Topic Introduction

Ethanol Behavioral Responses in *Drosophila*

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Drosophila melanogaster is a powerful genetic model for investigating the mechanisms underlying ethanol-induced behaviors, metabolism, and preference. Ethanol-induced locomotor activity is especially useful for understanding the mechanisms by which ethanol acutely affects the brain and behavior. Ethanol-induced locomotor activity is characterized by hyperlocomotion and subsequent sedation with increased exposure duration or concentration. Locomotor activity is an efficient, easy, robust, and reproducible behavioral screening tool for identifying underlying genes and neuronal circuits as well as investigating genetic and molecular pathways. We introduce a detailed protocol for performing experiments investigating how volatilized ethanol affects locomotor activity using the fly Group Activity Monitor (flyGrAM). We introduce installation, implementation, data collection, and subsequent data-analysis methods for investigating how volatilized stimuli affect activity. We also introduce a procedure for how to optogenetically probe neuronal activity to identify the neural mechanisms underlying locomotor activity.

BACKGROUND

The last 20 years of *Drosophila* addiction research have revealed remarkable similarities between flies and mammals in ethanol-induced behavior and the neural and molecular mechanisms underlying this behavior (Park et al. 2017; Engel et al. 2019; Petruccelli and Kaun 2019; Lathen et al. 2020). Flies not only show parallels in acute ethanol-induced behaviors such as hyperactivity and tolerance, but also self-administer ethanol to pharmacologically relevant concentrations, and will consume ethanol even when it is paired with an extremely bitter substance (Devineni and Heberlein 2009). Flies also share many molecular and neural correlates associated with alcohol-induced behaviors. The quick life cycle and vast array of mutant libraries in *Drosophila* provide the distinct advantage of using unbiased genetic approaches to discover novel genetic and molecular mechanisms for alcohol-associated behaviors (Venken et al. 2011; Hales et al. 2015; Caygill and Brand 2016). For example, epidermal growth factor receptor (EGFR), Lim-only kinase, Rho GTPase-activating protein (RhoGAP), and Notch signaling were implicated in alcohol-induced behaviors in *Drosophila* before they were studied in mammals (Rothenfluh et al. 2006; Corl et al. 2009; Lasek et al. 2011; Petruccelli et al. 2018). New intersectional genetic tools also provide exceptional spatial resolution for exploring the contribution of individual neurons or groups of neurons to behavior, and for expressing RNA interference libraries to knock down target genes in adult cells (Luan et al. 2020; Zirin et al. 2020). The number of genetic tools

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available to manipulate and measure gene expression in vivo is unparalleled in *Drosophila* compared to those in other organisms. Using integrative genetic and behavioral approaches can therefore have a profound impact on our understanding of the mechanisms of ethanol-induced behaviors.

Drosophila and Ethanol Preference

In their natural environment, *Drosophila* readily respond to ethanol as an ecologically relevant cue that directs them toward potential food and oviposition sites (Gibson et al. 1981; Giang et al. 2017). Ethanol concentrations that flies readily encounter in natural environments are ∼5% or less (Ainsley and Kitto 1975; McKenzie and McKechnie 1979; Dudley 2000, 2002, 2004). Naive fruit flies are attracted to these lower concentrations of ethanol, typically on the order of 0%–12%, and avoid higher concentrations (Gelfand and McDonald 1980; Parsons 1980a,b; Depiereux et al. 1985). Food–odor mixtures containing ethanol are preferred to food–odor mixtures that lack ethanol (Schneider et al. 2012). Female flies prefer ethanol-containing food as an oviposition substrate for egg laying (Eisses 1997; Azanchi et al. 2013). This ovipositional preference for fruits containing low-dose ethanol is a protective behavior to defend against larval infections from parasitic wasps (Milan et al. 2012; Lynch et al. 2017). However, there is also evidence that early ethanol exposure can be detrimental to larval development in a manner reminiscent of fetal alcohol syndrome (McClure et al. 2011; Logan-Garbisch et al. 2014).

Flies metabolize alcohol with the alcohol dehydrogenase (ADH) enzyme (David et al. 1976) and use the resulting metabolites as caloric substrates (Libion-Mannaert et al. 1976; van Herrewege and David 1980; Daly and Clarke 1981; Geer et al. 1985; Pecsenye et al. 1994). When given a choice between non-ethanol- and ethanol-containing foods, flies prefer to consume food with ethanol (Cadieu et al. 1999; Ja et al. 2007; Devineni and Heberlein 2009). This preference is also dependent on the overall content of the food substrate (Ja et al. 2007; Pohl et al. 2012; Xu et al. 2012). Whether it is the caloric or attractive odorant quality of ethanol, absolute ethanol intake is one of the best predictors of ethanol preference (Park and Ja 2020). Moreover, ethanol-consumption preference can extend well past the concentrations of ethanol typically found in fermenting fruit (Devineni and Heberlein 2009; Xu et al. 2012), and this preference can be influenced by preexposure to ethanol (Peru Y Colón de Portugal et al. 2014). Flies also willingly choose to consume ethanol in conjunction with bitter quinine, suggesting that they are willing to overcome an aversive stimulus to obtain ethanol (Devineni and Heberlein 2009). Together, these studies highlight that ethanol functions as a food cue, provides calories, and has intrinsic rewarding properties at pharmacological concentrations that drive preference.

Drosophila are also an effective model to study the neural and genetic correlates underlying the rewarding pharmacological effects of ethanol. Behaviors typically measured include ethanol-induced hyperactivity, sedation, sensitization, tolerance, loss-of-righting, and seeking/preference. The acute intoxicating effects of ethanol are typically evaluated using locomotor assays that are detailed further in the associated Protocol: **Methods for Exploring the Circuit Basis of Ethanol-Induced Changes in *Drosophila* Group Locomotor Activity** (Nuñez et al. 2023). Studying the long-term effects of ethanol requires more complex behavioral assays like ethanol-tolerance and olfactory conditioning paradigms to parse out the progression of maladaptive responses and to understand ethanol's rewarding properties (Scholz et al. 2000, 2005; Berger et al. 2004; Ogueta et al. 2010; Kaun et al. 2011; Nuñez et al. 2018). As with alcohol consumption, conditioned preference is dose-dependent (Nuñez et al. 2018). Further, flies will willingly overcome an aversive stimulus like electric shock to reach the ethanol-conditioned odor cue, highlighting the maladaptive nature of this preference (Kaun et al. 2011). Thus, *Drosophila* has emerged as a model for studying several aspects of alcohol use and its progression toward alcohol-use disorder, which has been made possible with constant technical innovations made throughout the years.

Ethanol-Induced Locomotion Assays

Among assays to study ethanol-induced behavior in *Drosophila*, the ability to track locomotion has made the greatest strides. Ethanol induces biphasic dose-dependent responses that are stimulatory at

low doses and sedative at high doses (Pohorecky 1977; Babor et al. 1983; Van Etten et al. 1995; Tomie et al. 1998). To parse out some of these dose-dependent differences, locomotor activity is a quick behavioral readout that can be used to identify genes or neural pathways underlying behavior, dissect developmental effects, and assess an animal's vitality and age. Locomotor-based assays using *Drosophila* have led to major discoveries regarding ethanol intoxication, sedation, sensitivity, tolerance, low-dose hyperactivity, and several social behaviors (Scholz et al. 2000, 2005; Berger et al. 2004; Lee et al. 2008; Ogueta et al. 2010; Shohat-Ophir et al. 2012; Kliethermes 2013, 2015; Aranda et al. 2017; Park and Ja 2020; Park et al. 2021).

The "inebriometer," which comprises a vertical column with several baffles, is one of the earliest tools used to measure postural control in response to an odorant or volatilized stimulus (Cohan and Graf 1985; Cohan and Hoffmann 1986; Krishnan and Nash 1990; Weber and Diggins 1990; Nash et al. 1991; Allada and Nash 1993; Campbell and Nash 1994; Leibovitch et al. 1995; Walcourt and Nash 2000; Walcourt et al. 2001). This apparatus capitalizes on negative geotaxis, a fly's natural tendency to migrate against gravity, to measure the influence of various volatized stimuli on locomotor activity. In response to volatilized ethanol, flies' motor functions, including negative geotaxis, become increasingly compromised. The inebriometer is also used to parse out dose–response relationships with intoxication, sedation, and sensitivity. The inebriometer was used to identify the first *Drosophila* mutant associated with ethanol sensitivity, which was originally named *cheapdate* and later identified as an allele of *amnesiac,* part of the cyclic AMP signal-transduction pathway (Moore et al. 1998). Additionally, it is still being used for the identification of natural variation in the gene pathways necessary for ethanol tolerance and sedation (Morozova et al. 2015).

Behavioral assays that measure horizontal locomotion were first applied to ethanol research in the early 2000s in the form of line-crossing assays (Singh and Heberlein 2000). Line-crossing assays consisted of a chamber lined with a grid for measurements. Ethanol vapor was perfused throughout the chamber, and the number of line crossings was measured as a function of time to provide a readout of locomotion in response to ethanol intoxication (Singh and Heberlein 2000). This line-crossing assay was paired with a narrow tube assay used to assess turning behavior. These two complementary assays and the inebriometer were used to isolate *barfly* and *tipsy*, which inversely affected ethanol-intoxication sensitivity and increased turning behaviors in response to ethanol (Singh and Heberlein 2000).

In an effort to obtain high-throughput quality hyperlocomotion data in response to ethanol intoxication, the "inebri-actometer" was developed (Parr et al. 2001). This apparatus tested 128 individual flies in narrow tubes and provided an automated readout of their locomotor activity through infrared beam crossings. The inebri-actometer beautifully combines the ease of automated analysis with the added power of being able to obtain a large sample size very quickly. Similar assays were developed around the same time to increase the throughput and to increase the resolution of the automated data obtained. The "booz-o-mat" was developed to acquire automated velocity readouts from a group of flies in eight individual vials isolated within narrow cylinders (Wolf et al. 2002). Tracking single flies in a horizontal chamber was used to obtain an accurate readout of the fly's path and velocity, which complemented data from the booz-o-mat (Wolf et al. 2002). The booz-o-mat led to the discovery of several genes and signaling pathways that play a role in ethanol tolerance and sensitivity (Kong et al. 2010a), as well as the role that dopaminergic neurons have in mediating enhanced locomotor activity in response to ethanol (Kong et al. 2010b). Other incremental improvements throughout the years have led to advancements in understanding different aspects of ethanol-induced behaviors. A refined version of a negative geotaxis assay with refined tracking capabilities was developed and called the ethanol rapid iterative negative geotaxis (eRING) assay (Bhandari et al. 2009). This assay revealed the role of integrins, *myospheroid*, and *scab* in ethanol-induced sedation and tolerance (Bhandari et al. 2009). Tracking of angular velocity and other high-content analyses of tracked behaviors following drug exposure became possible with advancements in computer vision, which led to the use of more open-field assays with *Drosophila* to investigate complex behaviors induced by intoxication (Gakamsky et al. 2013). Together, these assays have furthered our understanding of the genetic basis and behavioral dynamics underlying ethanol intoxication.

Most recently, automated tracking systems and high-resolution video tracking have provided the ability to study discrete behavioral features over time (Branson et al. 2009; Kabra et al. 2013; Nath et al. 2013; Berman et al. 2014; Robie et al. 2017). These technological advancements have led to a number of assays that can be used to parse out the behavioral components that underlie ethanol use. In the accompanying protocol, we detail the use of the fly Group Activity Monitor (flyGrAM; Scaplen et al. 2019; see Protocol: **Methods for Exploring the Circuit Basis of Ethanol-Induced Changes in *Drosophila* Group Locomotor Activity** [Nuñez et al. 2023]). The flyGrAM is a behavioral apparatus that provides a high-throughput assessment of group locomotor activity across several concurrently run groups of adult flies. flyGrAM's automated output provides real-time feedback that can help experimenters running behavioral screens to quickly assess drug doses and experimental manipulations, and plan follow-up experiments. Furthermore, flyGrAM simultaneously provides video recordings of experiments to allow for video post-processing using high-resolution tracking methods. In the accompanying protocol, we provide the necessary information to set up, install, and implement flyGrAM, and analyze its data output (see Protocol: **Methods for Exploring the Circuit Basis of Ethanol-Induced Changes in *Drosophila* Group Locomotor Activity** [Nuñez et al. 2023]).

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