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Genetic Targets of Acute Toluene Inhalation in *Drosophila melanogaster*

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ABSTRACT

Interpretation and use of data from high-throughput assays for chemical toxicity require links between effects at molecular targets and adverse outcomes in whole animals. The well-characterized genome of *Drosophila melanogaster* provides a potential model system by which phenotypic responses to chemicals can be mapped to genes associated with those responses, which may in turn suggest adverse outcome pathways associated with those genes. To determine the utility of this approach, we used the *Drosophila* Genetics Reference Panel (DGRP), a collection of ~200 homozygous lines of fruit flies whose genomes have been sequenced. We quantified toluene-induced suppression of motor activity in 123 lines of these flies during exposure to toluene, a volatile organic compound known to induce narcosis in mammals via its effects on neuronal ion channels. We then applied genome-wide association analyses on this effect of toluene using the DGRP web portal (http://dgrp2.gnets.ncsu.edu), which identified polymorphisms in candidate genes associated with the variation in response to toluene exposure. We tested ~2 million variants and found 82 polymorphisms located in or near 66 candidate genes that were associated with phenotypic variation for sensitivity to toluene at $P < 5 \times 10^{-5}$, and human orthologs for 52 of these candidate *Drosophila* genes. None of these orthologs are known to be involved in canonical pathways for mammalian neuronal ion channels, including GABA, glutamate, dopamine, glycine, serotonin, and voltage sensitive calcium channels. Thus this analysis did not reveal a genetic signature consistent with processes previously shown to be involved in toluene-induced narcosis in mammals. The list of the human orthologs included Gene Ontology terms associated with signaling, nervous system development and embryonic morphogenesis; these orthologs may provide insight into potential new pathways that could mediate the narcotic effects of toluene.

Key words: fruit fly; volatile organic compound; motor activity; narcosis; genome-wide association; DGRP.

Toxicity information regarding thousands of untested environmental chemicals is needed to evaluate their potential impacts on human health. Only about 20% of chemicals considered of high priority by the EPA have been tested (Judson *et al.*, 2008). This lack of information was a significant factor driving a recent recommendation by National Research Council (NRC., 2007) to develop an alternative toxicity testing strategy that would link effects of chemicals on molecular targets to adverse outcomes via hypothesized "adverse outcome pathways" (AOPs).

To implement this strategy, a broad range of mechanistically informative and high-throughput (*in vitro* or model system) assays have been developed to generate dose–response data from assays of thousands of chemicals. These assays comprise major components of the Computational Toxicology Research

Plan (EPA, 2009), the 2010 EPA Strategic Research Plan (Firestone *et al.*, 2010), and the ToxCast (Dix *et al.*, 2008; Judson *et al.*, 2009; Kavlock *et al.*, 2012) and Tox21 (Collins *et al.*, 2008; Shukla *et al.*, 2010; Tice *et al.*, 2013) programs.

The success of high-throughput toxicity testing relies on application of mechanism-based AOPs that link signals from screening tests to adverse outcomes in whole animals. The existence of extensive databases of genetic information for many species now make it possible to execute genome-wide approaches to identifying potential candidate genes that participate in, and thus identify, AOPs for priority chemical toxicants. To make this approach useful, methods are needed to determine whether adverse outcomes in the whole animal can be related quantitatively to effects at the genomic level.

Recent work on the genetic basis of behavior in *Drosophila* has developed a model system that may be useful for identifying AOPs. This "*Drosophila* Genetic Reference Panel" (DGRP: (Huang *et al.*, 2014; Mackay *et al.*, 2012) provides a publicly-available resource by which the genetic determinants of sensitivity to chemicals or other challenges can be assessed. The DGRP consists of 205 wild-type inbred lines for which extensive information on genotypes and phenotypes is available (http://dgrp2.gnets.ncsu.edu). Significant variation among lines and between sexes has been found for many phenotypes, including responses to acute (Weber *et al.*, 2012) and chronic oxidative stress (Jordan *et al.*, 2012), odor-guided behavior (Brown *et al.*, 2013) and olfactory avoidance (Swarup *et al.*, 2013). The DGRP lines reflect natural polymorphisms within a standard population, and are expected to vary in response to airborne chemical exposures. Genome-wide association methods can link variation across phenotypic responses to a chemical with genotypic correlates of sensitivity to the chemical; gene ontology methods may then suggest AOPs that are engaged by chemical exposure. Using the DGRP, this approach has identified genetic correlates of olfactory behaviors (Swarup *et al.*, 2013), resistance to chemical-induced oxidative stress (Weber *et al.*, 2012) and alcohol sensitivity (Morozova *et al.*, 2015).

We previously used 'Flyland', a hybrid of 40 DGRP lines, to quantify the toxicity of 3 airborne chemicals including toluene (Tatum-Gibbs *et al.*, 2015). This work revealed that toluene-induced narcosis in flies, defined as acute suppression of motor activity during exposure, was directly related to the concentration of toluene in the air, with an EC20 of 736 ppm in males and 595 ppm in females. Motor activity was not suppressed by exposure to 2 other tested volatiles, acrolein and vinyl chloride.

These effects of toluene on motor activity in *Drosophila* are remarkably similar to those observed in mammals. In rodents and humans, toluene has been shown to increase motor activity at low exposure levels and suppresses activity at higher levels (Benignus, 1981; Bushnell *et al.*, 1985); at very high concentrations it can suppress activity completely (Bowen *et al.*, 2006). Effective concentrations in rodents (1000–15 000 ppm) are somewhat higher than those in flies (700–1800 ppm) (Tatum-Gibbs *et al.*, 2015). As with ethanol and inhaled anesthetic gases, the acute effects of toluene are mediated by complex changes in several neurotransmitter systems, including GABA, glutamate, dopamine, glycine and serotonin (Bowen *et al.*, 2006; Bushnell *et al.*, 2005). While the precise set of interactions among these systems that produce narcosis is not presently known, it is clear from *in vitro* tests that ethanol, anesthetic agents and solvents including toluene enhance inhibitory effects of GABAergic and glycinergic pathways, inhibit activating effects of glutamatergic and nicotinic cholinergic pathways (Bale *et al.*, 2005a; Bale *et al.*, 2005b; Beckstead *et al.*, 2000; Cruz *et al.*, 1998; Mihic *et al.*, 1997) and interfere with voltage-sensitive calcium channels (Tillar *et al.*, 2002; Okuda *et al.*, 2001; Mullikin-Kilpatrick and Treistman, 1993; Kamatchi *et al.*, 1999). The fact that narcosis can be induced by drugs with specific action on these neurotransmitter systems (Bushnell *et al.*, 2005; Bowen *et al.*, 2006) suggests an important role for these neurochemical targets in toluene-induced narcosis.

The present work was designed to determine whether genome-wide association analysis of the variation in the narcotic effect of toluene across lines of the DGRP would reveal genes associated with these neurotransmitter pathways. The guiding hypotheses of these experiments include (a) that the panel of flies in the DGRP will vary in the magnitude of narcosis induced by a given concentration of toluene and (b) that the genes associated with variation in sensitivity to toluene across the panel will point to one or more signaling pathways reflecting mechanisms associated with narcosis in mammals. Our analysis focused on the acute effect of toluene on motor activity, given the abundant evidence regarding molecular mechanisms associated with toluene-induced narcosis in mammals and the relative paucity of information about its other effects.

Given that individual lines of the DGRP tend to be less robust than the Flyland hybrid, behavioral assessments were conducted at a concentration of 750 ppm, a value close the EC20 for toluene in male Flyland flies. Behavioral responses were determined for males of 123 lines of the DGRP, and were used as the phenotype in a genome-wide association analysis to identify genetic variants associated with variation in the narcotic response to toluene. Females were not tested because time and resources limited the study to one sex; males were selected because they are consistently more active than females in this system (Tatum-Gibbs *et al.*, 2015).

METHODS

Drosophila Husbandry and Collection for Behavioral Testing

Cultures of 123 lines of DGRP flies were obtained from the Department of Biological Sciences at North Carolina State University. Flies were maintained in an incubator at 23–25°C and relative humidity of ~35% under a 12:12-h light:dark cycle with lights (100 lux) off at 10 am. Flies were fed a standard corn meal-molasses-agar-based medium provided by the National Institute of Environmental Health Sciences (NIEHS). Tegosept, propionic acid and antibiotics were added to inhibit bacterial growth, and baker's yeast was sprinkled on the medium after cooling. Paper (Kimwipe®) was added to each breeding bottle to control humidity and assist with the breeding process. Flies were transferred from stock bottles to fresh half-pint bottles on Fridays for experiments the following week. Adults were discarded in the mornings after 10–12 days (Monday, Tuesday and Wednesday), and newly eclosed adults were collected 6 h later each day in 8-dram glass vials (25 × 95 mm O.D. x H, volume = 47 cm^3, capped with air-permeable foam plugs). Each vial typically contained ~ 20–30 adult flies of each sex. For each behavioral assay, the flies in the collection vials were combined, mixed together on the fly bed under CO_2 anesthesia, and sorted by sex. The females were discarded and the males were placed into the activity monitoring tubes. Flies were tested on Wednesday, Thursday and Friday of each week, and were 42–48 h old at the start of exposure.

Toluene Exposure System

Toluene (>99.5%, A.C.S. spectrophotometric grade) was purchased as a liquid from Sigma-Aldrich, St. Louis, MO. Toluene vapor was generated and its concentration was monitored during each exposure in real time by infrared spectroscopy as described previously (Tatum-Gibbs *et al.*, 2015). For the present studies, toluene was delivered to each of 4 activity-monitoring devices (see Section 2.3.1 below), and clean air was simultaneously delivered to 4 other identical devices. Total flow through the system was maintained at 50 cc/min (~ 0.2 cc/min for each individual fly).

Behavioral Testing

Equipment. Locomotor activity was measured in each of 8 fly exposure units using *Drosophila* Activity Monitors (DAM2, TriKinetics® Inc., Waltham, Massachusetts), each of which reported counts of photobeam breaks simultaneously from 32 individual flies. Glass tubes (65 mm long, 3.5 mm i.d., 5.0 mm o.d.) were placed in each of the 32 ports of the monitor and sealed at both ends during exposure with gas-distribution manifolds (MAN2, TriKinetics® Inc.) that were connected with stainless steel and Teflon tubing to the exposure system.

General methods: loading flies and measuring activity in monitors. Our previous work (Tatum-Gibbs *et al.*, 2015) indicated that activity of the flies increased rapidly for about an hour after the anesthesia required for loading them into the testing equipment, and either leveled off or continued to rise more slowly for several hours thereafter. The effects of toluene were clearly evident within minutes after the onset of exposure, and persisted until exposure was terminated. Based on these observations and evidence of a similar time-course of acute toluene-induced changes in motor activity in rodents (Bushnell *et al.*, 1985; Hinman, 1987; Tegeris and Balster, 1994), we selected a 1-h period for recovery from anesthesia, a 4-h period for toluene exposure, and a 15-h post-exposure observation period (overnight) to document recovery from the exposure.

Male flies were anesthetized with CO_2 and loaded individually into the monitor tubes using a pneumatic pipette designed to minimize injury to the animals. The tubes were placed into one manifold and held vertically in place during loading. A slight vacuum drew air downward through the tubes to keep the flies in place during loading. When all tubes were loaded, the monitor array was slipped over the tubes and a second manifold was pressed onto the open ends of the tubes to seal the flies in the tubes.

Eight monitors were loaded for each experimental run; loading required about 45 min. The loaded monitors were placed with the fly tubes aligned horizontally in a second incubator for testing, in which the temperature was 25°C and the light intensity was 6–8 lux, between 9:30 and 10:00 am. The dim light at this time, coinciding with prior entrainment to a light cycle that switched from light to dark at 10 am, was designed to maximize activity in these crepuscular animals (Rieger *et al.*, 2007). Locomotor activity was recorded in 10-min intervals beginning within 5 min after the last monitor was loaded. Photobeam breaks were summed in successive 10-min intervals for 20 h. Flies were removed from the tubes and discarded the following morning. The tubes were washed, dried and re-used for subsequent exposures.

Experimental design. Sixteen flies of a given line were tested each day: 8 flies per line received toluene in one monitor and 8 received clean air in a paired monitor. Paired toluene and air flies were loaded into corresponding cells in the 2 monitors to control for potential differences in activity associated with different locations in the monitor. Four pairs of monitors were loaded each day, and each monitor contained 4 lines of flies, thus accommodating 16 lines of flies. Each such cohort of 16 lines was tested at least 3 times, usually in the same week, with the location of the lines within monitors counterbalanced across days. Final sample sizes for the 123 lines of the DGRP averaged 18 in air and 19 in toluene, with totals of 3234 flies tested in air and 3420 in toluene. Because of variation in the robustness of the different lines, n's per line varied from 11–60 in air and 17–61 in toluene.

Data Analysis

Photobeam breaks per 10-min interval across the 20-h observation period were collected for each individual fly. These data were sorted by DGRP line and treatment (toluene vs. air); group assignment was verified by careful tracking of numbers of exposed flies per line in each cohort and sorting based on identifiers coded in the data files. Activity counts per time interval were averaged across flies within lines and treatment groups and plotted for visual inspection. Based on the patterns of activity during exposure (Tatum-Gibbs *et al.*, 2015), the narcotic effect of toluene was observed as the difference between the total activity counts during the 4-h period of exposure to toluene and the total counts during the corresponding time period in control flies.

The analytic process is shown schematically in Figure 1. Total activity counts during the 4-h period for the control and toluene flies were submitted to the DGRP web portal (Huang *et al.*, 2014) (http://dgrp2.gnets.ncsu.edu), which conducted a genome-wide association analysis of the 2 phenotypes and the difference between them (toluene–air). Polymorphisms associated with the difference scores at $P \leq 5 \times 10^{-5}$ were selected for further examination. This slightly relaxed criterion was used to increase the number of polymorphisms for analysis, since just 9 SNPs met the standard criterion of $P \leq 1 \times 10^{-6}$.

A list of *Drosophila* genes with annotations was created from these polymorphisms using the gene models in Flybase release 5.49 (http://flybase.org, McQuilton *et al.*, 2012) (Supplementary File, Table 1). Duplicates were removed from the list, leaving 66 candidate genes Supplementary File, Table 2). Human orthologs were obtained from the Drosophila RNAi Screening Center (DRSC) Integrative Ortholog Prediction Tool (DIOPT) (http://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl), using the same list of 66 candidate genes. Human orthologs with DIOPT scores greater than 2 were retained, leaving 52 human orthologs (Table 1).

These orthologs were analyzed for their functional relationship to the expected narcotic effect of toluene in mammals. The Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC) (www.genenames.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.genome.jp/kegg/pathway.html) databases were queried for gene and pathway functions. Pathways were selected from these databases based on involvement of processes known to mediate mammalian narcosis. The pathways from the HUGO database are shown in Table 2 and those from the KEGG database in Table 3. In addition, Ingenuity Pathway Analysis was performed on the human ortholog list to identify any unbiased association of the ortholog list with biological processes.

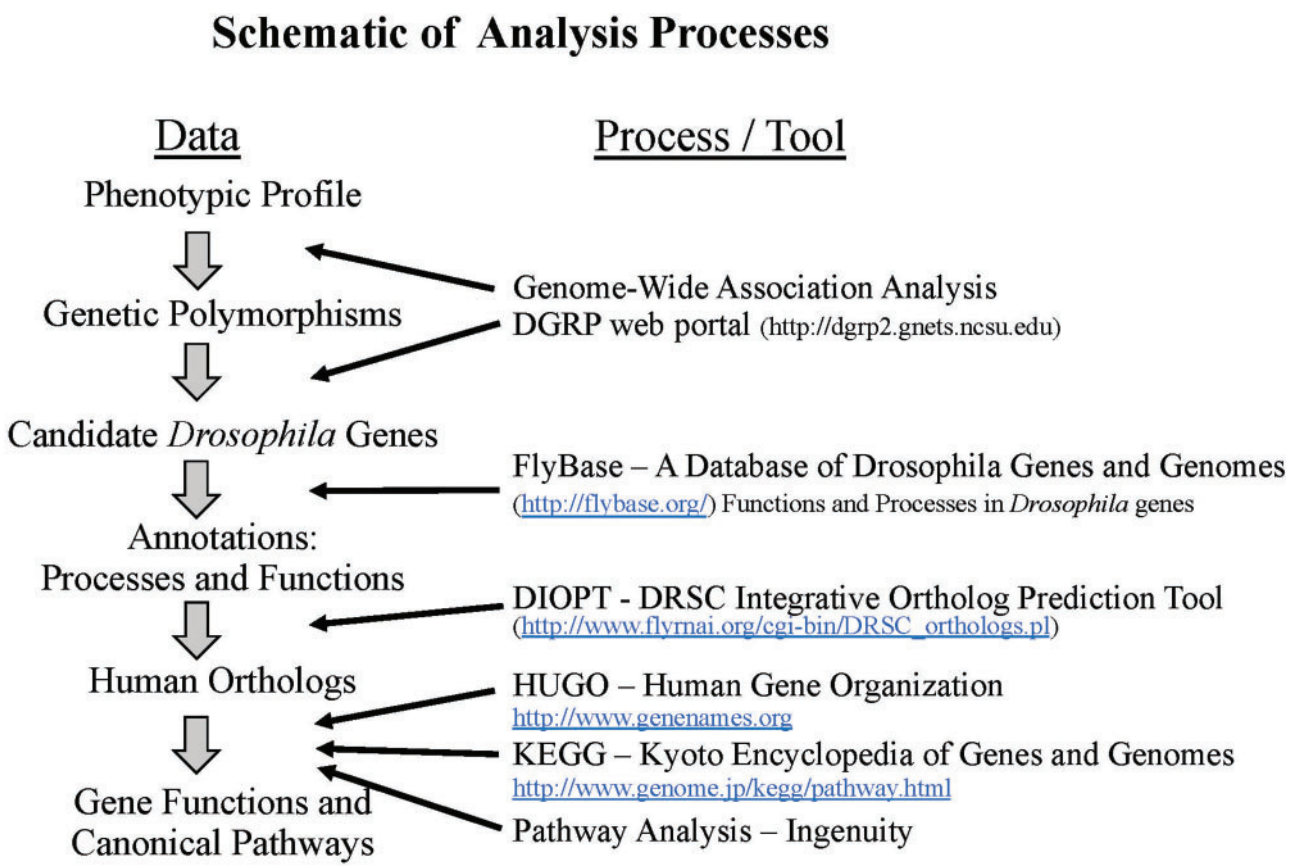

FIG. 1. Schematic description of data analysis process.

RESULTS

Effect of Toluene on the Time Course of Activity

Figure 2 shows activity patterns across time for 4 representative lines of flies in the DGRP. Inspection of these activity patterns reveals considerable variation across lines. This variation is evident in the overall activity level, the degree to which toluene suppresses activity during the 4-h exposure period, activity levels after exposure terminated, and survival times.

Difference scores between activity counts in toluene and in air showed substantial variation across the tested lines (Figure 3). Negligible differences occurred before toluene exposure (Figure 3A), indicating the essential replicability of measurement across lines when no treatment is applied. Toluene suppressed activity in 104 of the 123 lines during exposure (Figure 3B); after exposure, toluene increased activity in 75 of the 123 lines (Figure 3C). Toluene exposure increased survival time in 77 of the 123 lines (Figure 3D). Because narcosis is defined here as suppression of activity during exposure, only the data shown in Figure 3B were subject to a genome-wide association analysis.

Candidate Drosophila Genes and Human Orthologs for the Narcotic Response to Toluene

The genome-wide association analysis revealed 82 polymorphisms that were associated with phenotypic variation for sensitivity to toluene-induced narcosis at $P < 5 \times 10^{-5}$ (Supplementary Tables S1–S3). Fifteen of these SNPs were located in intergenic chromosomal regions, and 8 genes contained multiple SNPs (total duplicates = 12). In addition, we found 11 SNPs located in 2 different genes, adding 11 more genes to the candidate list. Removing the intergenic polymorphisms and multiple SNPs, and including the 11 pairs of genes identified by single polymorphisms left 66 unique candidate genes. The Gene Ontology categories for these genes were associated *inter alia* with olfactory learning, neuron differentiation and pattern formation (Supplementary Table 3). The DIOPT identified 52 unique human orthologs of the *Drosophila* candidate genes; these orthologs contain Gene Ontology terms associated *inter alia* with signaling, nervous system development and embryonic morphogenesis. Table 1 shows the candidate *Drosophila* genes, their known human orthologs, and biological processes known to be associated with the orthologs.

Relationships between Human Orthologs and Narcosis Pathways in Mammals

The HGNC database was queried with 6 terms that represent mediators of narcosis in mammals: glutamate, GABA, dopamine, glycine, serotonin, and voltage sensitive calcium channel. The number of genes in the database returned for each of these queries was 87, 30, 9, 35, 23, and 27, respectively. None of the 52 human orthologs identified here were among the genes related to any of the ion channel systems that have been shown to mediate the narcotic response to toluene in mammalian systems (Table 2). Similarly, for the 8 pathways selected from the KEGG database for their likely participation in mediating the mammalian narcotic response, there was at most a single gene in common between any KEGG pathway and the human orthologs (Table 3).

Table 1. List of Candidate *Drosophila* Genes and Human Orthologs Associated with Those *Drosophila* Genes

Drosophila Candidate Gene	Human Ortholog
Ppn	AMBP
chm	KAT7
upSET	MLL5
upSET	SETD5
dnc	PDE4D
dnc	PDE4B
Rgk1	RRAD
Rgk2	REM1
msn	TNIK
msn	MAP4K4
tun	WDYHV1
tun	IDB3
CG18208	ADRA2A
RhoGEF64C	ARHGEF3
RhoGEF64C	ARHGEF8
cato	ATOH7
VhaSFD	ATP6V1H
boi	CDON
cnk	CNKSR2
CG42342	COL23A1
CG1632	CORIN
CG5946	CYB5R1
Cyp6a21	CYP3A4
wry	DNER
CG10051	ERMP1
CG9416	ERMP1
Gen	GEN1
shn	HIVEP2
Abd-B	HOXD9
5-HT1B	HTR1B
htt	HTT
Toll-4	IL18R1
Fili	LINGO1
mbi	MBNL1
msn	MINK1
CG5334	MKRN1
Vap-33B	MOSPD2
oc	OTX1
CG11597	PPP4C
Prosalpha5	PSMA5
otk2	PTK7
shep	RBMS3
spri	RIN2
Dri-2	RYK
Sema-2a	SEMA3A
Prosap	SHANK1
Rim2	SLC25A33
Shawn	SLC25A40
Tyler	SLC25A40
sty	SPRY3
Srp54k	SRP54
qin	TDRD1
Swim	TINAGL1
Trxr-2	TXNRD2

A list of the biological processes associated with the *Drosophila* genes can be found in Supplementary Table 1.

Ingenuity Pathway Analysis of the Human Orthologs

To identify what biological processes have been associated with the 52 human orthologs identified here, the gene symbols for this list were submitted to Ingenuity Pathway Analysis. This analysis yielded 15 canonical pathways associated with these orthologs (Table 4). The table shows the pathways with a statistically significant ($P < .05$) probability of including any human ortholog identified in this experiment, and the orthologs that were members of each pathway. None of the resulting pathways specifically involve ion channel functions. Furthermore, omitting the 2 pathways containing fewer than 10 genes, the mean proportion of listed genes in a particular pathway was less than 4% and at most 7.7%. Thus any relationship between any of these pathways and the narcotic response to toluene is very weak.

Table 2. Results of a Query of the HUGO Database for Genes Associated with 6 Ion Channel Systems Involved in Toluene-Induced Narcosis

Narcosis Mediating System	Number of Genes in Mediating System	Number of Overlapping Human Homologs
Glutamate	87	0
GABA	30	0
Dopamine	9	0
Glycine	35	0
Serotonin	23	0
Voltage Sensitive Calcium Channels	27	0

The number of annotated genes in each of the 6 ion channel systems ranged from 9 to 87, but none of these genes was found among the human orthologs identified in this experiment.

DISCUSSION

Acute exposure to toluene vapor reliably altered motor activity in most of the DGRP lines, an effect that we observed previously with the hybrid "Flyland" fly (Tatum-Gibbs *et al.*, 2015). These changes in motor activity closely mimic those observed in mammals inhaling toluene (Benignus, 1981; Bushnell *et al.*, 1985), including the prolonged suppression of activity after an initial short period of activation, which is a hallmark of narcosis. The DGRP lines differed substantially in their sensitivity to this suppression of behavior (Figure 3B), and a genome-wide association analysis identified polymorphisms associated with the differential sensitivity of the lines to toluene. Examination of these polymorphisms revealed sets of *Drosophila* genes and human orthologs associated with the behavioral effect of toluene.

The Changes in Gene Expression Did Not Reveal a Signature of Acute Toluene Narcosis

The set of human orthologs identified here does not appear to play any clear role in processes known to be involved in the acute toluene-induced suppression of behavior in mammals. The primary neurobiological substrates for the acute effects of toluene, other volatile narcotic chemicals (including anesthetic gases), and ethanol are the various neuronal ion channels, including those gated by the neurotransmitters glutamate, GABA, dopamine, glycine and serotonin, and those calcium channels sensitive to neuronal membrane voltage. Thus, queries of the HGNC and KEGG databases for genes with documented relationships to these substrates yielded zero overlap with the genes identified from this experiment. That is, none of the human orthologs associated with the effect of toluene in flies were among the genes catalogued in these databases as

Table 3. Results of a Query of the KEGG Database for Genes Associated with CYP450-Related Drug Metabolism, Neuroactive Ligand Receptor Interaction, and 6 Ion Channel Systems Involved in Toluene-Induced Narcosis

KEGG Class	Pathway Name	Human Genes in Pathway	Number of Genes in Pathway and on List of Human Orthologs Associated with Acute Effect of Toluene	Ortholog Gene Name
1.11	Drug Metabolism–cytochrome P450	142	1	CYP3A4
3.3	Neuroactive ligand–receptor interaction	294	1	ADRA2A
5.6	Glutamatergic synapse	83	1	SHANK
5.6	Cholinergic synapse	86	0	
5.6	GABAergic synapse	63	0	
5.6	Dopaminergic synapse	81	0	
5.6	Serotonergic synapse	82	1	HTR1B
5.6	Synaptic vesicle cycle	40	1	ATP6V1H

The number of annotated genes in each of the 6 ion channel systems ranged from 40 to 294; at most, one of these genes was found in any of these pathways among the human orthologs identified in this experiment.

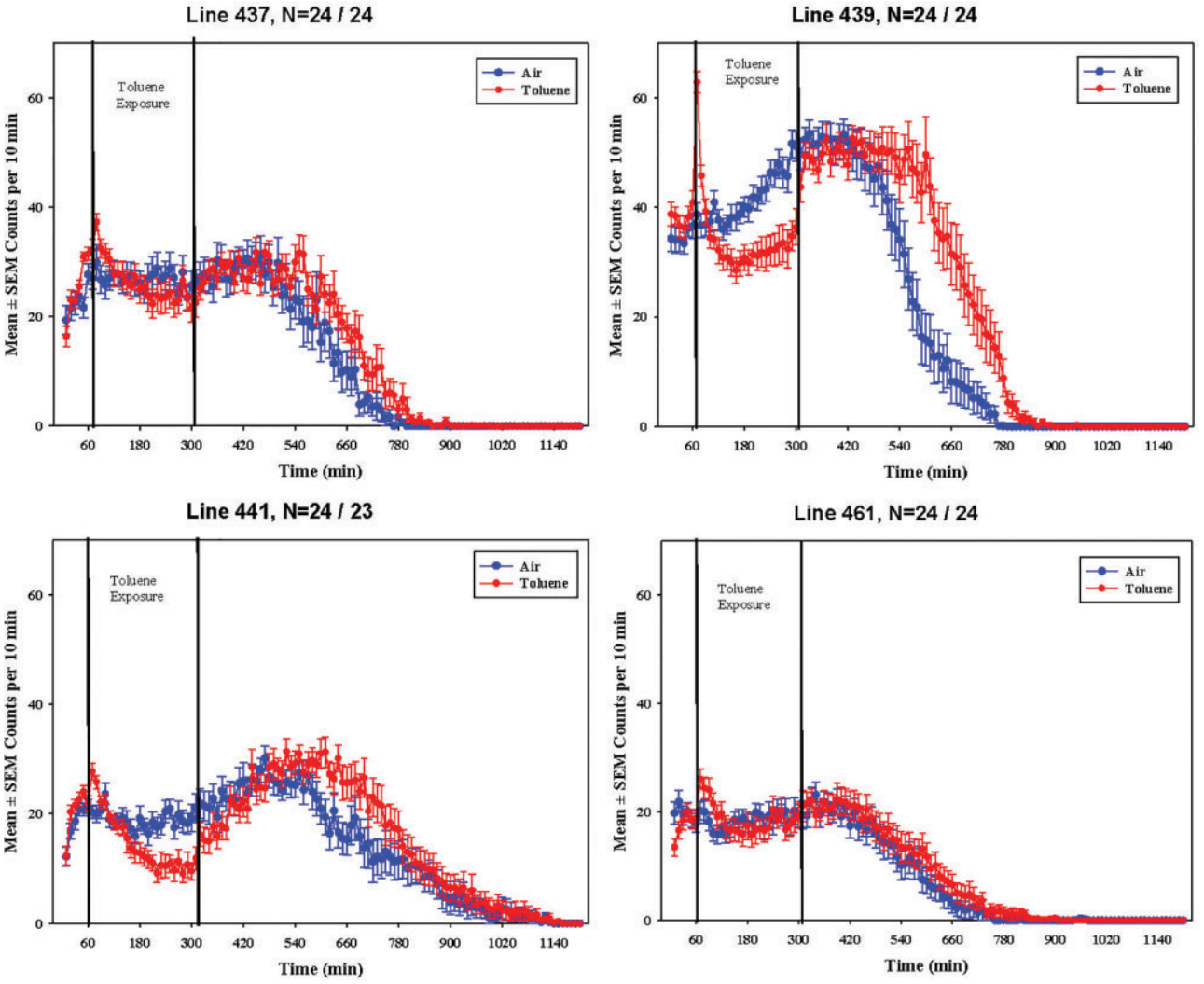

FIG. 2. Profiles of the time course of motor activity of 4 representative strains of flies from the DGRP. Activity counts per 10-min block are shown for flies in air (blue) and toluene (red) across the 120 ten-min blocks (20 h) of measurement. Toluene exposure began after a 60-min acclimation period and ended 4 h later. Activity reached a maximum after exposure in each strain and declined late in the observation period as the flies died. The N values above each panel represent the numbers of flies in the air and toluene groups respectively.

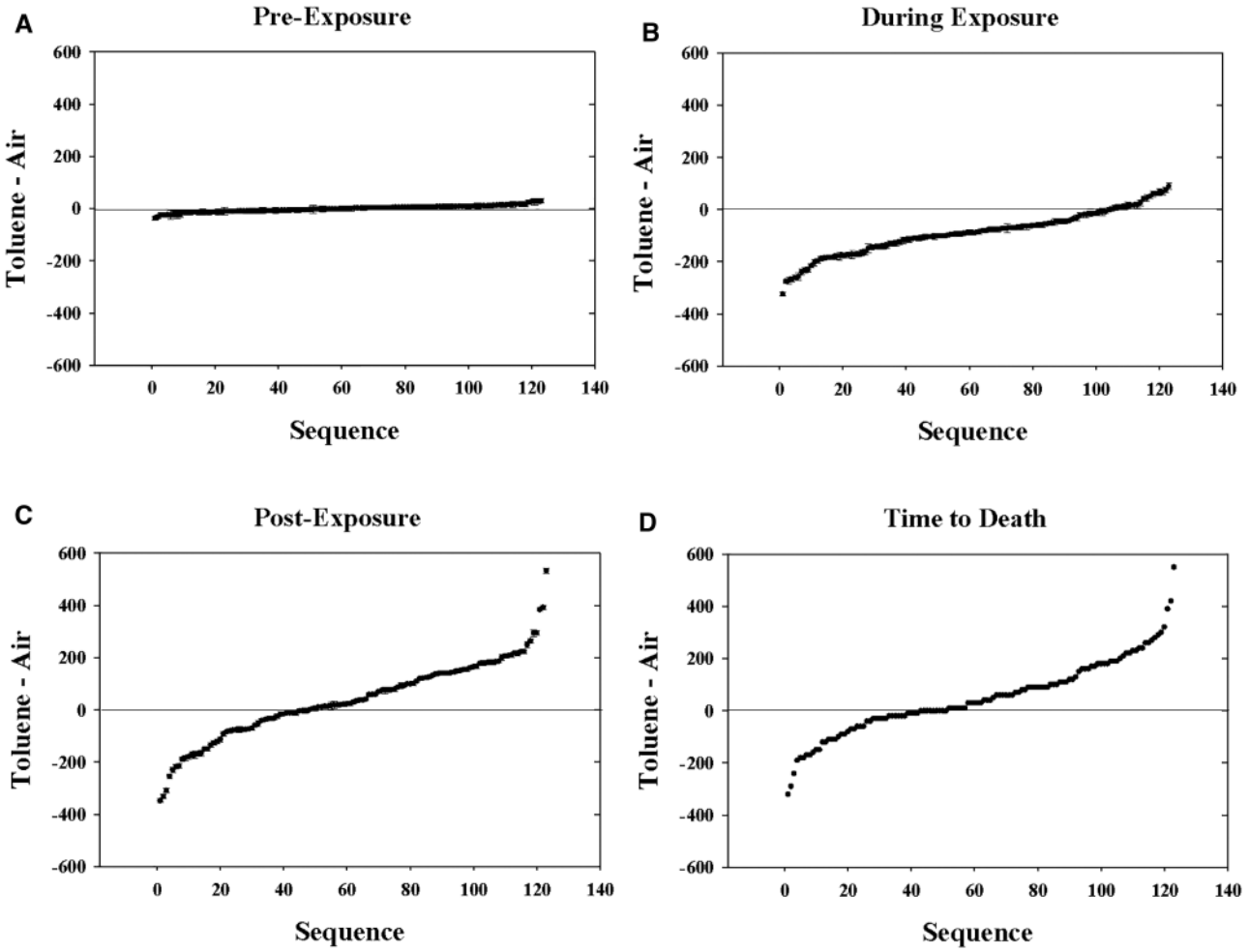

FIG. 3. Four effects of toluene distributed across the 123 lines of DGRP flies, ordered by the magnitude of the effect. The difference scores on the ordinate are displayed in rank order for each endpoint to show the proportions of flies that were more (values > 1) or less (values < 1) active in toluene than in air. Relative to activity in air, activity was suppressed by toluene in 104 of the 123 lines during exposure, and was increased after toluene exposure in 75 of the 123 lines. The rank-ordered sequences of the lines differed across endpoints.

being involved with any of the toluene-sensitive neuronal ion channels.

Similarly, an unbiased query of the Ingenuity Pathway database revealed 15 pathways associated significantly with the human orthologs identified in this study. However, the strength of the association of the ortholog list with any specific pathway is weak, indicating that the behavioral response to toluene cannot be linked closely to any particular pathway. In addition, and more pertinently, none of these pathways involve ion channels, and just 2 seem likely to be involved in activity of the nervous system (cAMP-mediated signaling and G-Protein coupled receptor signaling), but are by no means unique to the nervous system.

Examination of specific candidate *Drosophila* genes and human orthologs reveals a similarly weak connection to processes involving either ion channels or adult behavior. Four of the 66 candidate *Drosophila* genes have been associated with nervous system functions potentially relevant to the behavioral response to toluene. For example, a GO biological process associated with *5-HT1B* involves serotonin receptor signaling, and toluene has been shown to affect serotonin receptors. Less pertinently, *Or63a* is involved in the sensory perception of smell; *Sema-2a* is involved with drinking behavior; and *tun* with learning or memory.

Human orthologs have been identified for 3 of these candidate genes (not for *Or63a*). Examination of the biological processes associated with these 3 orthologs shows involvement with: G-protein coupled receptor signaling (*5HT1B*/HTR1B), apoptotic processes and axon guidance (*Sema-2a*/SEMA3A), and cellular protein modification (*tun*/WDYHV1). These general processes are not obviously linked to ion channel functions specifically. However, 2 other human orthologs on the list involve processes that could be related to the narcotic effect of toluene: HOXD9 is associated with "adult locomotory behavior" (as well as "anterior/posterior pattern specification"), and SHANK1 with "adult behavior, associative learning".

Several reasons may be proposed for this failure to support the hypothesized relationship between the genes associated with toluene-induced suppression of motor activity in *Drosophila* and the primary mechanism of toluene-induced narcosis in mammals. First, the associations in the present analyses are statistical, and include substantial uncertainty. Specific mechanisms of toluene-induced effects on motor activity in insects were not investigated here, and it is possible that

Table 4. Results of a Query of the Ingenuity® Database for Canonical Pathways Associated at $P < .05$ With the Human Orthologs Identified in this Experiment

Ingenuity Canonical Pathway	Number of Molecules in Pathway	Number of Molecules from List	*P* value	Molecules from Human Ortholog List in Pathway	List/Path
cAMP-mediated signaling	218	4	.0017	HTR1B,ADRA2A,PDE4D,PDE4B	0.018348624
Sperm Motility	121	3	.0029	PDE4D,PDE4B,PTK7	0.024793388
G-Protein Coupled Receptor Signaling	256	4	.0030	HTR1B,ADRA2A,PDE4D,PDE4B	0.015625
tRNA Splicing	35	2	.0030	PDE4D,PDE4B	0.057142857
Creatine-phosphate Biosynthesis	5	1	.0117	MAP4K4	
Thioredoxin Pathway	6	1	.0141	TXNRD2	
SAPK/JNK Signaling	94	2	.0204	MINK1,MAP4K4	0.021276596
Bile Acid Biosynthesis, Neutral Pathway	13	1	.0302	CYP3A4	0.076923077
Gαi Signaling	120	2	.0324	HTR1B,ADRA2A	0.016666667
DNA Double-Strand Break Repair	14	1	.0324	GEN1	0.071428571
Gustation Pathway	121	2	.0324	PDE4D,PDE4B	0.016528926
Vitamin-C Transport	15	1	.0347	TXNRD2	0.066666667
Cellular Effects of Sildenafil	129	2	.0363	PDE4D,PDE4B	0.015503876
Cardiac β-adrenergic Signaling	133	2	.0389	PDE4D,PDE4B	0.015037594
Relaxin Signaling	135	2	.0398	PDE4D,PDE4B	0.014814815
Mean					0.033135127
SD					0.024733503

Few orthologs are known to participate in any of the pathways; none of the pathways specify ion channels or involve functions uniquely related to functions of ion channels.

associations pointing to genes involved in ion channel function were missed.

Second, the power of the present GWAS was limited by the use of only 123 of the 205 existing lines of the DGRP. Adding more lines would likely reveal more significant polymorphisms associated with the effect of toluene and alter the ranking of those that were identified in this experiment, but would not eliminate them. On the other hand, the lines that were included in this study were the most robust of 128 DGRP lines that were selected specifically to be free of heterozygous inversions and highly related pairs (Wen Huang, personal communication). Thus it is unlikely that an entire suite of polymorphisms associated with mechanisms of narcosis was missed in this GWAS.

Furthermore, the lack of association with mammalian narcosis pathways cannot be attributed to insufficient sequence variation among *Drosophila* genes linked to those pathways. A review of the toluene literature revealed 20 mammalian genes associated with specific ion channels that toluene has been shown to affect. *Drosophila* orthologs were found for 11 of these genes (or groups of genes). The number of SNPs in these orthologs ranged from 8 to 539 across the 123 lines of the DGRP that we tested, demonstrating substantial variation in most cases. In addition, none of these orthologs were associated significantly (at $P < 5 \times 10^{-5}$) with toluene inhalation in the GWAS (Supplementary File 2).

Third, it is possible that the mechanisms by which toluene suppresses behavior in insects may differ from those that do so in mammals. However, the close correspondence of the behavioral changes, and similarities in concentration-response and time course of the effects of toluene (Tatum-Gibbs *et al.*, 2015), as well as the known presence of ion channels in the insect nervous system (Frolov *et al.*, 2012; Salkoff *et al.*, 1992), suggest the contrary.

Fourth, the fact that a relatively small number of polymorphisms, *Drosophila* genes, and human orthologs were identified by this approach suggests that the mechanisms by which toluene alters motor activity may not engage biological processes at the genetic level. That is, variation in the genome may not be associated with the observed variation in this phenotype. This possibility is supported by the small and limited number of changes in gene expression observed in rats after acute (Hester et al., 2011; Royland *et al.*, 2012) or repeated (Hester *et al.*, 2012) exposure to toluene.

Fifth, it is possible that the 4-h exposure time was not sufficient to elicit significant genomic changes associated with the narcotic effect of toluene in fruit flies. Whereas the effect of varying the duration of exposure to toluene has not to our knowledge been studied in *Drosophila*, alterations in gene expression increase with exposure duration in humans (Hong *et al.*, 2015). On the other hand, a far greater number of differentially expressed genes were observed in rat brain after a single 6-hr exposure to toluene than after 13 weeks of daily 6-h exposures (Hester et al., 2011; 2012). In any case, a 4-h exposure was sufficient to induce narcosis in the flies in this study, but did not reveal genes associated with any of the neurochemical mechanisms known to mediate that effect in mammals.

Finally, the currently available information about the functions of the candidate *Drosophila* genes and the human orthologs identified here may not be complete enough to reveal the hypothesized relationship between exposure to toluene and actions on ion channels. Further annotations will reveal more processes involving the genes that were identified here, some of which may link more directly to likely mechanisms of toluene-induced changes in motor activity.

Genetic Effects of Acute Toluene Exposure

That said, the genes and orthologs revealed by this analysis represent the genomic markers of the acute narcotic effect of toluene in *Drosophila*. Directly comparable studies in mammals have not to our knowledge been conducted. In a study of the effects of toluene in aging rats, Royland *et al.* (2012) dosed Brown Norway rats at 4, 12, or 24 months of age orally with

toluene and examined changes in gene expression using Affymetrix Rat GeneChips. Hippocampi were obtained from the animals at sacrifice 4 hr after exposure, a time when the toluene would have cleared the animal (Kenyon *et al.*, 2008). Royland *et al.* found 183 genes associated significantly (with > 1.25-fold difference in expression) as a function of age, 2 genes that varied significantly with toluene dose (*Lrpap1* and *Ralgps2*), and 56 genes associated with the dose-age interaction. IPA analysis of all statistically-significant genes, regardless of fold change, yielded 2 networks of genes, one containing down-regulated transcripts associated with cellular assembly/morphology and cell-mediated immune responses, and the other containing transcripts involved with neuronal growth and plasticity.

Whereas both *Lrpap1* and *Ralgps2* are "associated with dementia and Alzheimer's disease" according to Royland *et al.*, *Lrpap1* is known to be involved with lipoprotein expression in the brain, and *Ralgps2* with small GTPase-mediated signal transduction, rather than signaling via ion channels. Relaxing the criterion of fold change >1.25 revealed no further overlap between the genes identified by Royland *et al.* and those in the present study.

In a study of the effects of narcotizing concentrations of toluene on hippocampal neurogenesis in rats, Yoon *et al.* (2015) exposed adult rats to 7000 ppm of toluene for 4 hr and assessed clinical effects during exposure and effects on the brain 1, 2, 5 and 8 days after exposure. Two animals died during exposure and the others showed clear signs of intoxication, including "moderate irregular respiration, subdued behavior, prone position, and abnormal gait," which disappeared within 24 h after the end of exposure. Reductions in 2 markers of adult neurogenesis, Ki-67 and doublecortin, were found in hippocampi of exposed rats that persisted for 8 days after exposure.

It is intriguing that this narcotizing exposure affected neurogenesis in rats, and one of the *Drosophila* genes identified in the current study, *Vap-33B*, is associated with neurogenesis in flies, though the human ortholog *MOSPD2* has not been similarly annotated. Thus some connection between the current results and an acute effect of high-level exposure is apparent in a mammalian model of toluene intoxication, despite the fact that the changes in the markers in the CNS were observed after exposure had ceased.

In conclusion, whereas this method succeeded in identifying genes associated with the narcotic response of fruit flies to toluene, no clear and compelling relationship between those genes and mechanisms known to mediate narcosis in mammals was found. Thus, given the present state of knowledge about gene function, this approach does not appear to be useful for the identification of adverse outcome pathways for acute exposure to narcotic substances like toluene. Nevertheless, while the candidate genes identified here may not be linked to narcosis via the predicted ion channel mechanisms, they may suggest other currently unrecognized processes involved in narcosis.

The broader implication of these results suggests that high-throughput screening approaches to understanding, predicting, and managing effects of environmental chemicals on the nervous system lack the mechanistic framework necessary for use in risk assessment. In this case, even a simple assay of motor activity appears to involve a complexity of biological processes that is beyond the scope of current knowledge. Given that the mechanisms mediating more complex effects of other classes of compounds are even less well understood, the general applicability of these screening approaches must be regarded very cautiously. Therefore, calls to eliminate mammalian bioassays from toxicity testing protocols appear to be premature.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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REFERENCES

U.S. EPA. (2009). The U.S. Environmental Protection Agency's Strategic Plan for Evaluating the Toxicity of Chemicals. The ORD Future of Toxicology Working Group.

Bale, A. S., Meacham, C. A., Benignus, V. A., Bushnell, P. J., and Shafer, T. J. (2005a). Volatile organic compounds inhibit human and rat neuronal nicotinic acetylcholine receptors expressed in Xenopus oocytes. *Toxicol. Appl. Pharmacol.* **205**, 77–88.

Bale, A. S., Tu, Y., Carpenter-Hyland, E. P., Chandler, L. J., and Woodward, J. J. (2005b). Alterations in glutamatergic and gabaergic ion channel activity in hippocampal neurons following exposure to the abused inhalant toluene. *Neuroscience* **130**, 197–206.

Beckstead, M. J., Weiner, J. L., Eger, E. I., 2nd, Gong, D. H., and Mihic, S. J. (2000). Glycine and gamma-aminobutyric acid(A) receptor function is enhanced by inhaled drugs of abuse. *Mol. Pharmacol.* **57**, 1199–1205.

Benignus, V. A. (1981). Health effects of toluene: a review. *Neurotoxicology* **2**, 567–588.

Bowen, S. E., Batis, J. C., Paez-Martinez, N., and Cruz, S. L. (2006). The last decade of solvent research in animal models of abuse: mechanistic and behavioral studies. *Neurotoxicol. Teratol.* **28**, 636–647.

Brown, E. B., Layne, J. E., Zhu, C., Jegga, A. G., and Rollmann, S. M. (2013). Genome-wide association mapping of natural variation in odour-guided behaviour in Drosophila. *Genes Brain Behav.* **12**, 503–515.

Bushnell, P. J., Evans, H. L., and Palmes, E. D. (1985). Effects of toluene inhalation on carbon dioxide production and locomotor activity in mice. *Fundam. Appl. Toxicol.* **5**, 971–977.

Bushnell, P. J., Shafer, T. J., Bale, A. S., Boyes, W. K., Simmons, J. E., Eklund, C., and Jackson, T. L. (2005). Development of an exposure-dose-response model for the acute neurotxocity of organic solvents: Overview and progress on in vitro models and dosimetry. *Environ. Toxicol. Pharmacol.* **19**, 607–614.

Collins, F. S., Gray, G. M., and Bucher, J. R. (2008). Toxicology. Transforming environmental health protection. *Science* **319**, 906–907.

Cruz, S. L., Mirshahi, T., Thomas, B., Balster, R. L., and Woodward, J. J. (1998). Effects of the abused solvent toluene on recombinant N-methyl-D-aspartate and non-N-methyl-D-aspartate receptors expressed in Xenopus oocytes. *J. Pharmacol. Exp. Ther.* **286**, 334–340.

Dix, D. J., Kavlock, R. J., Houck, J., Martin, M. T., Judson, R., (2008). "ToxCast." Retrieved 8 August, 2008, from www.epa.gov/ncct/toxcast.

Firestone, M., Kavlock, R., Zenick, H., and Kramer, M. and U.S. EPA (2010). The U.S. environmental protection agency strategic plan for evaluating the toxicity of chemicals. *J. Toxicol. Environ. Health B* **13**, 139–162.

Frolov, R. V., Bagati, A., Casino, B., and Singh, S. (2012). Potassium channels in Drosophila: historical breakthroughs, significance, and perspectives. *J. Neurogenet.* **26**, 275–290.

Hester, S. D., Johnstone, A. F. M., Boyes, W. K., Bushnell, P. J., and Shafer, T. J. (2011). Acute toluene exposure alters expression of genes associated with synaptic structure and function. *Neurotoxicol. Teratol.* **33**, 521–529.

Hester, S. D., Johnstone, A. F. M., Boyes, W. K., Bushnell, P. J., and Shafer, T. J. (2012). Transcriptional responses in rat brain associated with sub-chronic toluene inhalation are not predicted by effects of acute toluene inhalation. *Neurotoxicol. Teratol.* **34**, 530–533.

Hinman, D. J. (1987). Biphasic dose–response relationship for effects of toluene inhalation on locomotor activity. *Pharmacol. Biochem. Behav.* **26**, 65–69.

Hong, J. Y., Yu, S. Y., Kim, S. Y., Ahn, J. J., Kim, Y., Kim, G. W., Son, S. W., Park, J. T., and Hwang, S. Y. (2015). Association analysis of toluene exposure time with high-throughput mRNA expressions and methylation patterns using in vivo samples. *Environ. Res.* **146**, 59–64.

Huang, W., Massouras, A., Inoue, Y., Peiffer, J., Ramia, M., Tarone, A. M., Turlapati, L., Zichner, T., Zhu, D., Lyman, R. F., *et al.* (2014). Natural variation in genome architecture among 205 Drosophila melanogaster Genetic Reference Panel lines. *Genome Res* **24**, 1193–1208. doi: 10.1101/gr.171546.113.

Jordan, K. W., Craver, K. L., Magwire, M. M., Cubilla, C. E., Mackay, T. F., and Anholt, R. R. (2012). Genome-wide association for sensitivity to chronic oxidative stress in Drosophila melanogaster. *PLoS One* **7**, e38722.

Judson, R., Richard, A., Dix, D. J., Houck, K., Martin, M., Kavlock, R., Dellarco, V., Henry, T., Holderman, T., Sayre, P., Tan, S., Carpenter, T., and Smith, E. (2008). The toxicity data landscape for environmental chemicals. *Environ. Health Perspect.* **117**,

Judson, R. S., Houck, K. A., Kavlock, R. J., Knudsen, T. B., Martin, M. T., Mortensen, H. M., Reif, D. M., Rotroff, D. M., Shah, I., Richard, A. M., and Dix, D. J. (2009). *In vitro* screening of environmental chemicals for targeted testing prioritization: The ToxCast Project. *Environ. Health Perspect.* **118**,

Kamatchi, G. L., Chan, C. K., Snutch, T., Durieux, M. E., and Lynch, C. III, (1999). Volatile anesthetic inhibition of neuronal Ca channel currents expressed in Xenopus oocytes. *Brain Res.* **831**, 85–96.

Kavlock, R., Chandler, K., Houck, K., Hunter, S., Judson, R., Kleinstreuer, N., Knudsen, T., Martin, M., Padilla, S., Reif, D., *et al.* (2012). Update on EPA's ToxCast program: providing high throughput decision support tools for chemical risk management. *Chem. Res. Toxicol.* **25**, 1287–1302.

Kenyon, E. M., Benignus, V. A., Eklund, C. R., Highfill, J. W., Oshiro, W. M., Samsam, T. E., and Bushnell, P. J. (2008). Modeling the toxicokinetics of inhaled toluene in rats: Influence of physical activity and feeding status. *J. Toxicol. Environ. Health A* **71**, 249–265.

Mackay, T. F. C., Richards, S., Stone, E. A., Barbadilla, A., Ayroles, J. F., Zhu, D., Casillas, S., Han, Y., Magwire, M. M., Cridland, J. M., *et al.* (2012). The *Drosophila melanogaster* Genetic Reference Panel. *Nature* **482**, 173–178.

McQuilton, P., St Pierre, S. E., and Thurmond, J. (2012). FlyBase 101–the basics of navigating FlyBase. *Nucleic Acids Res.* **40**, D706–D714.

Mihic, S. J., Ye, Q., Wick, M. J., Koltchine, V. V., Krasowski, M. D., Finn, S. E., Mascia, M. P., Valenzuela, C. F., Hanson, K. K., Greenblatt, E. P., *et al.* (1997). Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. *Nature* **389**, 385–389.

Morozova, T. V., Huang, W., Pray, V. A., Whitham, T., Anholt, R. R. H., and Mackay, T. F. C. (2015). Polymorphisms in early neurodevelopmental genes affect natural variation in alcohol sensitivity in adult drosophila. *BMC Genomics* **16**, 865.

Mullikin-Kilpatrick, D., and Treistman, S. N. (1993). Electrophysiological studies on calcium channels in naive and ethanol-treated PC12 cells. *Alcohol Alcohol* **Suppl. 2**, 385–389.

Okuda, M., Kunitsuga, I., Kobayakawa, S., and Hobara, T. (2001). Inhibitory effect of 1,1,1-trichloroethane on calcium channels of neurons. *J. Toxicol. Sci.* **26**, 169–176.

Rieger, D., Fraunholz, C., Popp, J., Bichler, D., Dittmann, R., and Helfrich-Forster, C. (2007). The fruit fly Drosophila melanogaster favors dim light and times its activity peaks to early dawn and late dusk. *J. Biol. Rhythms* **22**, 387–399.

Royland, J. E., Kodavanti, P. R. S., Schmid, J. E., and MacPhail, R. R. (2012). Toluene Effects on Gene Expression in the Hippocampus of Young Adult, Middle-Age, and Senescent Brown Norway Rats. *Toxicol. Sci.* **126**, 193–212.

Salkoff, L., Baker, K., Butler, A., Covarrubias, M., Pak, M. D., and Wei, A. (1992). An essential 'set' of K+ channels conserved in flies, mice and humans. *Trends Neurosci.* **15**, 161–166.

Shukla, S. J., Huang, R., Austin, C. P., and Xia, M. (2010). The future of toxicity testing: a focus on in vitro methods using a quantitative high-throughput screening platform. *Drug Discov. Today* **15**, 997–1007.

Swarup, S., Huang, W., Mackay, T. F., and Anholt, R. R. (2013). Analysis of natural variation reveals neurogenetic networks for Drosophila olfactory behavior. *Proc. Natl. Acad. Sci. USA* **110**, 1017–1022.

Tatum-Gibbs, K. R., McKee, J. M., Higuchi, M., and Bushnell, P. J. (2015). Effects of toluene, acrolein and vinyl chloride on motor activity of *Drosophila melanogaster*. *Neurotoxicol. Teratol.* **47**, 114–124.

Tegeris, J. S., and Balster, R. L. (1994). A comparison of the acute behavioral effects of alkylbenzenes using a functional observational battery in mice. *Fundam. Appl. Toxicol.* **22**, 240–250.

Tice, R. R., Austin, C. P., Kavlock, R. J., and Bucher, J. R. (2013). Improving the human hazard characterization of chemicals: a tox21 update. *Environ. Health Perspect.* **121**, 756–765.

Tillar, R., Shafer, T. J., and Woodward, J. J. (2002). Toluene inhibits voltage sensitive calcium channels expressed in pheochromocytoma cells. *Neurochem. Int.* **41**, 391–397.

Weber, A. L., Khan, G. F., Magwire, M. M., Tabor, C. L., Mackay, T. F., and Anholt, R. R. (2012). Genome-wide association analysis of oxidative stress resistance in Drosophila melanogaster. *PLoS One* **7**, e34745.

Yoon, J-h., Seo, H. S., Lee, J., Moon, C., and Lee, K., (2015). Acute high-level toluene exposure decreases hippocampal neurogenesis in rats. *Toxicol. Industr. Health.* [Epub ahead of print] PMID: 26475279.