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Insights from intoxicated Drosophila

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ABSTRACT

Our understanding of alcohol use disorder (AUD), particularly alcohol's effects on the nervous system, has unquestionably benefited from the use of model systems such as *Drosophila melanogaster*. Here, we briefly introduce the use of flies in alcohol research, and highlight the genetic accessibility and neurobiological contribution that flies have made to our understanding of AUD. Future fly research offers unique opportunities for addressing unresolved questions in the alcohol field, such as the neuromolecular and circuit basis for cravings and alcohol-induced neuroimmune dysfunction. This review strongly advocates for interdisciplinary approaches and translational collaborations with the united goal of confronting the major health problems associated with alcohol abuse and addiction.

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Introduction

For over a century, *Drosophila melanogaster* (Greek for "dew-loving black-bellied"), hereinafter flies, have reigned as a premier genetic model organism. Over 200 human diseases can be modeled in flies, including alcohol use disorder (AUD) (http://flybase.org/lists/FBhh/). Within the past 20 years, flies have proven to be a promising system in which to discover the genetic underpinnings of alcohol sensitivity, tolerance, and addiction (Devineni & Heberlein, 2013; Kaun, Devineni, & Heberlein, 2012; Park, Ghezzi, Wijisekera, & Atkinson, 2017; Rodan & Rothenfluh, 2010). Fly research continues to provide exceptional genetic tools, bioinformatic approaches, and behavioral analyses that extend and refine our knowledge of alcohol abuse and addiction.

In nature, flies consume and lay their eggs on fermenting fruit (up to 4.5% ethanol) (Dudley, 2004; Gibson, May, & Wilks, 1981). Despite this ecological routine, flies are still susceptible to alcohol's intoxicating effects. Similar to mammals, as internal alcohol levels rise, flies become hyperactive, uncoordinated, and eventually sedated (Wolf, Rodan, Tsai, & Heberlein, 2002). Flies also show both rapid (pharmacokinetic) and long-term (pharmacodynamic) tolerance upon multiple alcohol exposures (Berger, Heberlein, & Moore, 2004; Scholz, Ramond, Singh, & Heberlein, 2000). To screen for genes underlying these behaviors, researchers have designed clever high-throughput assays. For example, alcohol-induced loss-of-righting and sedation are readily quantified by the Inebriometer, a baffled fly elution column, or in the Booze-o-mat, an array of transparent open-field vapor tubes (Cohan & Graf, 1985; Wolf et al., 2002). Numerous other apparatuses and paradigms have also been ingeniously engineered to measure various alcohol-associated behaviors – odor preference (olfactory trap assay) (Reed, 1938), volitional consumption (CAFÉ assay) (Devineni & Heberlein, 2009; Ja et al., 2007), egg-laying choice (Azanchi, Kaun, & Heberlein, 2013; Richmond & Gerking, 1979), addiction-like behavior (cue-induced memory of intoxication) (Kaun, Azanchi, Maung, Hirsh, & Heberlein, 2011), and a fetal alcohol syndrome model (McClure, French, & Heberlein, 2011). These efforts have collectively led to interesting correlations between alcohol-associated genes and molecular pathways associated with specific behaviors, including feeding (Landayan & Wolf, 2015), memory (Berger et al., 2008; Robinson & Atkinson, 2013; Zars, 2009), and circadian regulation (De Nobrega & Lyons, 2016; van der Linde & Lyons, 2011).

The ease and inexpensive maintenance of flies perpetuates the creation and implementation of remarkable genetic and neurobiological technologies. With these advances, and vast wealth of

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accrued knowledge, flies afford unprecedented scientific perspective from higher order behavior in whole organisms to single synapses in complex neural circuitry. This unrestricted experimental flexibility has and will continue to shape basic genetic and neurobiological principles in alcohol research. Here, we review how genetic, neuroanatomical, and neuroimmune approaches in flies can aid in our understanding of human AUD (Fig. 1).

Fly genetics and contributions to alcohol research

Despite having diverged from humans over 780 million years ago (Hedges, Dudley, & Kumar, 2006; Shih, Hodge, & Andrade-Navarro, 2015), the fly genome contains functional orthologs of roughly 75% of known human disease-related genes (Chien, Reiter, Bier, & Gribskov, 2002; Lloyd & Taylor, 2010; Reiter, Potocki, Chien, Gribskov, & Bier, 2001). Because of this, there exists a dizzying array of fly genetic resources, including countless mutant and transgenic stocks, as well as compilations of datasets (Mohr, Hu, Kim, Housden, & Perrimon, 2014). Unprecedented genetic specificity, from specific populations of cells down to single neurons (Pfeiffer et al., 2008, 2010), permits unparalleled access for molecular control (gene knock-out, knock-down, overexpress, etc.) via several genetic engineering and expression system techniques (Cre, CRISPR/Cas, Gal4/UAS, LexA/LexOp, etc.) (Bier, Harrison, O'Connor-Giles, & Wildonger, 2018; McGuire, Roman, & Davis, 2004).

The compact fly genome — roughly 120 million base pairs vs. ~3 billion in mouse and human — has also been considerably leveraged for high-throughput whole-genome and transcriptomic sequencing. Work in fly neurobiology, for instance, now includes single cell RNA-seq (Crocker, Guan, Murphy, & Murthy, 2016; Croset, Treiber, & Waddell, 2017; Karaiskos et al., 2017) and RNA-seq from specific cell types (Cleary, 2018), and isolated nuclei of target cell-types (INTACT) (Henry, Davis, Picard, & Eddy, 2012; Pankova & Borst, 2016). Furthermore, a recent transcriptomic study found extensive similarities between human and fly extracellular vesicles, such as exosomes, suggesting conserved mechanisms for RNA-mediated intercellular communication (Lefebvre et al., 2016).

Relevance to alcohol research

The application of model organism research is particularly relevant for elucidating some of the heritable genetic predispositions for developing an AUD. Unfortunately, large-scale forward genetic screening in mammals poses financial, practical, and ethical drawbacks. Flies, however, are ideal for forward genetic approaches because of their rapid development (~12 days), robust fecundity (>100 offspring/female), and genetic toolkit for large-scale exhaustive approaches. To date, several genetic discoveries in fly screens have been translated to rodent and human alcohol models (Corl et al., 2009; Kapfhamer et al., 2013; Lasek, Gesch, Giorgetti, Kharazia, & Heberlein, 2011; Lasek, Giorgetti, Berger, Tayor, & Heberlein, 2011; Lasek, Lim et al., 2011; Maiya, Mangieri, Morrisett, Heberlein, & Messing, 2015; Maiya et al., 2012). While human genome-wide association studies (GWAS) for alcohol sensitivity and tolerance have discovered several significant variants that contribute to addiction (Hancock, Markunas, Bierut, & Johnson, 2018), GWAS research may be hampered by selection bias, confounding environmental factors, low power of infrequent alleles, and limitation to SNP linkage disequilibrium. Genomic studies in flies, however, have addressed these challenges using artificially selected and inbred lines to yield qualitative and quantitative translational insights (Morozova, Mackay, & Anholt, 2014; Morozova et al., 2009).

Like many other areas of science, the alcohol field is striving to interpret and apply information from bioinformatic RNA-seq data, particularly from brain tissue (Farris & Mayfield, 2014). A small number of fly microarray studies have examined alcohol-induced changes in gene expression. They have identified differentially expressed genes corresponding to alcohol-regulated genes in mammalian alcohol models, as well as implicated new genes whose biological functions still remain unknown (Awofala, Davies,

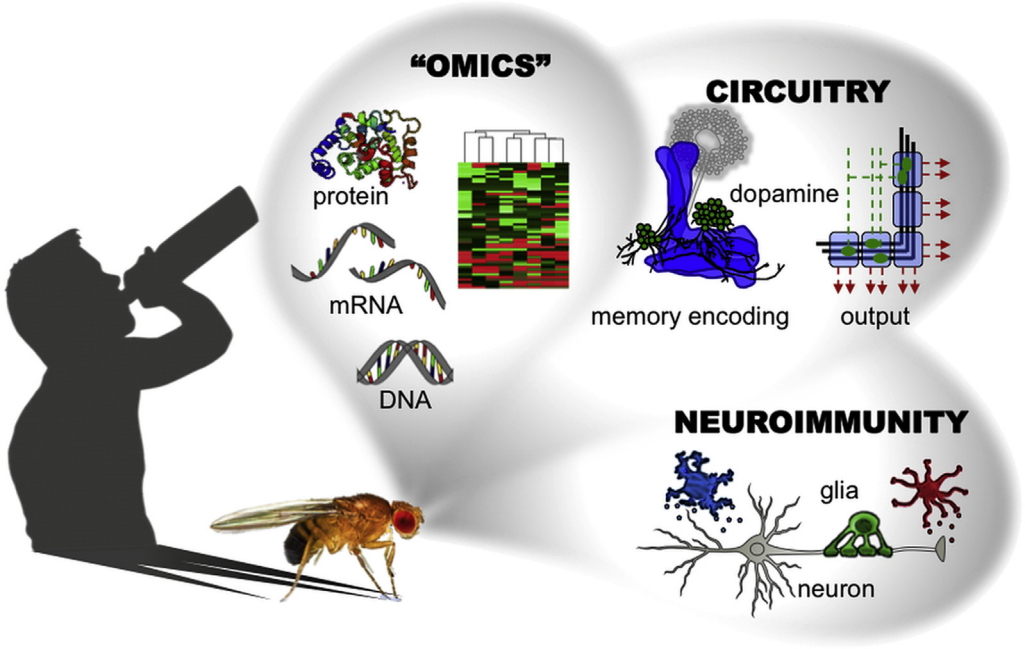


Fig. 1. Alcohol research in flies complements our understanding of human alcohol use disorder (AUD). The fly's small and refined genome provides integration and translational perspective for big data ("omics"). Precise neuroanatomical manipulation in flies provides insight into the basic principles underlying modulation of complex, yet tractable, reward circuitry. The fly's powerful genetic tools and highly conserved innate immune signaling system sheds light on complex neuron-glia interactions associated with AUD.

& Jones, 2012; Kong, Allouche et al., 2010; Morozova, Anholt, & Mackay, 2006, 2007; Urizar, Yang, Edenberg, & Davis, 2007). The limited detection capacity of microarrays for modestly expressed transcripts, such as neurotransmitter receptors and ion channels, suggests that both fly and mammal alcohol research will benefit more from high-throughput sequencing approaches. Due to the heterogeneity of mammalian neuronal tissues and complex spatiotemporal dynamics of alcohol responsivity, extensive bioinformatic projects are likely to be more feasible in flies.

Alcohol-induced changes in epigenetic and post-translational regulation are also widely appreciated in mammalian and fly alcohol models (Berkel & Pandey, 2017; Pandey, Kyzar, & Zhang, 2017; Ponomarev, 2013). In flies, genome-wide changes in histone acetylation, and the interplay between HDAC and HAT activity, underlie alcohol tolerance (Engel et al., 2016; Ghezzi, Li, Lew, Wijesekera, & Atkinson, 2017; Ghezzi et al., 2013; Park et al., 2017). Flies have long been used to understand the role of chromosome architecture in genome function, and recent epigenomic mapping of histone modifications and chromatin factors has partitioned genomic domains that contribute to chromosome folding and the regulation of gene expression (Schwartz & Cavalli, 2017). The ability to rapidly identify and test candidate genes makes flies an opportune system in which to advance our understanding of epigenetic and post-translational mechanisms underlying alcohol responses.

Neural circuitry and behavior

The remarkable conservation of neurobiological function has enabled translational wisdom from unexpected model systems such as *Caenorhabditis elegans* (roundworm), *Doryteuthis pealeii* (squid), *Aplysia californica* (sea slug), and others. The fly nervous system has also become a leading model in neuroscience with its small, but impressive, central nervous system comprised of ~10,000 larval and ~100,000 adult neurons capable of performing complex behaviors (feeding, sleep, courtship, aggression, memory, etc.). The fly's genetic arsenal affords, among many things, the ability to label, ablate, or temporarily silence or activate neural cells of interest (Mohammad et al., 2017; Sivanantharajah & Zhang, 2015).

Recent intersectional genetic methods in flies have further refined the spatiotemporal control of transgenes, particularly in the nervous system (Dolan et al., 2017; Luan, Peabody, Vinson, & White, 2006; McGuire, Mao, & Davis, 2004; Ting et al., 2011). This unprecedented, nearly single-cell, access allows for precise definition and control of complex neural networks underlying behavior. Additionally, in contrast to the invasive procedures and limited genetic flexibility encountered in mammalian neurobiological research, entire flies are receptive to induction techniques such as optogenetics, thermogenetics, and drug-inducible gene switches. Recent pioneering work has even mapped neural substrates of behavior by characterizing the effect of systematically activating thousands of individual neural-labeled lines (Robie et al., 2017). This work has not only generated an easy-to-use online interface for perusing the rich repertoire of corresponding behavior-neural subsets, but has also produced open source machine-vision learning software applicable for any model organism research (http://research.janelia.org/bransonlab/FlyBowl/BehaviorResults/ and https://github.com/kristinbranson/BABAM).

To reveal stereotyped connectivity patterns in the fly nervous system, exciting new trans-synaptic mapping tools have just debuted, such as trans-Tango (Talay et al., 2017), TRACT (Huang et al., 2017), or the synthetic Notch receptor (synNQ) system (He, Huang, & Perrimon, 2017). Computational registration of neuroanatomical images to a common fly brain then facilitates neuronal track identification and categorization for future hypothesis-driven research (Ostrovsky, Cachero, & Jefferis, 2013; Peng et al., 2011). Fly brain connectomes have also recently become available, which closely complement anatomy obtained from light microscopy (Schlegel, Costa, & Jefferis, 2017). Combining a functional behavioral approach with the connectome of the first instar larva (Schneider-Mizell et al., 2016) has revealed circuit properties for touch (Takagi et al., 2017), learning and memory (Eichler et al., 2017), response to a mechanosensory stimulus (Jovanic et al., 2016), locomotion (Fushiki et al., 2016; Ohyama et al., 2015), and food intake (Schlegel et al., 2016). Similar approaches with the adult fly visual system (Takemura, Nern, et al., 2017) have revealed circuit mechanisms underlying visual motion (Strother et al., 2018). With the connectome of the adult mushroom body, an important memory-encoding structure, now available (Eichler et al., 2017; Takemura, Aso, et al., 2017), understanding the circuit basis of memory formation and expression is imminent. An electron microscopy dataset of an entire adult fly brain has also been completed (Zheng et al., 2017), and within a few years, anatomical framework will be fully annotated in order to guide functional studies (Schlegel et al., 2017).

Relevance to alcohol research

The importance of the dopaminergic system has long been appreciated in understanding drugs of abuse. Various aspects of alcohol-associated phenomena are linked to dopaminergic neuron tonic/phasic firing and alteration of D2-like receptor sensitivity (Volkow, Fowler, Wang, Swanson, & Telang, 2007). The heterogeneity, complex interconnectivity, and temporal dynamics of mammalian dopaminergic circuitry are technical research challenges for achieving non-invasive and precise manipulation of circuit networks (Ichinose, Tanimoto, & Yamagata, 2017). As in mammals, the fly dopaminergic system is important for determining internal state and behavior, including playing a prominent role in appetitive and aversive stimuli perception (Cognigni, Felsenberg, & Waddell, 2017; Kaun & Rothenfluh, 2017; Scaplen & Kaun, 2016). Similarly, dopamine in flies is also required for alcohol's acute locomotor and rewarding effects (Bainton et al., 2000; Kaun et al., 2011; Kong, Woo et al., 2010).

In the fly adult central nervous system, there are 11 clusters of dopaminergic neurons that project throughout the brain to various neuropil structures (Aso, Hattori, et al., 2014; Mao & Davis, 2009; Milyaev et al., 2012). The mushroom body is a well-defined neuropil structure required for many forms of learning and memory, and is extensively innervated by two clusters of dopaminergic neurons in a compartmentalized fashion (for a more in-depth review, please see Kaun & Rothenfluh, 2017; Scaplen & Kaun, 2016). These dopaminergic projections align with cholinergic, GABAergic, or glutamatergic mushroom body output neurons that project to other brain regions (Aso, Hattori, et al., 2014). This neuroanatomical framework provides a tractable foundation for investigating the basic principles underlying reward memory.

The mushroom body has been implicated in alcohol-induced hyperactivity (King et al., 2011), tolerance (Engel et al., 2016), and in the acquisition and retrieval of alcohol-cue association memories (Aso, Sitaraman, et al., 2014; Kaun et al., 2011; Robinson, Khurana, & Atkinson, 2013). Recent neuroanatomical and functional studies are enhancing our understanding of dopaminergic/mushroom body circuitry (Aso & Rubin, 2016; Berry, Cervantes-Sandoval, Nicholas, & Davis, 2012; Cohn, Morantte, & Ruta, 2015; Waddell, 2016; Yamagata et al., 2015), and will provide a detailed anatomical framework for exploring the synaptic and molecular mechanisms by which alcohol regulates complex reward networks. These efforts can help determine the basic neurobiological and circuitry principles governing alcohol addiction.

Fly research has also identified molecular mechanisms linking internal state and alcohol consumption. Shohat-Ophir et al. showed that repeated sexual rejection in male flies increases alcohol consumption, and that the balance of neuropeptide Y-like (NPF) signaling is critical for perceiving the rewarding properties of sex and alcohol (Shohat-Ophir, Kaun, Azanchi, Mohammed, & Heberlein, 2012). The volitional consumption of alcohol in the absence of natural reward was later localized to a small subset of NPF neurons that project to several brain regions implicated in regulating reward responses (Shao et al., 2017). This finding is particularly important for alcohol research since, like many drugs of abuse, alcohol has both aversive and appetitive properties. In flies, some alcohol concentrations and treatments are perceived as aversive in the short-term, but appetitive as a long-lasting memory (Kaun et al., 2011). This duality may alter the delicate balance between the highly interconnected neural circuits that encode valence and influence decision-making, thus producing lasting memories underlying alcohol cravings. Future work in flies is, therefore, ideal for studying the circuit and molecular mechanisms by which alcohol experience is valued and for studying the way social experiences contribute to the development and progression of alcohol-associated behavior.

Understanding the effect of alcohol on neuronal activity will be critical for defining alcohol-associated circuitry mechanisms. Dynamic neuronal activity can be recorded in intact behaving flies (Chamberland et al., 2017; Grover, Katsuki, & Greenspan, 2016; Lin & Schnitzer, 2016; Riemensperger, Pech, Dipt, & Fiala, 2012; Royer et al., 2016). The genetic precision and improved genetically encoded calcium (Chen et al., 2013; Ohkura et al., 2012) and voltage (Han et al., 2013; St-Pierre et al., 2014) indicators afforded in flies provide notable advantages over mammalian approaches. Although live imaging work has not yet been applied in fly alcohol research, it will undoubtedly aid in the investigation of how alcohol influences neural dynamics in a behavioral context.

Unresolved neuroimmune relationships

Despite being implicated in the prevention, progression, and treatment of neurodegenerative diseases, the cause and consequence of neuroimmune responses remains a hotly debated issue. The highly conserved Toll innate immune pathway (first discovered in flies), and downstream nuclear factor-κB (NF-κB) signaling control the expression of antimicrobial peptides and cytokine defense molecules (Zhang & Ghosh, 2001). Glial cells serve as the nervous system's resident immune cells, and compose roughly 50% of human brain volume (Reemst, Noctor, Lucassen, & Hol, 2016). Fly glia are morphologically and molecularly similar to their mammalian counterparts, and play crucial roles in neuronal maintenance and neuropathology. Artificial activation of NF-κB in fly glia alone, for instance, can sufficiently cause neurodegeneration (Petersen, Katzenberger, & Wassarman, 2013; Petersen, Rimkus, & Wassarman, 2012). Furthermore, suppressing or enhancing glial immune responses in flies can stave off or speed up age-dependent neurodegeneration, respectively (Kounatidis et al., 2017).

There are, unfortunately, many challenges in determining specific time-course, tissue-specific, and stimulus-dependent neuroimmune responses. Nearly all transcriptomic studies studying stressors in flies demonstrate an overrepresentation of immune-related genes, suggesting a generalized role for the immune system during stress responses (Cantera & Barrio, 2015). Additionally, many immune-related proteins tend to serve other non-immune-related functions. The Toll pathway, for example, is not only activated by gram-negative and fungal pathogens, but also has essential roles in the basic development and maintenance of axons and synapses. Alternatively, many stress-induced genes, such as heatshock proteins, can have neuroprotective or neurodegeneration effects without being necessary for immune responses. There are also, presently, undetermined neuroimmune signals whose effects likely switch from being acutely neuroprotective and chronically neurodegenerative, or vice versa. Future work in flies will help to resolve the complex relationship between neuroimmune signaling and neuropathology.

Relevance to alcohol research

Alcohol exposure increases innate immune signaling in flies, rodents, and humans (Crews, Qin, Sheedy, Vetreno, & Zou, 2013; Liu et al., 2006; Troutwine, Ghezzi, Pietrzykowski, & Atkinson, 2016). In human alcoholic brain tissue, glial cell death often precedes the death of neurons (Cui, Shurtleff, & Harris, 2014). Furthermore, in both human and rodent models of addiction, moderate alcohol exposure induces glial immune responses, whereas excessive alcohol exposure is associated with immunosuppression and neurodegeneration (Cui et al., 2014). New approaches for treating AUD will benefit from a better understanding of neuroimmune interactions and alcohol-associated behaviors.

Recent studies in flies have emphasized the importance of innate immune signaling in alcohol responses. Activation of the fly Toll pathway by parasitic wasp infection causes larvae to therapeutically increase alcohol consumption (Kacsoh, Lynch, Mortimer, & Schlenke, 2013; Milan, Kacsoh, & Schlenke, 2012). Similarly, activation or suppression of Toll signaling in adult flies respectively increases resistance or sensitivity to alcohol-induced sedation (Troutwine et al., 2016). Excitingly, it was recently shown that fly glia alter their shape and function in response to ethanol, and regulate ethanol tolerance via conserved signal transduction pathways (Parkhurst et al., 2018). Furthermore, D-serine, a putative glial neurotransmitter, was up-regulated in flies exposed to alcohol (Kong, Allouche et al., 2010). More studies are clearly warranted for a better understanding of glia's role in alcohol-induced fly behaviors.

The fly's neurogenetic accessibility can be used to address unanswered questions regarding alcohol-associated neuroimmune responses. What specific immune signals are induced from the various subtypes of glia in response to alcohol exposure? How does neuroimmune activity change following acute and repeated alcohol exposures? Are particular types of neurons more or less receptive to alcohol-associated immune signals? What targeted immune-related pharmacologic approaches are therapeutically viable for treating AUD? Future neuroimmune research in flies exposed to alcohol will likely help advance our understanding of alcohol-associated neuroimmune responses.

Conclusion

Insights from intoxicated *Drosophila* help complement mammalian research and extend our understanding of AUD in various ways. The genetic conservation and robust neurogenetic tools available in flies offer valuable experimental approaches that address the heritable predisposition and progression of alcohol sensitivity, tolerance, consumption, cravings, dependence, and maladaptive behavioral decisions. Moreover, the integration of "-omics" level data (i.e., genomics, transcriptomics, proteomics, etc.) within and across model systems is sure to provide clinically relevant information for treating AUD. In flies, the resolution and function of precise neurocircuits is being rapidly elucidated. Concerning alcohol research, this knowledge can help establish neurobiological principles governing basic alcohol-associated plasticity and behavioral responses. Finally, we propose here that work in flies may also shed light on alcohol-associated neuroimmune dysregulation. Elucidating the complex dynamic

interactions between neurons and glia in flies can provide directed hypotheses for mammalian research.

Celebrating the unique tools, experimental tractability, and practical suitability of model organisms such as flies fosters interspecies collaborations that enhance our scientific perspective. Collaborative approaches between scientists using various models will undoubtedly inform tailored clinical approaches and provide key insights into the underlying neurobiology that suggest novel pharmacologic targets for treating AUD.

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