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Drosophotoxicology: the growing potential for Drosophila in neurotoxicology

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Abstract

Understanding neurotoxic mechanisms is a challenge of deciphering which genes and gene products in the developing or mature nervous system are targeted for disruption by chemicals we encounter in our environment. Our understanding of nervous system development and physiology is highly advanced due in large part to studies conducted in simple non-mammalian models. The paucity of toxicological data for the more than 80,000 chemicals in commercial use today, and the approximately 2,000 new chemicals introduced each year, make development of sensitive and rapid assays to screen for neurotoxicity paramount. In this article I advocate the use of *Drosophila* in the modern regimen of toxicological testing, emphasizing its unique attributes for assaying neurodevelopment and behavior. Features of the *Drosophila* model are reviewed and a generalized overall scheme for its use in toxicology is presented. Examples of where the fly has proven fruitful in evaluating common toxicants in our environment are also highlighted. Attention is drawn to three areas where development and application of the fly model might benefit toxicology the most: 1) optimizing sensitive endpoints for pathway-specific screening, 2) accommodating high throughput demands for analysis of chemical toxicant libraries, 3) optimizing genetic and molecular protocols for more rapid identification toxicant-by-gene interactions. While there are shortcomings in the *Drosophila* model, which exclude it from effective toxicological testing in certain arenas, conservation of fundamental cellular and developmental mechanisms between flies and man are extensive enough to warrant a central role for the *Drosophila* model in toxicological testing of today.

Introduction

A principal goal in neurotoxicology research is to identify and characterize discrete mechanisms by which a given chemical or mixture induces detrimental effects on formation and/or function of the nervous system. Advancing this goal is integral to risk assessment for toxicants to which people are exposed. There are more than 80,000 chemicals in commercial use today, and the approximately 2,000 new chemicals introduced each year for which there is little to no toxicological data (<http://ntp.niehs.nih.gov/>). Furthermore, we continue to come in contact with persistent environmental toxicants, such as mercury, lead and arsenic, with insufficient knowledge of their mechanism of action, particularly in the context of the

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developing fetus. Understanding toxic mechanisms relies on identifying genes and gene products (e.g. genomic DNA, mRNA transcripts and/or proteins) that are targeted for disruption by the xenobiotics we are likely to encounter in our world. Equally important is characterization of genes, and their products, that act in defense toward individual or classes of toxic compounds. Developing informative, sensitive and rapid assays to screen for neurotoxicity has therefore become a priority.

Fortunately, our understanding of nervous system development and physiology is highly advanced, thanks in large part to decades of genetic, molecular and behavioral studies conducted in simple non-mammalian models. The roundworm (*Caenorhabditis elegans*), the fruit fly (*Drosophila melanogaster*) and the zebrafish (*Danio rerio*) are the predominant alternative models that have been perpetuated through the genomic and post-genomic revolution of the last decade. Each of these models has its distinct advantages with respect to generation time, laboratory expenses, genetic manipulability, efficiency of screening methods and conservation with higher organisms. In this article I advocate the use of *Drosophila* in the modern regimen of toxicological testing, emphasizing its unique attributes for assaying neurodevelopment and behavior. Genetic manipulability and ease of detecting phenotypes made *Drosophila* the model of choice for mutagenesis screens of the 1980's and 90's. These same features make *Drosophila* ideal for toxicological screens. Indeed, flies have been, and continue to be, used routinely in mutagenicity tests. Recent investigations have propagated a number of powerful assay methods with *Drosophila* in developmental and behavioral toxicology.

In this article I present a brief summary of the features of the *Drosophila* model and a generalized overall scheme for its use in toxicology studies. As well, I allude to examples from the literature where the fly has proven fruitful in evaluating common toxicants in our environment. Finally, I summarize three areas where development and application of the fly model might most benefit toxicology. This review does not attempt to be a comprehensive survey of *Drosophila* in toxicology. For instance, *Drosophila* have proven highly effective in modeling a number human neurodegenerative diseases and have found a niche in drug discovery, which is reviewed in depth elsewhere [11,64,74,76,86]. Yet, the rationale for using flies in these other arenas holds true for its application to conventional toxicology. Furthermore, incorporating fly-based assays into toxicological testing is a direct answer to the call for a paradigm shift in testing proposed jointly by the EPA, National Toxicology Program (NTP) and National Institute of Health Chemical Genomics Center (NCGC) [23]. Moreover, recommendations put forth by the National Research Council Committee on Toxicology proclaim *Drosophila*, along with *C. elegans* and zebrafish, as models of choice to advance the science behind risk assessment of developmental toxicants [75].

The relatively unique life cycle, form, function and experimental manipulations of the fly, with respect to worms and fish, encompass an area of research for which I suggest the name "Drosophotoxicology".

Biology of the fly

Flies are cheap and easy to maintain. They are propagated in small (20–50mL) vials or bottles on a simple solid food medium of cornmeal, molasses, yeast and agar (see Fig. 1C). Under standard culture conditions at 25°C generation time is 12–14 days from egg to adult. The life cycle of the fly is comprised of four distinct stages: embryo, larva, pupa and adult. Each of these stages present unique opportunities to assess susceptibility of the nervous system to xenobiotics.

The embryo develops in a period of approximately 24 hours at 25°C. During this time neurogenesis and differentiation within discrete regions of the ventral neurectoderm give rise

to a fully functioning nervous system capable of executing the motor and sensory behaviors of the larvae including foraging, chemo- and phototaxis. In the ventral nerve cord the position and timing of birth of each of the 30 individual neuroblasts (NBs) within each hemisegment has been mapped in great detail [13,31,89]. In addition, the architecture of the neurons and glia that are propagated from each of these NB lineages has been resolved ([89] and <http://www.neuro.uoregon.edu/doelab/lineages/overview/grasshopper-entres.html>). Neurons and glia of the developing PNS have similarly been mapped with great detail [77], thus presenting an exceptional template for evaluating fundamental defects in neurogenesis and neuronal and glial morphology.

The larval stage progresses over four days and is characterized by a period of growth punctuated by two molts and results in a 10-fold increase in body size. While neural cell numbers in the CNS also increase 10-fold in larval stages, due to a secondary wave of NB proliferation, neurons and glia remain relatively undifferentiated [97]. The following 5–6 days of pupal metamorphosis is a dramatic period of tissue reorganization, culminating in the fusing together of the adult structures from the precursor imaginal disc tissues. Larval-derived CNS and PNS neurons undergo dramatic pruning and re-growth while newly born adult neurons migrate to their final position and elaborate their processes to succinct targets [98,99].

The newly hatched adult fly will rapidly acquire characteristic behaviors of flight, chemo-, photo- and geotaxis, foraging and mating. The adult CNS consists of large bilateral optic lobes, a central brain consisting of supra- and subesophageal ganglia, and a large cervical connective nerve that attaches to the ventral nerve cord with three enlarged thoracic neuromeres [98]. In many cases the explicit circuits controlling visual [96], olfactory [45], mechanosensory [54] and chemosensory [95] inputs from the peripheral organs (eye, antennae, bristle organs and maxillary palps) have been mapped both physically and functionally. In addition, the central Mushroom Body of the brain has been elucidated as a center for memory and conditioned behaviors [26]. The sum of these well-documented developmental and behavioral aspects of *Drosophila* make it an especially informative and adaptable model to investigate a wide variety of toxicological endpoints relevant to human biology and behavior.

Features of the fly model

Drosophila has been regarded primarily as a model for the study of genetics. Indeed, some of the first principles of genetics, such as the chromosome theory of heredity and the mutagenicity of X-rays were elaborated in the fly (summarized in [85]). The large polytene chromosomes isolated from the salivary glands served as a template for physical mapping of genes that were revealed through banding patterns and eventually via hybridization methods. Mutagenesis screens, that exploited the easily interpretable endpoints of lethality, or disrupted embryo, wing or eye morphology, rapidly advanced the knowledge of gene function. In addition, these mutants propagated the unique, sometimes humorous, nomenclature for their respective phenotypes, such as Hedgehog, Wingless and Notch, which have subsequently become synonymous with the underlying signaling pathway.

The relatively simple genetic make up of the fly consists of four chromosomes encoding approximately 13,600 genes, roughly half the number in humans [2]. More than 95% of its genetic content is on three of its four chromosomes [the sex (first or I) chromosome and two autosomes (II and III)], which has made conventional genetic analyses straightforward. Early fly genetic studies were aided by the ease of distinguishing phenotypes in the wing or eye, which are non-essential appendages for viability. Now, the finest cellular details within developing tissues of the embryo, larval and pupal stages are accessible for routine phenotypic analyses due to advancements in immunohistochemistry and expression of vital fluorescent dyes (e.g. GFP) and improved microscopy techniques (e.g. confocal and multiphoton

microscopy). Ironically, it is the highly toxic compound ethyl methanesulphonate (EMS) that was used as the mutagen in a number of the classic mutagenic screens in *Drosophila* that led to identification of fundamental genes in developmental processes (e.g., the Hox genes that organize the body plan and the Slit/Robo signaling partners that direct axonal outgrowth and targeting [46,91]). The emergence of molecular cloning and recombinant DNA technology led to sequence identification of genes and ultimately opened the door to functional analyses of transgenes in vivo.

The tools of the *Drosophila* trade are as comprehensive for genomic, proteomic, bioinformatic and functional molecular studies as any model organism currently in use. Important online starting points are the Virtual Library: *Drosophila* (<http://ceolas.org/fly/>) and Flybase (<http://flybase.org/>). The Virtual Library: *Drosophila* contains a number of informative links to sites covering topics ranging from a basic introduction to *Drosophila* to specific neurobiological resources. Flybase (the database of *Drosophila* genes and genomes) contains annotations of all known genes including information on related mutant phenotypes, published and unpublished references, and links to reagent sources.

The method of creating transgenic flies through P-element transformation revolutionized molecular studies in *Drosophila* in the 1980's [24]. This method, a means for stably integrating foreign DNA into the chromosome, opened the door to manipulating and monitoring expression of endogenous or introduced genes over the course of development. Variations in this method have been used to create reporter gene constructs that are uniquely powerful for determining the activity of a given gene in response to a xenobiotic. For example, a premier tool in fly manipulations is the Gal4-UAS gene expression system [16,17]. This two-part system exploits heterologous DNA sequences from yeast that encode: 1) the Gal4 transcription factor and 2) the Upstream Activating Sequence (UAS) promoter to which Gal4 binds. In a typical application Gal4 coding sequence is linked to an endogenous fly promoter in one fly while the UAS sequence is linked to a downstream reporter gene (e.g. GFP) in a second fly. Combining these elements through mating the flies creates a strain that expresses GFP wherever the promoter of interest is turned on. This methodology has been pivotal, for example, in creating "humanized" versions of flies that express disease genes that invoke neuropathology mimicking human disease in the fly [11,64,74,76,86].

Drosophotoxicology: the overall scheme

In Figure 1 I summarize modes in which *Drosophila* can be used in toxicological assays. Components of the scheme are: 1) the variety of chemical toxicants to be investigated; 2) the mode of delivery to the organism; 3) the developmental stage of the nervous system; 4) the endpoints to be measured in determining biological/toxicological effects.

Compounds and mixtures for which toxicological information is needed are varied ranging from longstanding environmental contaminants to libraries of molecules derived from combinatorial chemistry from various industries including pharmaceuticals. In the case of known environmental contaminants, such as mercury, arsenic and lead, there is a need for more diverse assays to elucidate the specific mechanisms of their respective toxicities. On the other hand, the Molecular Libraries Program (MLP, <https://mli.nih.gov/mli/>) has compiled over 300,000 compounds for which it seeks high throughput assays to unveil unique biologically active reagents for the biomedical research community. Of particular importance is the ability to evaluate toxicity of the more than 2,000 new chemicals introduced each year that are added to the list of 80,000 chemicals registered with the National Toxicology Program (<http://ntp.niehs.nih.gov/>).

The mode of delivery is an important consideration for getting toxicants exposed to the cells or organ of interest in flies. Exposure to embryonic tissues can be achieved through maternal

feeding or through injection or in vitro incubation (Fig. 1A,B,D). Maternal feeding is effective for embryo exposures but requires determination of dosage empirically due to the unknown characteristics of metabolism and delivery. Embryo injection methods have been employed for decades for gene delivery and have recently been optimized for high throughput screening protocols suitable for small scale molecular library screens [105]. Chemical exposure by direct incubation of fly embryos has proven effective in several cases, yet has been limited by the technical difficulty of permeablizing the vitelline membrane in the shell (Fig. 1A). In some cases small molecules have been shown to make it into embryos with minimal manipulations giving distinct neural developmental outcomes [67,82]. Methods to enhance shell permeability should open the door to a variety of applications for embryos in toxicology assays. Larvae and adult flies are readily exposed to chemicals through dosages in the food medium (Fig. 1C, D). Injection methods have also been employed for compound delivery in larvae and adults [10, 28]. Adult flies are also easily exposed to vapors and aerosols in a controlled environment [79].

Mode of delivery must be considered with respect to the developmental stage of the nervous system and the anticipated activity of the toxicant(s). In the embryo, ontogeny of essentially every neuroblast, neuron and glia cell in the CNS and PNS has been carefully mapped out and is thus desirable for assaying effects on neurogenesis and neurite outgrowth (Fig. 1E, top panel). The larval nervous system contains several fully formed circuits with stereotypical synaptic profiles (e.g. neuromuscular junctions) that underlie characteristic behaviors such as foraging for food. The subsequent pupal stages invoke periods of neuronal remodeling and new patterns of synaptogenesis (Fig. 1E, middle panel). The fully formed adult nervous system has an elaborated network of inputs from the periphery that are well mapped physically and functionally (Fig. 1E bottom panel). This stage is best suited to assay effects on nerve physiology and behavioral traits of the whole organism. Toxic effects can be elicited by influencing earlier developmental events (Fig. 1J) or by acute affects upon the adult fly (Fig. 1K).

The requirements for high throughput screening (HTS) make choosing a toxicological endpoint a balance between the complexity of the anticipated outcome (e.g. molecular composition versus cellular or organ morphology versus behavioral phenotype) and the ease of detection and quantification. Disruption of neurogenesis and neuronal and glial patterning in the embryonic nervous system is a very powerful endpoint for screening (Fig. 1F, G). In this case microscopy is essential for detection, and thereby introduces technical challenges for adaptation to high throughput analyses. Yet, recent advancements in genetically encoded fluorescent reporters and automated imaging make this approach very attractive and amenable to HTS methods ([9,104] and see discussion below). Peripheral neurons in larvae and pupae are relatively accessible for imaging and exhibit many features common to mammalian structures, such as synaptic boutons at the neuromuscular junction (see Fig. 1H and [20]). Larvae also display quantifiable behaviors in motility, odor response and memory that can be used to determine fitness of nervous system function (Fig. 1I and [38,88]). These latter events are readily adaptable to higher throughput with image tracking systems akin to those commonly used in assays of *C. elegans* locomotion [35]. Eclosion (hatching of adults from the pupal stage) is perhaps the simplest lethality endpoint and can be done without the aid of a microscope. A wide variety of assays for adult flies have been devised to determine morphological defects and neurological fitness. The most common assays examine changes in adult structures such as the eye or wing (Fig. 1J). These are useful proxies for effects upon underlying molecular pathways, as discussed below. Common adult behavioral assays assess simple locomotive behaviors including overall activity, geotaxis (upward climbing), photo- or chemotaxis (see Fig. 1K and [8,37,59]). More complex behaviors such as courtship, circadian rhythm and even associative learning and memory are now understood to have a genetic component in *Drosophila* and are rapidly being established as phenotypes for screening strategies [94].

Examples of measurable endpoints in *Drosophila* toxicology

Lethality

Historically, *Drosophila* has been used in tests of genotoxicity. One of the earliest tests for genotoxicity is the sex-linked recessive lethal (SLRL) test. The SLRL has been employed for decades to test for the mutagenicity of compounds toward the X chromosome in male germline cells [93]. The test entails feeding parental (P1) males with the test compound and assaying for lethality of males of the second (F2) generation and thus requires two crosses and more than four weeks for a determination. The SLRL test was used in tests of over 300 compounds for the National Toxicology Program (NTP) over a ten-year period, and in the end demonstrated a high specificity but low sensitivity in identifying carcinogenic compounds [36]. For genotoxicity screening today the SLRL test has largely been substituted with more efficient and less time consuming in vitro cell assays, namely the *Salmonella* mutagenicity (or Ames) test, where positive results for a chemical in these assays are highly correlated with carcinogenicity in multiple species/sexes of rodents and at multiple tissue sites [7].

Lethality of adult flies subsequent to larval or adult chemical exposure has proven highly effective in analysis of heavy metals and, importantly, in screening for genetically encoded resistance traits [21,34,63]. More than 20 years ago Magnusson and Ramel [63] used *Drosophila* in analyses of heavy metal toxicity, notably with inorganic mercury and methylmercury (MeHg). Their studies showed that tolerance to MeHg, as assayed by adult hatching after larval exposure, has a genetic component that displays a polygenic, yet dominant trait. Furthermore, this tolerance trait could be artificially selected for in the laboratory [63]. While these studies were not pursued to the level of isolating the genes involved, a similar experimental approach taken by the Jacobson lab found unique dominant traits in cadmium and strontium tolerance that resided on different chromosomes [21,34]. Muniz Ortiz, et al. [73] have recently re-invoked this approach of using natural variation in tolerance and susceptibility to screen for mechanisms of arsenic toxicity. With methods developed since the Magnusson and Ramel studies, such as microsatellite recombination mapping, chromosomal deficiency lines and RNAi methodology, these investigators identified genes in the glutathione synthesis pathway that are functionally linked to arsenic tolerance [73].

The larval to adult transition in flies is complex and perhaps the least homologous developmental event with respect to mammals. As such, lethality assays of chemical exposure at this stage are not likely to return meaningful LD50 values relative to mice or man for a given agent. Nonetheless, adult lethality remains a powerful screening tool for tolerance or susceptibility to a given chemical or genetic stress and thus has many applications to toxicological investigations.

Adult morphology

Eye and wing phenotypes are a hallmark of *Drosophila* assays. For genotoxicity the somatic mutation and recombination test (SMART) continues to provide sensitive, rapid and inexpensive assays for mutagenic and recombinogenic properties of chemicals [40,84]. The SMART is based on the principle that a loss of heterozygosity through mutation or recombination of suitable recessive marker genes can lead to the formation of mutant clones of cells appearing as “spots” on the wings or eyes of the adult flies [40]. The test requires treatment of larvae with the candidate compound and subsequently examining spot frequency in the adult tissue. The SMART test is useful in investigation of both mutagenic and anti-mutagenic properties of compounds, the latter being conducted in combination with a proven mutagen and assaying for suppression of mutagenic activity [1,83]. It has been touted as a high throughput screening method by virtue that it is a single generation test requiring minimal handling of flies. The SMART test continues to be used extensively in assays of compounds

and mixtures ranging from polycyclic aromatic hydrocarbons (PAHs) to polluted river water [30]. Yet, the SMART assay has limitations with respect to the requirements of HTS needed for chemical libraries of today due to the materials and time needed to treat and culture larvae and subsequently determine adult wing spot frequency. Furthermore, the SMART assay is best suited for assaying chromosomal effects, specifically recombination, point mutation, large deletions and non-disjunction. Yet, the SMART assay has found an application in characterization of human drug metabolizing enzymes [53].

Direct assessment of teratogenic effects of chemicals in flies has been employed in a limited number of studies. The merit of this approach is the fact that a number of cell signaling mechanisms that direct neural development also act in a mechanistically conserved manner in morphogenesis of the wing and eye. Thus, an abnormal endpoint, such as wing notching, can indeed reveal an activity of the xenobiotic in question upon the Notch pathway. In an effort to develop a rapid, economical screening assay of developmental toxicants Lynch, et al. [60] described the effects of larval exposure to 32 chemicals from the NTP repository by monitoring induced malformations in adult flies. In a summarized test-system protocol two endpoints, wing notches and bent humeral bristles, were identified as useful for scoring. However, this approach has seen little general use in toxicology, likely due to the low frequency of induced phenotypes that occur and labor intense manual scoring. However, this method warrants re-investigation. Assessing teratology in the larval-adult transition could be enhanced by the rationale used in conventional enhancer/suppressor screens for genetic analysis. In these screens flies carrying mutations that partially debilitate function in a known signaling pathway become exceptionally susceptible to chromosomal and extra-chromosomal influences that alter signaling in that pathway. The potential for such genetically sensitized fly strains in toxicology is discussed below.

Behavioral traits

Flies exhibit a wide array of behaviors relevant to understanding human response to environmental challenges. These behaviors include locomotion, circadian rhythm, sleep patterns, courtship and mating, aggression, and grooming. Many of these are under the control of genetic and molecular mechanisms that were first resolved in *Drosophila* [42,94]. Furthermore, at a physiological level the underlying neurotransmitter systems in the fly are conserved including serotonin, dopamine, GABA, glutamate and acetylcholine (reviewed in [74]). To date, behavioral endpoints in *Drosophila* have been used primarily to isolate genes that specifically support a given trait rather than as a tool for screening vast numbers of chemicals. This is exemplified by an initiative to find genes that regulate ethanol (EtOH) tolerance which led to identification of the “cheapdate” mutant [69]. In a conventional forward genetic approach the Heberlein lab first established that ethanol intoxicated flies exhibit analogous traits to inebriated humans, e.g., an initial period of hyperactivity and disorientation followed by a period of sedated, uncoordinated locomotion (see Fig. 1K and [69]). The loss of postural control upon EtOH exposure was then assayed using an “inebriometer”, an ingenious column device able to separate flies based on their latency to intoxication after EtOH administration [69]. The cheapdate mutant, which is a defective allele of the fly pituitary adenylate cyclase activating peptide (PACAP) homolog known as amnesiac, was one of 12 mutants identified from screening ~5000 mutant alleles. Further genetic and molecular analysis has resolved that activation of the cAMP signaling pathway is central to the behavioral response to EtOH intoxication, not only in flies but in mice [61]. Thus, in this example, a defined behavioral trait in flies has fostered dissection of a conserved toxic mechanism for ethanol.

Behavior based assays have also contributed to studies of lead toxicity. Courtship, fecundity and locomotion are reliable behavioral outputs that respond to lead dosages to developing larvae [49]. Interestingly, courtship behavior, assayed by the number of matings occurring

within 20 minutes of pair introduction, shows an increase at low lead dosage and a decrease at higher dosage [49], an example of hormesis, a common non-linear response exhibited with pollutants and stress exposure on mammals. Parallel studies demonstrated morphological differences in the relationship of muscle area and motor terminal size at the neuromuscular junction in lead exposed larvae [71]. Ongoing research implementing quantitative trait loci mapping (QTL) of lead-induced behavior traits aims to resolve gene candidates underlying lead toxicity mechanisms [50].

Embryonic development

Fly embryos are highly effective for in vitro analysis of developmental toxicity. Capitalizing on the developmental potential of early gastrulating embryos the Bournias-Vardiabasis lab has devised a cell culture assay to evaluate teratogenicity [14]. Cells from homogenized 3.5 hour old embryos subsequently cultured for 24 hours display a quantifiable degree of differentiation revealed by the formation of two structures: neuronal ganglia and myotubes. These structures, once highlighted by fixation and staining, are quantified with an automated image capture and analysis system. Assay of potential teratogenicity is revealed by a decrease in the number of one or both of these differentiated cell structures. A systematic evaluation of 100 chemicals demonstrated 45 agents that affected embryonic cell differentiation with statistical significance [15]. Of these 45 agents, only two showed false positive activity compared to their teratogenic potential in mammalian species. As well, only four of the 55 other agents were determined as false negatives [15]. While this assay has not seen widespread use or incorporation into regulatory protocols it highlights the conserved response to toxic exposure in embryonic cells of the fly. Further development of this assay exploiting a variety of available transgenic reporter systems could tailor this approach to investigate pathway-specific sensitivity to chemicals.

Embryonic exposure via maternal feeding has supported investigation of MeHg toxicity and identification of the Notch pathway as a target of MeHg. Flies fed MeHg in the medium show a dose dependent increase in mercury accumulation in the embryo which correlates with developmental defects as determined by a reduced rate of hatching and disrupted nervous system development ([81], also see Fig. 1F,G). Using microarray analysis of transcript levels a significant upregulation of Notch receptor target genes was found in MeHg exposed embryos [81]. These in vivo effects on transcription were then resolved in a defined cell culture system using a *Drosophila* neural cell line. This line of inquiry has led to the observation that Notch target genes can be upregulated independent of the Notch receptor, evoking new hypotheses for MeHg action on transcriptional events [81].

Mellerick and Liu [67] have taken the approach of directly incubating embryos on cotton saturated with various concentrations of methanol (MeOH) to examine toxicity. MeOH induces defects in morphological cell movements with severe defects resulting in the embryonic CNS and PNS, as revealed with immunostaining using a variety of available neural and glial specific antibodies [67]. It is of note that this study arose from initial attempts to look at polychlorinated biphenyl (PCB) toxicity delivered in MeOH solvent, where PCB effects were only seen at excessively high concentrations. This latter effect likely stems from the difficulty of transporting chemicals across the vitelline membrane in the embryo shell. Overcoming this permeability issue has been demonstrated in a number of cases giving the fly embryo great potential for more widespread screening applications, as discussed below.

Reporter gene assays

The Chowdhuri group has made extensive use of a transgenic flies carrying a lacZ reporter gene under the control of the heat shock protein 70 promoter (hsp70-lacZ) [72]. Hsp70 is a highly responsive stress gene with fairly low specificity with respect to environmental stressor. Nonetheless, this reporter has served as a rapid readout of toxicity in larval and adult feeding

assays of specific organophosphates as well as complex mixtures of industrial leachates [43, 92]. In addition, immunohistochemical analysis of the reporter reveals tissue specific patterns of toxic insult that expose sex-linked differences in response [43]. As with the embryonic cell assays mentioned above, application of more specific promoter-based reporter genes in transgenic flies to toxicological screens will prove highly effective for identifying chemical-pathway interactions in vivo.

Targeted mechanistic studies

The fly model has also been implemented in efforts aimed at managing toxicity in drug administration. Methotrexate (MTX) is an anti-folate drug commonly used in chemotherapy due to its inhibitory action on dihydrofolate reductase (DHFR) and the subsequent lethality imposed on rapidly dividing cells. Its use has expanded to treatment of rheumatoid arthritis, asthma, Crohn's disease and lupus as well as in termination of early pregnancies [33,48,78, 102]. MTX elicits skeletal defects and developmental abnormalities in the fetus, which conflict with its use in chemotherapy. The Walker lab has exploited the high degree of conservation of DHFR and folate metabolism in the fly to screen for MTX-resistant variants of DHFR in a laboratory selection paradigm [3]. Interestingly, MTX elicits analogous defects on gestation and embryogenesis in the fly, causing reduction of egg production in females with corresponding deformities in the ovaries and a variety of embryonic defects in the progeny [4]. These investigations have led to identification of a L30Q mutant that confers high resistance to MTX in vivo in flies [5]. Mutation of the homologous amino acid in mammalian DHFR shows MTX resistance as well [27,66], thus establishing a complete model of functional homology. Future efforts will exploit the fly system to screen additional MTX-resistant variants and dissect the mechanism of DHFR-independent MTX toxicity, all of which advance novel strategies for avoiding unwanted MTX toxicity in a clinical setting [101].

Drosophotoxicology for the future

The *Drosophila* model can contribute to today's demands of toxicology through advancement of existing assay approaches as well as through application of newly emerging methods. Three areas ripe for development with flies are: 1) optimizing sensitive endpoints for pathway-specific screening, 2) accommodating HTS demands for analysis of chemical libraries, 3) optimizing existing genetic and molecular protocols for more rapid identification of genes underlying toxicant-by-gene interactions. In the big picture, development of Drosophotoxicology models must complement mammalian models and other alternative models and have an overall enhanced efficacy for identifying chemical toxicity and vulnerable cellular and molecular pathways in humans.

Genetically sensitized *Drosophila* strains

Using genetically sensitized strains of flies is endorsed in the recommendations put forth by the National Research Council Committee on Toxicology [75]. The underpinnings of multicellular development is encompassed by 17 intercellular signaling pathways [75]. While a majority of these pathways exist in flies, six prominent signaling pathways are used repeatedly in early development and are highly conserved in *Drosophila* and vertebrates: the Wnt, TGF β , Hedgehog, EGF, cytokine and Notch pathways. It follows that strains of flies sensitized to report effects on each of these pathways individually could prove invaluable for identifying pathway-specific interactions of chemicals. This could be achieved via two methods: 1) using transgenic reporter genes tailored to respond to signaling changes in each pathway and 2) using fly strains that are genetically sensitized to reveal xenobiotic activity in a given signaling pathway through common morphological phenotypes.

The concept of using reporter gene flies is illustrated by studies using the Hsp70-lacZ flies discussed above. A similar approach could be implemented, for example, with the Notch pathway. The enhancer of split (E(Spl)) target genes are direct transcriptional targets of the activated Notch receptor. In fact, engineered flies with a lacZ reporter downstream of E(spl) genes are reported and have been shown to respond to genetic manipulations of Notch activity [25,39]. Reporter gene response could be monitored at any stage of development using modes of delivery summarized in Figure 1. For example, a simple assay would involve feeding E(spl)-lacZ larvae on media containing various concentrations or compositions of agents for a defined time and determining β -galactosidase levels in homogenates of larvae by standard chemical substrate assays [92]. Through optimization of larval sample handling techniques this approach could be adapted to a multi-well format for high throughput. Implementing this approach will require a concerted effort by the research community to develop standardized strains of flies carrying the appropriate reporter constructs for each signaling pathway referred to above.

Alternatively, strains sensitized in a select pathway can be used for screening, analogous to the enhancer/suppressor screening method used for identifying genetic interactions with Notch [103]. This can be done by feeding compounds to larvae of, for example, heterozygous Notch null mutants and scoring wing notching phenotype in adults. Furthermore, application of an automated wing image analysis system, the “Wingmachine”, described by Houle, et al. [51] could facilitate analysis of numerous chemicals in a medium throughput platform. Proof of principle for this approach can be seen whereby larval exposure to DAPT, a small molecule gamma-secretase inhibitor that inhibits Notch activity, gives a pheno-copy of the Notch wing ([68] and see Fig. 1J). Fly stocks useful for a sensitized-strain approach for screening in the Notch [103], Wnt [41], Hedgehog [44], TGF β 3 [52] and EGF [80] pathways are reported.

Potential for *Drosophila* in high throughput assays: toxicity in the embryo

Drosophila have traditionally been considered a model for high throughput assays due to the low cost of resources, rapid generation time, easily identified mutant phenotypes and the ability to process on the order of 10,000 flies for a given screen. However, toxicological screening today demands a throughput able to analyze a library of 10,000–100,000 chemicals in a period of a day to a week. This becomes logistically challenging where, for example, manual scoring of a wing notching phenotype is required. As with assays in mammalian models, the information content of a given assay has to be balanced with throughput logistics when implementing a *Drosophila*-based assay. Nonetheless, there is considerable room for development of fly assays to address today’s HTS demands which can capitalize upon automated methods for scoring adult traits, ranging from wing morphology to social behavior [18,51].

Perhaps the greatest potential for HTS applications is harbored in the fly embryo. While fly embryonic cells in culture are highly sensitive to teratogens [15], the intact embryo presents an even more relevant platform. First, the embryo offers the advantage of small size (150×500 μ m), thus 50 or more embryos can be reared in the well of a 96-well plate. Next, similarly staged embryos can easily be obtained through timed laying and controlled incubation methods. Commercially available instrumentation (e.g., the COPAS Select large particle sorter (Union Biometrica, <http://www.unionbio.com>)) is capable of automated embryo sorting based on size, shape and fluorescence intensity. This instrument is also capable of distributing embryos in a multiwell format. Additional commercially available technology is capable of high-resolution image capture and analysis (e.g. BD Pathway High-Content Bioimager, <http://www.bdbiosciences.com>), also in a multiwell format. The combined application of transgenic fluorescent reporter genes and improved methods of introducing xenobiotics to the embryo (see below) has high potential for a robust platform for toxicology assays.

The fly embryo is ideally suited for neurotoxicology assays. Embryonic neural development processes have been documented in exquisite detail with respect to the overall morphology of CNS and PNS and the cytoarchitecture of individual motor, sensory and interneurons [13,19, 32,89]. Immunoreagents and genetically encoded fluorescent dyes have been constructed that are extremely effective in revealing cell morphology in fixed or live tissue (see Fig. 1F,G). Gene networks and signaling pathways responsible for discrete neural developmental events are known and genetically accessible via reporter genes or mutant alleles that alter their activity. Existing knowledge of how these pathways regulate cell fates, neural patterning and neurite outgrowth explicitly in the fly embryo prepares the investigator to correlate phenotypes with pathways affected by exposure to a toxin.

The embryo model is not without challenges for practical use. Administration of drugs to the fly embryo must overcome the barriers presented by the shell that encases the developing organism. Of particular note is the vitelline membrane, which is highly impermeable to water and solutes [58], making it difficult to achieve high throughput application of chemicals to the embryo. To date, delivery to the embryo has predominantly relied on manual microinjection methods. An automated microinjection system has been developed capable of fairly high throughput, yet requires somewhat sophisticated instrumentation and skilled manipulations for set up [105]. In limited cases, maternal dosage has been demonstrated with, for example, methylmercury, which results in distinct neural developmental phenotypes and alteration of signaling events [81]. Much effort has been directed toward permeabilization methods, particularly with respect to the goal of introducing cryopreservatives to the embryo [65]. The most effective methods have relied on organic solvents, such as heptane or octane, to remove the waxy layer of the vitelline membrane [58,65]. Delivery of drugs and small molecules to embryos in vitro has been successful in a few instances, most notably in the delivery of bromodeoxyuridine (BrdU) to the developing peripheral nervous system in lineage analysis studies [12]. However, this analysis used octane to permeate the vitelline membrane, which was suitable since a limited degree of development was needed (1–4 hours) for an endpoint subsequent to exposure. In one example dimethyl sulfoxide (DMSO) was shown to facilitate delivery of RNA inhibitors to the embryo [6] and may prove useful for delivery of other similar sized chemical compounds through the embryo shell. Refinement of methods to delivery xenobiotics to embryos in vitro will likely open the door to a wide array of assays in this well-established model.

Genetic approaches identification of genes underlying toxic mechanisms

The ability to resolve bona fide gene-environment interactions in model organisms could rapidly advance our understanding of toxicity mechanisms for compounds of concern in human health. The conventional forward-genetic approach with flies is very effective for identifying genes, e.g. in EtOH toxicity, yet is time consuming and can prove frustrating where toxic response traits are polygenic in nature. The methods of quantitative trait loci (QTL) analysis hold great promise for elaborating genetic components of pathways in toxicology [50]. QTL has been used extensively in analyses of natural variations in behaviors and morphology among fly strains [62]. Complex traits such as longevity and thermotolerance have been mapped to distinct loci and, in some cases, candidate genes [70,100]. The QTL method involves recombination and complementation tests as well as deficiency mapping and has been optimized in *Drosophila*. For example, genetic crosses are carried out between multiple individual recombinant inbred fly strains, which display variation in a given trait (e.g. lead tolerance) and an array of fly strains carrying defined chromosomal deficiencies. The offspring are tested and scored for the trait of interest. Genetic combinations resulting from these matings that show quantitative differences in the trait unveil chromosomal regions (loci) that harbor genes affecting that trait. This approach, together with genomic sequencing and transcriptional profiling (e.g. microarray analyses) [57] has the potential to resolve candidate genes rapidly

by pinpointing positions of polymorphisms correlating to the trait of interest. The method will likely become more efficient with the recent development of speedmapping protocols using a modified microarray approach [56]. This latter approach utilizes pooled DNA probe sets derived from fly lines that differ in the trait of interest (e.g. tolerance to a toxicant). DNA regions that differ between the fly lines by a single feature polymorphism (SFP) are identified and correlated with the trait using a competitive hybridization protocol with Affymetrix array chips. The general utility of this method is the ability to map quantitative traits to <900kb regions of the genome [56], which was validated against flies with known QTLs identified by conventional complementation methods. Utility of this approach awaits testing on traits with no previous QTL data.

Integration with mammalian models

Collins et. al. [23] propose a model in which *Drosophila*, and other alternative model organisms, will contribute to toxicology studies of the future. Behind this model is a proposed paradigm shift to change toxicology from an “observational” science to a “predictive science focused on broad inclusion of target-specific, mechanism based biological observation in vitro” [23]. Efforts are now focused on HTS, employing in vitro biochemical and cell-based assays that are specific to individual pathways and that can process on the order of 10,000 chemicals per day. While this kind of throughput is not plausible with an in vivo adult *Drosophila* platform, a high degree of pathway specificity can be maintained in fly-based assays. Targeted, in vivo testing in alternative non-mammalian models is essential to graduate lists of prioritized toxic agents from cell-based assays to the level of rodent testing and human clinical experience. The Zebrafish model has been selected as a proxy for vertebrate response to developmental toxicity in the EPA’s Toxcast program ([29] and <http://www.epa.gov/ncct/toxcast/assays.html>). Yet, the *Drosophila* embryo model presents an ability to obtain a higher throughput with shorter development times and lower costs, while at the same time granting access to a comprehensive system for reporter gene, morphological and lethality endpoints. Together, the two models are likely to compose a supportive and logical progression of complexity in the realm of pathway-targeted toxicological studies. For example, an agent’s proclivity toward the Notch pathway may be quickly identified in *Drosophila*, in which there is one gene encoding the Notch receptor (and likewise for several of the core genes of the Notch pathway). With the multiple paralogs of the Notch genes that are present in Zebrafish, a more tissue specific context of agent activity toward Notch signaling (e.g. in cardiac development) is likely to be resolved. The potential for cooperativity among *Drosophila*, zebrafish and *C. elegans* models calls for a concerted effort on behalf of the experts using these models to coordinate assays suited for today’s emerging toxicological science goals.

Pitfalls and limitations

There are a number of non-conserved properties in flies that preclude its use for certain assays. Fly metabolism of xenobiotics can differ significantly from that of mice and man. This becomes important where metabolism is responsible for transforming chemicals to toxic isoforms. In the case of arsenic, both the arsenate and arsenite ions are methylated in mammalian systems [22,55]. The relevance of methylation of arsenic remains a foremost question in the mechanism of arsenic toxicity. Arsenic is not methylated in *Drosophila* [84], which makes it difficult to investigate the role of endogenous methylating enzymes in this model. Nonetheless, this “methylase-deficient” background could prove exceptionally powerful for screening of mammalian gene candidates for arsenic methylation activity. Such an approach has proven informative for investigation of other drug metabolizing enzymes. For example, transgenic expression of the rat cytochrome CYP2B1 gene in flies invokes hypersensitivity to the pro-carcinogen cyclophosphamide in the SMART assay [53]. Nonetheless, where screening of

libraries of chemical with unknown toxicity is undertaken use of the *Drosophila* model must be weighed against possible discrepancies with mammalian counterparts. Along the same line, evidence suggests methylation of DNA is not a robust regulatory epigenetic mechanism in *Drosophila* as it is in mouse and man. As many xenobiotics can affect DNA methylation as a mode of action, such a mechanism might not be revealed in a fly model.

The utility of flies for determining a relevant dose response for mammals is not well established. Flies are not likely to be useful for this determination because of anticipated differences in transport, metabolism and toxicokinetics compared to mammalian systems. Nonetheless, the model is likely to accurately reflect a comparative toxicity between two agents, which could corroborate parallel studies in other invertebrate and vertebrate models.

Summary

The *Drosophila* model has been behind many of the fundamental advances that have occurred in genetics, molecular and developmental biology in the last century. Features that have made this organism so well suited to uncover gene identity, sequence and function are equally well suited to reveal biological activity of chemicals encountered through environmental exposure. There are some shortcomings in the *Drosophila* model, which preclude its use for testing in certain arenas. Nonetheless, the level of conservation of fundamental cellular and developmental mechanisms between flies and man are extensive enough to warrant a more central role of *Drosophila* toxicology in neurotoxicology testing of today.

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Figure 1. Drosophotoxicology: an overall scheme for flies in toxicology

Chemicals to be tested range from longstanding environmental contaminants, such as mercury, arsenic and lead, to molecular libraries, to the growing list of chemicals of concern identified by the National Toxicology Program (<http://ntp.niehs.nih.gov/>). Several modes of delivery are possible at the embryonic (A, B), larval (C) and adult (D) stages. Exposure to embryonic tissues can be achieved through in vitro incubation (A), injection (B) or maternal feeding (D). Larvae and adult flies are readily exposed to chemicals through dosages in the food medium (C, D). Injection methods have also been employed for compound delivery in larvae and adults. As well, controlled vapor phase exposure to adult flies is possible (B adapted from www.egr.msu.edu/~xbtan/SML/embryo.jpg; D adapted from James Waters, Arizona State University. See text for discussion). The mode of delivery dictates the developmental stage (E) at which exposure is incurred. The drastic changes in form of the CNS (gray) and PNS (blue) during the progression from the embryo to pupa to adult (E) illustrate outcomes of neurogenesis, neurite elaboration, cell migration and synaptogenesis that occurs at distinct time points in these developmental stages (adapted from Hartenstein [47], see text for discussion). Several endpoints are possible for determining outcomes of toxic exposure. Defects in the embryonic nervous system can be revealed by immunostaining (F) or with genetically encoded vital reporters such as GFP (G). (F, staining of stage 14 embryos with neural specific BP102 antibody, ventral view of CNS. Left panel, control; right panel, methylmercury exposed. G, lateral view of CNS and PNS revealed by pan-neural GFP expression. Left panel, control; right panel, methylmercury exposed [82].) Motor neurons in larvae form synaptic boutons at the neuromuscular junction (H) that are sensitive to disruption by toxicants (see [71]). Larvae also display quantifiable behaviors in motility such as rate of locomotion and chemotaxis (I, Rate of control larvae (CS) locomotion is compared to a tyramine β -hydroxylase mutant, the latter showing a shorter path length in the same interval of time (adapted from [87])). Common assays examine changes in adult structures such as the eye or wing (J, top panel, control wing; bottom panel, wing from γ -secretase inhibitor, DAPT, treated larvae with Notch-like phenotype (adapted from [68])). Adult behavioral assays are able to quantify complex behaviors such as courtship, circadian rhythm and associative learning and memory. Response and tolerance to ethanol exposure can be quantified by general locomotor activity monitored by video tracking (K, top panel: the normal burst in activity followed by sedation over after first EtOH exposure; bottom panel: the burst in activity does not subside in EtOH tolerance experienced with the second exposure (adapted from [90])).