chemical synapses to the dorsal longitudinal motorneurons (DLMns; yellow) that innervate the dorsal longitudinal muscles (DLMs). The relative positions of the stimulating and recording electrodes are indicated. (Adapted, with permission of Elsevier, from Allen et al. 2006.) (*B*) Artist's impression of the GFS showing the central nervous system within the fly's body. The neurons and muscles of the GFS are shown in their approximate positions and the best positions for the stimulating and recording electrodes are indicated.

central synapses including Glued, Rac1, Robo, Semaphorin 1a, and Neuroglian (Allen et al. 1999, 2000; Godenschwege et al. 2002a,b, 2006). The GFS has also been used to investigate the effects of aging, sensitivity to anesthetics, the effects of neurodegeneration, and the molecular basis of habituation (Engel and Wu 1996, 1998; Lin and Nash 1996; Martinez et al. 2007; Watson et al. 2008).

Stimulating and Recording from the GFS

The GFs can be activated directly with brain stimulation, and the two output pathways can be monitored by recording simultaneously from the TTM and DLMs. The original rationale was that by placing the stimulating electrodes into the brain and slowly increasing the stimulation voltage, a point would be reached where only the GF interneurons would propagate an action potential because their large size would mean they have the least resistance and thus the lowest threshold. Although this may theoretically be true, in practice, accurate positioning of the electrodes is hard to achieve, so the stimulation voltage given is much above threshold. This ensures that the GFs are activated directly and not by upstream neurons (unless that is desired, see below). Although many neurons in the brain may be activated, the only route to the TTMs and DLMs from the brain activated by this procedure seems to be via the GFs. This is supported by findings that genetic ablation of the GFs, or abrogation of the electrochemical synapses between the GF and the TTMn and PSI, results in total loss of TTM and DLM responses on brain stimulation (Allen et al. 2000; Allen and Murphey 2007). However, both TTMn and the DLMns have other unidentified inputs, one of which is triggered by looming stimuli (Fotowat et al. 2009). Once direct activation of the GFs is achieved, recordings from TTM monitor the function of the GF–TTMn central synapse along with the neuromuscular junction (NMJ) and recordings from DLM monitor the function of the GF–PSI and PSI–DLMns synapses as well as the NMJ.

Standard Tests of Synaptic Function

The most commonly used tests for the GFS are response latency, the refractory period, and the ability to follow high-frequency stimulation. These will be described in turn.

Response Latency: This is the time taken for the output muscle to respond to a single stimulus acti-vating the GFs. In the TTM of wild-type flies this is ~0.8 msec after GF activation and is via the monosynaptic pathway through the large electrochemical GF–TTMn synapse. The response in a DLM, through the disynaptic pathway, is seen ~1.2 msec after GF activation. These latencies corre-spond to the escape behavior in which the jump always occurs before the wing downbeat. This robust short-latency (SL) response is a good indicator of synaptic function, and any abnormalities in the synapses of the GFS will result in an increase in the latency or a loss of the response—for example, loss of gap junctions or structural malformations of the synapse that alter its shape or size (Thomas and Wyman 1984; Oh et al. 1994; Allen et al. 1999, 2000; Godenschwege et al. 2002a,b, 2006; Allen and Murphey 2007; Uthaman et al. 2008).

In addition to SL responses, intermediate-latency (IL) responses (TTM ~ 1.8 msec, DLM ~ 2.2 msec), and long-latency (LL) responses (TTM ~ 3.9 msec, DLM ~ 4.3 msec) can be elicited by sim-ply reducing the voltage during brain stimulation or providing a light-off stimulus to a tethered fly. All these responses are still conducted through the GF; note the delay between the TTM and DLM response is always ~0.4 msec, indicating the disynaptic pathway from GF to DLM via the PSI and DLMn. The longer IL and LL responses, during low-voltage electrical stimulation or a light-off stim-ulus, are attributed to indirect activation of the GF by the afferent neurons in the brain. These neu-rons still remain unidentified but have interesting properties as they show both sensitivity to anesthetics and habituation to repeated stimuli (Engel and Wu 1996, 1998; Lin and Nash 1996).

Refractory Period: In this test, twin stimuli are given, initially 10 msec apart, and the responses from both TTM and DLM are recorded. The interval between the two stimuli is then gradually reduced until the second stimulus fails to elicit a response. The shortest time between two stimuli that still produces two responses is defined as the refractory period. For TTM this is ~3 msec and for DLM it is ~5 msec because of the greater time needed for the PSI–DLMn chemical synapses to replenish their synaptic vesicles. This test is less common than the other two as similar information can be gleaned if you observe the responses to the first two stimuli in the "following at high frequencies" test.

Following at High Frequencies: In this test a train of 10 stimuli are given to the preparation at high frequency and the number of responses is recorded. These trains of stimuli are usually given at 100, 200, and either 250 or 300 Hz. At 100 Hz (stimuli 10 msec apart) both TTM and DLM should respond 1:1 and give 10 responses. At the higher frequencies—for example, 250 Hz (stimuli 4 msec apart)—TTM will still respond 1:1 because of the robust GF–TTMn electrochemical synapse; how-ever, DLM recordings will start to show failures as the time between stimuli is less than the refrac-tory period of the PSI–DLMns synapses. An alternative way of performing the test is to gradually increase the frequency of the stimuli until the response rates fall below 50% (5 out of 10). This is described as the Following Frequency$_{50}$ (FF$_{50}$) (Gorczyca and Hall 1984). This test will often reveal an abnormality in synaptic function that does not cause an abnormal response latency (Allen et al. 1999), although it usually confirms an aberrant response latency.

Protocol

Recording from TTM and DLM: The Outputs of the GFS

This protocol is a standard method for recording from the GFS of *Drosophila*. It is a relatively non-invasive method that allows the investigator to stimulate the giant fibers in the brain and assay the function of several central synapses within this neural circuit by recording from the thoracic musculature.

MATERIALS

CAUTION: See Appendix for proper handling of materials marked with <!>.
See the end of the chapter for recipes for reagents marked with <R>.

Reagents

CO_2 or ice (Step 1)
Dental wax, soft (available from most dental product suppliers)
Drosophila melanogaster wild-type/control flies (e.g., Oregon-R, *w^{1118}*, *bendless/+; shakB^2/+*) and mutant strains (e.g., *bendless*, *shakB^2*)
Forceps
KCl <!>, 3 M, or GFS saline <R>
Slide or mounting tray
    These can be made from a small Petri dish filled with tooth carding wax (shown in Fig. 2B), from a piece of Plexiglas or a coin, or from a small piece of wood.

Equipment

Electrodes, recording (glass with a resistance of 40–60 MΩ; need two of these)
    These are fabricated using a good glass microelectrode puller (e.g., a Sutter P-95). Again preformed microelectrodes can be purchased if desired.
Electrodes, tungsten and sharpened (one ground and two stimulation electrodes)
    These can be fabricated from 0.005-in-diameter tungsten wire sharpened electrolytically using 4 M NaOH. Alternatively, commercially available tungsten electrodes can be used. The electrophysiology rig is shown in Figure 2A,B (the figure legend contains equipment source information).
Faraday cage (optional)

METHOD

Mounting Flies

1. Anesthetize the fly on ice or with CO_2.

    The fly should be left for 20–30 min after mounting if CO_2 is used, because occasionally it can affect recordings. This is not a problem when using ice; however, the fly must be secured in the wax more quickly as recovery from cooling can be quite rapid.

2. Using forceps, transfer the anesthetized fly to the wax by its legs, and mount it into soft wax on a slide or tray with the ventral side down, pushing the legs into the wax to secure.
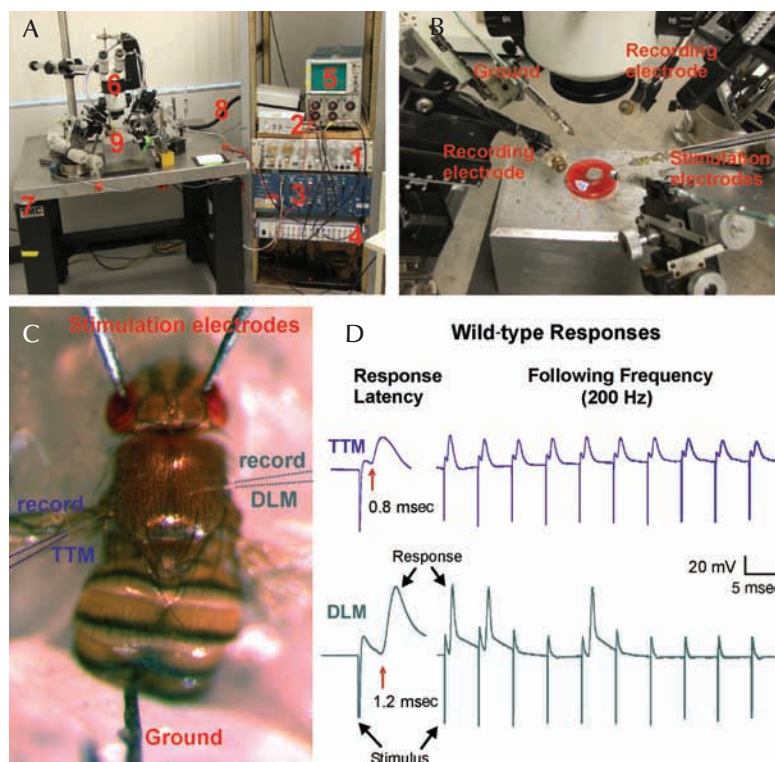
FIGURE 2. Electrophysiology of the GFS. (*A*) Components of the electrophysiological rig. 1: Stimulator (S48 Square Pulse Stimulator, Grass Instruments); 2: stimulation isolation unit (SIU5 RF Transformer Isolation Unit, Grass Instruments); 3: two-channel intracellular amplifier (Model 5A Microelectrode Amplifier, Getting Instruments); 4: data acquisition system (Digidata 1440A, Molecular Devices) and computer with software (not shown); 5: storage oscilloscope 5111A (Tektronix); 6: stereomicroscope (Wild M5) on a boom stand; 7: vibration isolation table (TMC); 8: light source (Fostec); 9: recording platform with five manual multi-axis micromanipulators (Narashigi, Sutter Instrument Company, and World Precision Instruments). (*B*) Magnification of the recording platform. Around the recording tray are arranged two stimulation electrodes (sharp tungsten electrodes), two recording electrodes (glass electrodes filled with saline), and one ground electrode (sharp tungsten electrodes). (*C*) *Drosophila melanogaster* impaled with stimulation electrodes through the eyes in the brain and a ground in the abdomen. Two glass electrodes are placed in the thorax for recording responses from the TTM and DLM. (*D*) Sample electrophysiological traces from recordings of the TTM and DLM on brain stimulation of a wild-type fly. The response latency of the GF-TTM pathway is 0.8 msec and it can follow stimuli 1:1 at 200 Hz. In contrast, the response latency of the GF-DLM pathway is 1.2 msec, and responses are not seen after every stimulus when given 10 stimuli at 200 Hz.

3. Pull the proboscis outward and push into the wax so that the head lies slightly forward and down on the surface.

> This step is important because the head needs to be secure and not move when the stimulating electrodes are inserted (Step 6). Keeping the head slightly stretched in front of the thorax will also help prevent inadvertent stimulation of the ventral nerve cord.

4. Pull the wings outward, away from the thorax, and secure. Ensure that the fly cannot move its thorax and that the areas of the DLM and TTM (Fig. 3, dotted areas) are visible and accessible.

> If the fly is mounted incorrectly or not securely, it becomes very difficult to obtain recordings, so it is advisable to practice these steps several times before proceeding with the protocol.

Placement of Electrodes

Successful recording from the GFS depends on being able to arrange the five micromanipulators so that the electrodes can be placed within several millimeters of each other. It is worth spending some time moving and adjusting these before a preparation is introduced so that minimal adjustment is required when recordings are needed.
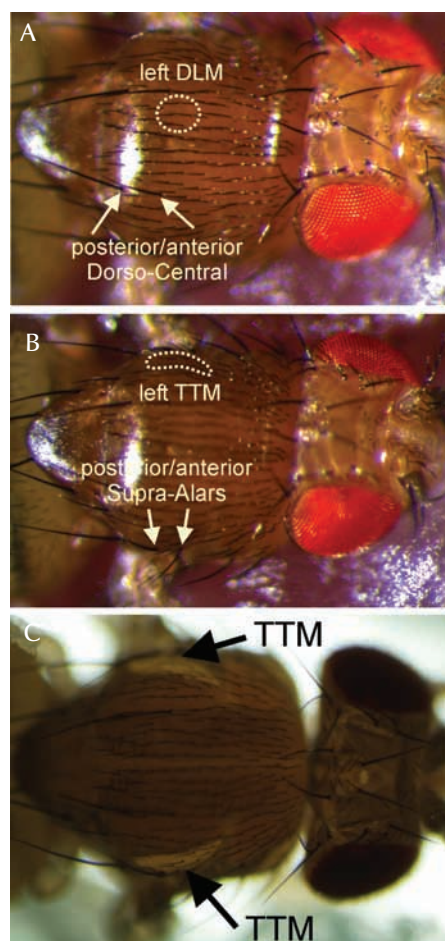
FIGURE 3. Localization of the TTM and DLM using bristles and illumination. (*A*) There are six pairs of indirect flight muscles, but only the dorsal longitudinal muscle pair 45a (also called the dorsal median muscle or muscle number 6) are innervated by the DLM motor neurons that receive input via the PSI from the contralateral GF (Demerec 1994). The attachment site of the DLM 45a muscles are under the cuticle between the anterior dorsocentral setae (yellow arrows) and the midline of the animal (yellow circle indicates site of left DLM). (*B*) The TTM is underneath the cuticle, just dorsal of the anterior and posterior supra-alar setae (yellow arrows) as indicated by the area circled by a dotted line (Demerec 1994). (*C*) As the TTM fibers are running along the dorsoventral axis they can be nicely visualized when a light source is placed underneath the fly (black arrows).

5. Place the ground electrode into the posterior end of the abdomen (Fig. 2C).

6. Place the stimulating electrodes through the eyes into the brain (Fig. 2C).

   The brain sits at the back of the head capsule, but electrodes pushed in too far may traverse the head capsule and enter the thorax where they may stimulate the ventral nerve cord directly.

7. Give single pulses of 30–60 V for 0.03 msec and check for successful activation of the GFS by looking for movement of the wings and/or TTM muscle on stimulation.

   See Troubleshooting.

8. Place the GFS saline (or 3 M KCl)-filled glass electrode for intracellular recordings into the left (or right) DLM muscle fiber 45a, which is immediately below the cuticle (see Figs. 2C and 3A).

   See Troubleshooting.

Stimulation and Recording

9. Give single stimuli as in Step 7 and modulate the stimulus strength by varying the voltage to determine the threshold for eliciting a response.

   The response of a good DLM recording is ~50–70 mV and has a latency of ~1.2–1.4 msec (Fig. 2D). Set the voltage 5–10 V above the determined threshold for the remainder of the experiment.

   See Troubleshooting.

10. Place the second intracellular recording electrode in the right (or left) TTM muscle on the contralateral side with respect to the recording electrode for the DLM (see Figs. 2C and 3B).

The TTM muscle fibers are much smaller than the DLM muscle fibers and hence it is more difficult to obtain and maintain a good recording. The response of a good recording from the TTM is ~30–50 mV and has a latency of ~0.8 msec (Fig. 2D). Protocols can be programmed in software such as pCLAMP to capture 10-msec sweeps to collect data.

See Troubleshooting.

11. Once good recordings have been obtained from the TTM and DLM, give 10 single stimuli with an interval of ~5 sec between the stimuli and determine the average response latency for both GF outputs.

    For this step a separate software protocol that captures 120-msec sweeps can be used to collect the data.

12. Finally, determine the following frequency by giving 10 trains of 10 stimuli at 100 Hz, with an intermittence of ~2 sec between the trains. Calculate the percentage of the total responses. Perform the same assay for trains of stimuli given at 200 Hz and 300 Hz.

13. Compare the TTM and DLM response latencies as well as the following frequencies at 100, 200, and 300 Hz between wild-type and mutant flies.

TROUBLESHOOTING

Problem (Steps 8 and 10): The recording electrodes are sliding on the cuticle and are unable to pierce it to impale the correct muscle.

Solution: The more perpendicular the electrode is to the cuticle, the easier it is for the electrode to get through the cuticle. Possible changes to make the electrode more perpendicular to the cuticle are to move the electrode to a slightly different location within the target area, change the angle of the micromanipulator, try for the muscle on the contralateral side, or remount the fly in a differently angled position.

Problem (Steps 8 and 10): The recording electrodes are indenting the cuticle or the recording electrodes are bending without piercing the cuticle.

Solution: Confirm that the electrode is not broken and has the appropriate shape. The tip of your electrode should have the approximate shape and size similar to the posterior supra-alar setae (Fig. 3B). If the electrode is not broken and has the appropriate shape, try gently tapping on the back of the forward-moving knob of the micromanipulator (once there is slight indentation) to encourage penetration through the cuticle.

Problem (Steps 7, 9, and 10): There is no stimulation artifact and no response.

Solution: Check whether all equipment is turned on. Double-check whether the fly is responding on stimulation (Step 7). If it does not, there is something wrong with your stimulation (check stimulation electrodes, ground and stimulator settings, etc.). If the fly does respond, then there is something wrong with your recording (check recording electrodes and amplifier settings).

Problem: The muscle response has an unusual shape with multiple peaks.

Solution: The microelectrode is not recording from a single muscle cell. This can occur in recordings from either muscle but is more common in TTM recordings, because this muscle is composed of many small fibers and maintaining the position of the electrode after several muscle contractions is problematic. An unusual shaped or multipeaked response trace does not affect the data, because response latencies and followings will still be preserved.

Problem: There is a very large stimulation artifact obscuring the muscle response and/or recordings of multiple stimuli are drifting on the recording monitor.

222 / *Chapter 13*

Solution: Consider the following.
1. Confirm that the ground electrode is properly in the fly.
2. Double-check the voltage and duration of the stimuli given.
3. Also, when the hemolymph dries up around the ground wire it results in a loss of conductance. This can be prevented and restored with a small drop of GFS saline on the fly where the ground electrode enters the abdomen.

Problem: There are long latencies or no responses in wild-type flies.
Solution: Consider the following.
1. Double-check whether the electrode is in the correct target area for the appropriate muscle.
2. The electrode might have pierced through the correct muscle. Both muscles are just underneath the cuticle. The cuticle is approximately no thicker than two to three times the thickness of a posterior supra-alar setae at it thickest visible point (Fig. 3B).
3. Stimulation is below threshold. Try increasing the voltage (duration).
4. If CO_2 was used to anesthetize the fly, either leave the fly to recover from CO_2 longer before testing or anesthetize flies using ice.
5. The wild-type fly may be a mutant.

Problem: Very short latencies are obtained for both TTM (<0.7 msec) and DLM (<1 msec).
Solution: This occurs if the ventral nerve cord, and thus the TTMn and DLMn motorneurons, are being activated directly. Check the position of the stimulating electrodes and replace them in the brain if necessary.

DISCUSSION

In wild-type flies average response latencies to a single stimulus are in the range 0.8 msec ± 0.1 msec for the GF-TTM pathway and 1.4 msec ± 0.3 msec for the GF-DLM pathway, depending on genotype and genetic background. Similarly, with respect to following frequencies the GF-TTM path is able to follow 10 stimuli 1:1 up to 300 Hz and the GF-DLM pathway is able to follow 10 stimuli up to 100 Hz, but variability between individual flies of different genotypes and genetic background has been observed. Hence, it is important to choose carefully the appropriate control flies when analyzing the electrophysiological phenotypes of mutants or targeted disruptions in the GFS. Two classic mutants that do affect the function of the GFS dramatically are *shakB*[2] and *bendless* (Thomas and Wyman 1984; Blagburn et al. 1999; Allen and Murphey 2007; Phelan et al. 2008; Uthaman et al. 2008). In *shakB*[2] flies, the GF–TTMn synapse lacks the gap junctions, but the chemical component is still present. The average response latency for the TTM in these flies is consistently increased to an average of 1.5 msec and it is not able to follow stimuli given at either 100, 200, or 300 Hz because of the weak labile nature of the resultant GF–TTMn synapse. In addition, no responses are obtained from the DLM when the GF is stimulated in the brain. Proof that the lack of responses is not due to a defect at the NMJ comes from the ability to record responses from the DLM muscle when the motorneurons are stimulated directly by stimulation electrodes placed in the thorax (Thomas and Wyman 1984).

In contrast, in *bendless* flies the GF-DLM pathway remains unaffected when compared to wild-type control flies. However, the GF-TTM connection is consistently increased to an average of >2 msec and is not able to following stimuli given at either 100, 200, or 300 Hz.

The reason that these indirect electrophysiological tests of these central synapses of the GFS are successful is that the NMJs at both TTM and the DLMs are large and extensive with many synaptic boutons. They rarely fail; the motorneurons can be stimulated directly at frequencies up to 500 Hz and the muscles will still show 1:1 responses to stimuli (MJ Allen and TA Godenschwege, unpubl.). Thus any effects seen on transmission through the pathways from the GF can be attributed to cen-

tral synaptic defects. If defects are seen when testing it is always prudent to stimulate the motoneurons directly to confirm that the NMJs are functioning correctly in at least a few flies of the same genotype, because some mutants do affect the adult NMJ (Huang et al. 2006).

RECIPE

CAUTION: See Appendix for proper handling of materials marked with <!>.
Recipes for reagents marked with <R> are included in this list.

GFS Saline

NaCl	101 mM
CaCl$_2$ <!>	1 mM
MgCl$_2$ <!>	4 mM
KCl <!>	3 mM
Glucose	5 mM
NaH$_2$PO$_4$	1.25 mM
NaHCO$_3$	20.7 mM

Adjust the pH to 7.2. From Gu and O'Dowd 2006.

ACKNOWLEDGMENTS

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Index

Page references followed by f denote figures; those followed by t denote tables.

526 / *Index*

530 / Index

532 / Index

534 / Index