

Chapter 2

Alternatives to Mammalian Pain Models 2: Using *Drosophila* to Identify Novel Genes Involved in Nociception

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Abstract

Identification of the molecules involved in nociception is fundamental to our understanding of pain. *Drosophila*, with its short generation time, powerful genetics and capacity for rapid, genome-wide mutagenesis, represents an ideal invertebrate model organism to dissect nociception. The fly has already been used to identify factors that are involved in other sensory systems such as vision, chemosensation, and audition. Thus, the tiny fruit fly is a viable alternative to mammalian model organisms. Here we present a brief primer on techniques used in screening for thermal and/or mechanical nociception mutants using *Drosophila*.

Key words: *Drosophila*, Larva, Multidendritic neuron, Thermal nociception assay, Mechanical nociception assay, Screen, Behavior

1. Introduction

An organism's ability to sense and respond to the sensory cues from the surrounding environment is essential for its survival. In particular, there has been significant research aimed at unveiling the molecular players that contribute to the detection of noxious stimuli and the development and maintenance of the pain-sensing neurons collectively called the nociceptors.

Drosophila melanogaster is emerging as a promising invertebrate model organism for the study of nociception. The highly branched *Drosophila* nociceptive MULTIDENDRITIC (md) neurons tiling the larval body wall are functionally and morphologically

analogous to the vertebrate nociceptive neurons whose branched, naked nerve endings likewise tile the skin (1). Although the evolutionary distance between flies and humans is vast, there is already some compelling evidence that suggests that the nociceptive molecular machinery is, at least in part, conserved among invertebrates and vertebrates (2, 3). The implication of these molecular players in nociception has laid the early groundwork for our understanding of the machinery at work in this specialized and essential sensory system. There are numerous other as yet unidentified molecules, both in vertebrates and invertebrates, that contribute to the normal function of the nociceptive neurons and *Drosophila* is emerging as a premiere system to uncover these players.

Two behavioral assays have been developed to probe nociceptive function in *Drosophila* larvae (1). These animals exhibit a robust nocifensive escape behavior in response to noxious mechanical or thermal challenges within physiologically and biologically relevant ranges. During escape locomotion, the larvae rotate around the long body axis in a corkscrew-like fashion. In contrast, normal locomotion of the undisturbed larvae involves rostral to caudal waves of muscle contraction. The nocifensive behavioral output is highly stereotyped and genetically encoded and can therefore be used as a measure of normal nociceptive neuron function. This, coupled with the fact that *Drosophila* possesses a simplified nervous system that is readily amenable to sophisticated, large-scale, high-throughput genetic dissection, makes it possible to rapidly determine the molecules responsible for conferring mechanosensory and thermosensory function to the nociceptors. With respect to this final point, we will briefly describe the major techniques to generate mutants in *Drosophila* that can be subsequently tested in the thermal and/or mechanical nociception assays (described below).

This mutagenesis primer is in no way exhaustive, but does highlight the most commonly used techniques that fall under three main categories: irradiation, chemical, and genetic mutagenesis. The specific details of these techniques are not included, as they would be far beyond the scope of this work, but a brief summary of the technique as well as the original citation(s) are included.

Irradiation. Flies can be irradiated with either γ - or X-rays to produce lesions in the genome (4). This technique has somewhat fallen out of favor since the resulting chromosomal aberrations can be quite large and complex. Nevertheless, large rearrangements or deletions can be cytologically mapped with the larval salivary gland polytene chromosome preparation (4) and further refined with Southern Blot. In the post-genome era, homozygous viable deletions can also be mapped using genomic tiling arrays.

Chemical Mutagenesis. Ethylmethanesulfonate (EMS) mutagenesis is well-established and routine form of chemical mutagenesis that is extraordinarily convenient because it simply involves feeding a standard dose of EMS to male flies and following a proscribed mating scheme (5). EMS is a powerful mutagen that causes G/C-to-A/T transition point mutations at a very high rate (75–100%) but other mutations, such as deletions, are possible although at a vastly low rate (4, 6–8). Similar to EMS mutagenesis, ethylnitrosurea (ENU) mutagenesis can likewise be used to generate mutants. ENU typically causes A-to-T transversion and A/T-to-G/C transition point mutations, but it is far less effective than EMS (4). The trade-off for efficient generation of mutants and subsequent screening for your phenotype of interest is the time-consuming task of mapping the mutation. Rough genetic mapping is typically performed with the *Drosophila* Deficiency Kit (<http://flystocks.bio.indiana.edu/Browse/df-dp/dfkit-info.htm>) and once the region is narrowed, finer mapping can be carried out with smaller deficiency strains available from the Bloomington Stock Center Kit, Exelixis Deficiency Kit or the DrosDel Kit (9–12) and, ultimately, PCR sequencing the gene or denaturing HPLC (8) on mutagenized flies must be used to identify the specific mutation. Finally, as the cost and speed of sequencing entire genomes has substantially improved, it is now possible that mutant strains can be fully sequenced to identify the causative mutation (13).

Genetic Mutagenesis. Transposable Elements (TEs), also known as mobile genetic elements, are perhaps a *Drosophilist's* most important genetic tool. There are literally thousands upon thousands of fly strains that harbor individual TEs – such as P-elements, piggyBac elements, and Minos elements (12, 14, 15), to name a few – that have been inserted randomly into the genome to disrupt many of the ~14,000 predicted *Drosophila* genes (16). Several groups have initiated large-scale projects (12, 17–22) to randomly insert different TEs into the *Drosophila* genome and altogether these transposable element strains are estimated to disrupt roughly 65% of the genes in the fly genome; there are also private collections that likely increase the coverage to a small degree.

These thousands of strains are publicly available and potentially mutate novel pain genes. Indeed, a screen of one such collection of 1,500 randomly inserted EP (20) P-element strains for thermal nociception defects was used to isolate the nociception gene *painless* (1). The advantage of transposable element mutagenesis is that the precise insertion site of a given TE has been pre-determined and therefore the gene underlying the phenotype is known. If the insertion site of a given TE is unknown, it can easily be determined, by inverse PCR using primers directed against the P-element sequence.

While these random TE insertions cause a loss-of-function phenotype, a clever design feature of the EP type P-element can be used to reveal a gain-of-function phenotype. In other words, with a routine genetic tool (the Gal4/UAS system, see Brand and Perrimon 1993 for details), the gene in which the TE is inserted can be over-expressed in a tissue-, cell- or stage-specific manner (18, 20, 23, 24).

If a given TE strain that is inserted into a gene has a mild nociception phenotype, it is likely that the insertion is only a hypomorphic allele. The advantage of P-elements, however, is that they can be readily mobilized by introduction of a transposase enzyme (25, 26) or, as discussed above, irradiation, to produce flanking deletions into a gene of interest (27, 28). These deletions vary in size, but it is possible to precisely define the breakpoints caused by the lesion using a combination of Southern Blot and PCR. Even though the frequency of flanking deletions generated by transposase-mediated imprecise excision from a P-element is fairly low, this is still the most efficient and commonly used technique to generate stronger or null alleles of a given gene; a few similar but less well known techniques (*cis* or *trans* hybrid-element insertion and hobo deletion-generator) have also been developed (11, 29, 30). With P-element mobilization, many excision events will be precise, and these events often revert to the original phenotype lending further support to the idea that the gene where the P-element is inserted is indeed responsible for the defect. Interestingly, the piggyBac TEs do not generate flanking deletions when mobilized (only precise excisions), but these elements are engineered with so-called FRT sites that allow for the rapid generation of precise custom-made deletions at or around a gene of interest that can easily be confirmed by PCR (9, 10, 12).

There are several other TE constructs that were used to create new collections and have features that can likewise uncover novel gene functions including: (1) gene disruption by insertion of GT1 (19, 31) or SUPor-P P-elements (these can also be used to make imprecise excisions) and (2) so-called “Gal4 enhancer trapping” (17, 23, 32, 33). In enhancer trap strains, candidate genes are isolated based on their expression in a tissue of interest.

The techniques discussed above are useful for performing unbiased screens for candidate genes, however, there are also genetic tools available for disrupting a specific gene. It is likely that a gene of interest – perhaps one that is a fly homologue of a human gene implicated in pain states – will have TEs inserted in or near it. This can be determined by searching FlyBase (<http://www.flybase.org>), and, if TEs are available, though a number of the public Stock Centers (http://flybase.org/static_pages/allied-data/stock_collections.html), the genetic mutagenesis techniques outlined above can be performed in those cases. Recall, however, that ~35% of the genes in the fly genome do not

have TE alleles, and in those cases a few additional approaches (discussed below) can be taken to generate mutants.

RNA interference (RNAi) is another powerful tool that can be used to knock down genes in *Drosophila*. In brief, gene regulation in vivo by RNAi involves the production of double stranded RNAs (dsRNAs) that are processed into small interfering RNAs (siRNAs) by the Dicer ribonuclease, and these siRNAs in turn guide degradation of their complementary mRNA through Argonaute, which is a catalytic component of RNA-Induced Signaling Complex. Initially, dsRNAs were generated and micro-injected into *Drosophila* embryos, but this technique is labor intensive and invasive, dosage is difficult to control, and the injected dsRNAs may not interfere with later development. In order to circumvent these pitfalls, inverted repeats – used to create dsRNAs – have been cloned into P-element vectors for nearly 90% of the *Drosophila* genes ((34) and <http://flyrnai.org/>). These dsRNA-containing P-element vectors were stably incorporated into individual fly strains (~22,000; some are duplicates for the same gene) and made publically available (<http://www.vdrc.at>). These P-elements have been designed to drive expression of a given dsRNA under strict tissue-, cell-, and stage-specific control, as alluded to above (23, 24, 34). We have used a subset of these RNAi strains to systematically knock out/knock down the various *Drosophila* ion channel subunits specifically in the larval md neurons and have screened this collection for defects in thermal and mechanical nociception. Potential disadvantage in this type of screen is that false-positives may occur due to promiscuous off-targeting of the processed siRNA and false-negatives may occur due to incomplete knock-down of a given gene. Nevertheless, the ease of screening with RNAi is a strong advantage to traditional screen and verification of RNAi phenotypes by creation of genetic mutants is relatively straightforward.

Homologous recombination has been used routinely in eukaryotic organisms to target and replace a wild type copy of a gene with a mutant version created in the lab or to simply delete the gene altogether. Until recently, gene targeting was not possible in *Drosophila* but two techniques, ends-in and ends-out (replacement) gene targeting, have been developed (35–39).

2. Materials

2.1. General

1. Distilled water.
2. 60 × 15 mm Polystyrene Petri Dish.
3. DVD Handycam Camcorder with DVD-R Recordable Media (Sony, Tokyo, Japan).

4. MZ6 Stereomicroscope (Leica, Wetzlar, Germany).
5. MM99 Adaptor S/N: 1658 (Martin Microscope, Easley, SC).
6. Ace Light Source with Dual Gooseneck Fiber Optic Light Guide (Schott, Elmsford, NY).

2.2. Thermal Nociception Assay

1. Variable Autotransformer/Digital Voltmeter (Variac, Cleveland, OH) 120 VAC, Single Phase Input, 0-120 VAC Output, 12A.
2. BAT-12 Thermocouple (Physitemp, Clifton, NJ).
3. MLT1402 T-type Ultra Fast Thermocouple Probe (IT-23).
4. Soldering iron with a copper tip shaped into a chisel 0.6 mm wide outfitted with the thermocouple lead wire under the copper tip.

2.3. Mechanical Nociception Assay

1. Glass pipettes.
2. Nylon Monofilament fishing line (Shakespeare Omniflex 6 lb test, diameter 0.009 inch [0.23 mm]).
3. Weighing scale.

3. Methods

Once a station to test nociceptive function in larvae has been built, and after some practice, the behavioral assays (1) are straightforward, rapid, and reproducible. It is important to empirically calibrate your thermal nociception probe such that 80–90% of control animals respond in one second or less.

3.1. Fly Husbandry

1. Flies are maintained on typical *Drosophila* media at 25°C with 70% humidity and 12:12 Light:Dark Cycle.

3.2. Thermal Nociception Assay

1. Six virgin females are mated to three males, and the adults are transferred into new food vials on the fifth day.
2. Third instar wandering larvae (those which are no longer in the food but are actively crawling on the walls of the vial) are gently washed from the food vials into Petri dishes with distilled water on the fifth day (see Note 1).
3. Larvae are tested at room temperature (21–23°C) in Petri dishes containing water shallow enough to allow the ventral cuticle of animals to make contact with the dish (see Note 2).
4. The soldering iron probe is heated to the desired temperature by adjusting the voltage on the Variac and is monitored on the digital readout on the thermocouple (see Note 3).

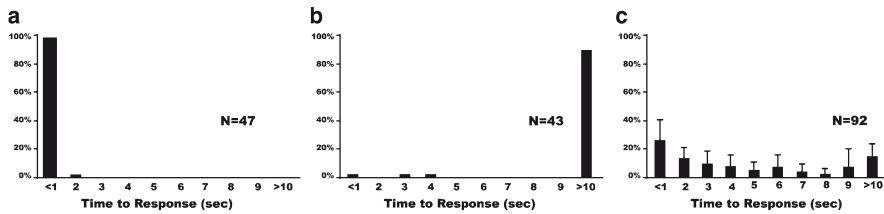


Fig. 1. In control animals (a), nearly 100% of the larvae initiate nocifensive rolling in response to a 46°C probe. In contrast, strains where the multidendritic nociceptive neurons are genetically silenced with tetanus toxin (b, md-Gal4; UAS-TNT-E), most of these animals do not respond to the thermal stimulus. Similarly, *painless* (1) mutants (c) exhibit significantly different average rolling latencies compared to controls at 46°C. Reprinted from reference (1)

5. With the video camera running (see Note 4), the heated probe is gently placed against the lateral surface of abdominal segments 4, 5, or 6 until the animal completes a 360° roll along the dorsal–ventral axis (see Note 5).
6. Videos are played back and the latency to roll in response to the probe is calculated with a digital stopwatch <http://tools.arantius.com/stopwatchanalyzed> (see Note 6). Response latencies are separated into 11 bins (<1 s, <2, 3, 4...10 s, >10 s).
7. Data are graphed as percentage of larvae that fall into each bin (Fig. 1) and are analyzed by comparing average latency of wild type controls and a given experimental genotype by *T*-test.

3.3. Mechanical Nociception Assay

1. Crosses and handling of larvae are performed as described above for the thermal nociception assay. Furthermore, the larva selected for testing should be relatively stationary and in an aqueous environment as described above.
2. The noxious mechanical stimulus is delivered to the dorsal midline of a wandering third instar larva. To deliver the mechanical stimulus, a 50 mN Von Frey Fiber (see Note 7) is used to rapidly jab the larva midway along the anterior posterior axis (see Note 8). The fiber is held perpendicular to the larva and rapidly depressed downward (effectively squashing the larvae against the surface of the Petri dish).
3. By varying the length of the Von Frey Fiber, it is possible to deliver forces of greater or lesser intensity and to generate a response curve (see Note 9).
4. Data are graphed as percent of larvae that initiate rolling in response to a particular intensity of mechanical stimulation. The data are analyzed by *T*-test comparing wild type controls to a given experimental genotype.

4. Notes

1. The stage of the larvae is important; they should be opaque wandering third instar (108–120 h after egg laying).
2. It is important to perform the behavioral assay in an aqueous environment (the larvae must be wet but not floating). For reasons that are not fully understood, escape locomotion is not readily elicited if the larvae are dry. One possible explanation may be that the selective advantage of rotational escape locomotion is most significant when the larvae are buried in the food (and wet).
3. Monitoring the temperature of the probe is very critical. The BAT-12 thermocouple from Physitemp is outfitted with an IT-23 thermocouple probe to the tip of the soldering iron (the thermocouple is attached using a small drop of solder). The temperature at the tip of the soldering iron is lower than the temperature on the shaft, so measuring temperature as close to the tip of the soldering iron as possible is very important. It is also important to “rest” the probe for a few seconds to let it reheat between animals as the temperature of the probe dips slightly when the tip is placed in the aqueous environment.
4. The *Drosophila* behavioral assay for thermal nociception is a population measurement. At 46°C, the probe is close to the threshold temperature for eliciting the behavior, therefore a distribution of response latency will be evident. To obtain a precise population measurement of latency, the behavior must be video recorded and then documented off-line.
5. It is also important to stimulate animals when they are relatively stationary (with mouth hooks moving as if feeding). If the animals are moving rapidly with peristalsis, they are reluctant to initiate escape locomotion in response to noxious heat. The stimulation should be applied near the middle of the anterior/posterior axis in abdominal segment 4, 5 or 6. The larvae appear to have behavioral options that depend upon the somatotopic location of the noxious heat stimulus. Stimulation near posterior does not robustly elicit escape locomotion in wild type since they can escape by moving forward. In addition, if the stimulation is applied too close to the head, the larvae have a tendency to turn away with their head to escape rather than rotate.
6. Stopwatch outputs of the response times of individual larvae are recorded in a spreadsheet format. Off-line analysis is performed by an observer and is the most time-consuming aspect of the assay.

7. Von Frey fibers can be homemade by attaching nylon monofilament to glass pipettes that have been bent in a Bunsen Burner to form a right angle. To make a 50 mN probe, the monofilament fishing line (Shakespeare Omniflex 6 lb test, diameter 0.009 inch [0.23 mm]) is first cut to make a short piece of 18 mm in length. The 18 mm long filament is then glued to the bent glass pipette such that 8 mm of the fiber protrudes from the end, and 10 mm anchors the fiber to the pipette. The exact force delivered by an individual fiber will vary slightly among fibers as the fiber becomes more flexible with prolonged use. To precisely measure the maximum force delivered by a particular Von Frey fiber, the fibers are used to depress a balance until the fishing line begins to bend. The force measured on the balance just prior to bending represents the maximum force exerted. The force in grams is converted to milliNewtons through multiplication by a factor of 9.8 (i.e., Gravity). For example, a fiber that generates a force of 1 g delivers a maximum force of 9.8 mN.
8. The stimulus should be delivered as instantaneously as possible, so that the larva is immediately released from it. Following such a stimulus, the wild type larvae will be observed to briefly pause movement (for approximately one second), and the nocifensive escape behavior will initiate after this pause. We typically observe nocifensive behavior in 75% of trials using a 50 mN Von Frey fiber in wild type strains. In contrast, strains that harbor mutations that interfere with mechanical nociception show infrequent escape behavior following the 50 mN stimulus.
9. Although the *painless* mutant, for example, shows significantly reduced nociception responses to a 50 mN stimulus, nocifensive responses can still be elicited by 100 mN of force. The latter result suggests that this mutant is not defective in the motor output.

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