Invertebrate Models of Drug Abuse

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ABSTRACT: Susceptibility to drug addiction depends on genetic and environmental factors and their complex interactions. Studies with mammalian models have identified molecular targets, neurochemical systems, and brain regions that mediate some of the addictive properties of abused drugs. Yet, our understanding of how the primary effects of drugs lead to addiction remains incomplete. Recently, researchers have turned to the invertebrate model systems *Drosophila melanogaster* and *Caenorhabditis elegans* to dissect the mechanisms by which abused drugs modulate behavior. Due to their sophisticated genetics, relatively simple anatomy, and their remarkable molecular similarity to mammals, these invertebrate models should provide useful insights into the mechanisms of drug action. Here we review recent behavioral and genetic studies in flies and worms on the effects of ethanol, cocaine, and nicotine, three of the most widely abused drugs in the world. © 2003 Wiley Periodicals, Inc. J Neurobiol 54: 161–178, 2003

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INTRODUCTION

Drug addiction is a chronic and relapsing mental illness characterized by compulsive drug use despite serious negative consequences. Several reasons have motivated neurobiologists over the years to study the mechanisms that underlie this complex condition. First, the social and medical costs to society are enormous; most people in the world have been affected, directly or indirectly, by the devastating consequences of drug addiction. Second, drugs of abuse subvert normal brain processes such as motivated behavior and learning and memory, and thus are tools for understanding these fascinating phenomena. Finally, at a practical level, drugs of abuse coordinately induce specific behavioral responses, making their analysis tractable and open for dissection at the level of molecular and neural circuits.

Complex genetic and environmental factors contribute to a predisposition for drug addiction. The genetic component is likely multigenic and heterogenous, making identification of specific "addiction genes" a difficult endeavor. These complexities together with the high cost of human studies have led to the development of animal models for various drug-induced behaviors. Most prominently, rodent models, amenable to genetic and pharmacologic approaches, have been used extensively to study how drugs of abuse exert their acute, chronic, and addictive effects. These studies have pointed to brain regions, neurotransmitter systems, and some molecules that mediate the behavioral effects of abused drugs. Interestingly, while most abused drugs have different primary effects and modes of action in the central nervous system, their positive reinforcing and rewarding properties appear to be mediated by at least partially overlapping mechanisms. For example, most drugs of abuse act on the mesolimbic dopamine system, a system that likely evolved to signal the positive reinforcing properties of so-called natural rewards, including food, sex, and social interaction (reviewed in Wise, 1996; Spanagel and Weiss, 1999). The mechanisms by which the acute reinforcing effects of abused drugs lead to the long-lasting changes in behavior that accompany addiction, and the identity of the genetic factors that modulate this transition, remain mysterious.

Recently, some researchers have turned their attention to the worm *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* as animal models for addiction research. This motivation is derived primarily from the fact that invertebrate nervous systems are many orders of magnitude simpler than those of mam-

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mals (flies have approximately 300,000 neurons, worms have 302) and the fact that invertebrate genetics are comparatively more sophisticated and rapid. Are worms and flies good models for drug addiction? Although at a first glance these organisms do not exactly resemble mammals, a closer examination reveals many similarities. For example, worms and flies must do many of the same things that higher organisms do: they search for food, reproduce, coordinate activity and rest periods with the appropriate time of day, and try to avoid death. Not only are some of these innate behaviors quite complex, but they are also modifiable by experience (reviewed in Sokolowski, 1998; Sokolowski, 2001; Thomas, 2001). These behaviors require sophisticated integration of sensory inputs and coordination with appropriate motor outputs. Although the complexity of decision making in worms and flies is clearly lower than that of mammals, these organisms have greater cognitive processing abilities than they are usually given credit for. For example, flies can make choices when faced with contradictory visual cues, and can generalize between related cues (Liu et al., 1999; Tang and Guo, 2001).

The molecular architecture of vertebrate and invertebrate nervous systems is mostly shared (reviewed in Bargmann, 1998; Littleton and Ganetzky, 2000; Lloyd et al., 2000; Rubin et al., 2000). *Drosophila* and *C. elegans* have most—if not all—major neurotransmitters, molecules involved in synaptic vesicle release and recycling, receptors and channels for neurotransmission (with the exception of voltage-gated sodium channels in worms), and signal transduction mechanisms involved in neural function in vertebrates. More specifically, genes implicated in the actions of drugs of abuse are, for the most part, conserved (discussed in detail below). These include the catecholamine reuptake transporters and nicotinic acetylcholine receptors, the main targets of cocaine and nicotine, respectively. Sites of ethanol action, such as certain ligand- and voltage-gated ion channels, and a G-protein–activated potassium channel (reviewed in Harris, 1999) are also present in flies and worms. Some drug targets may not be conserved, however. No opioid receptors have been identified in flies or worms, although there are receptors bearing sequence similarity in both organisms and opiate binding activity has been reported in the fly brain (Santoro et al., 1990). Additionally, neither organism appears to contain cannabinoid receptors (Elphick and Egertova, 2001; McPartland et al., 2001).

The range of experimental techniques available for invertebrate studies differs from that available in mammals. This opens new and complimentary approaches to old problems in addiction research, but also complicates direct comparisons of results. The major advantage of flies and worms is the simplicity and scale with which they can be manipulated genetically. Massive and unbiased forward genetic screens for mutants, coupled with mutant gene cloning and genetic pathway analysis, have made major contributions to our understanding of many basic developmental, physiologic, and behavioral processes. In addition, various methods for pinpointing the time and place of gene action have allowed high-resolution analyses of gene function at the organismal level. Most of these approaches have already been used to study the actions of ethanol, cocaine, and nicotine in *Drosophila* and *C. elegans* (see below). Another advantage of flies and worms is that large populations can be easily obtained; behavioral analysis of large numbers of genetically identical animals reduces variability and brings many observations within the realm of statistical significance. Finally, the availability of nearly complete genome sequences for both organisms (*C. elegans* consortium, 1998; Adams et al., 2000) has accelerated forward genetics and opened the door for systematic reverse genetics. Flies and worms also have major shortcomings: their behavioral repertoire is limited to fairly simple motor responses, and electrophysiologic techniques, widely used in mammals to study synaptic responses to drugs of abuse, have been essentially limited to the neuromuscular junction. Recordings from central neurons are becoming feasible however, particularly in *C. elegans* (e.g., see Brockie et al., 2001). In addition, recent technologic advances now allow monitoring of neural activity in intact animals or whole brains in worms and flies (Kerr et al., 2000; Rosay et al., 2001; Wang et al., 2001).

The extent of analogy between the effects of drugs of abuse on brain function and behavior in mammals and invertebrates remains to be determined. Although invertebrate studies are still nascent, at least some surprising parallels at the behavioral and molecular level have emerged already. In the following sections we will discuss specific findings for ethanol, cocaine, and nicotine in *Drosophila* and *C. elegans*, and draw parallels with findings in mammals. We also discuss some of the new techniques for measurement of behavior that were developed in these studies.

ETHANOL

Alcohol—specifically ethanol—is one of the most widely abused drugs in the world, yet our understanding of the mechanisms by which it regulates brain

function and behavior is rudimentary. Unlike other drugs of abuse, ethanol does not appear to act through a specific receptor and, in the past, its effects on the nervous system had been attributed primarily to non-specific disturbances in the properties of neuronal membranes. However, in the last few years, increasing evidence has shown that specific brain proteins are selectively sensitive to ethanol. These include receptors for γ-aminobutyric acid type A (Mihic et al., 1997), *N*-methyl-D-aspartate (Peoples and Weight, 1995), and 5-hydroxytryptamine$_3$ (Zhou et al., 1998; Lovinger, 1999), voltage-gated K^+ and Ca^{++} channels (Covarrubias and Rubin, 1993; Dopico et al., 1998), and G-protein-activated inwardly rectifying K^+ channels (Kobayashi et al., 1999; Lewohl et al., 1999). How ethanol acts on these proteins and how these effects relate to ethanol-induced behaviors is poorly understood.

There is strong evidence from family, twin, and adoption studies that genetic as well as environmental factors contribute to the risk for alcoholism (Schuckit, 2000). The genetic factors that contribute to an increased risk for alcoholism are quite complex and have so far eluded definitive identification. Upon exposure to low doses of ethanol, most humans exhibit responses such as disinhibition and euphoria. Higher doses cause incoordination and confusion, and in extreme cases, coma and death. The degree of response to ethanol appears to be influenced genetically and may be a good predictor of risk for alcoholism (Schuckit, 1994, 1996). It is therefore possible that an understanding of fairly simple behaviors induced by acute ethanol exposure may help gain insights into the more complex process of alcohol addiction.

Ethanol Intoxication in *Drosophila*: Behavioral Assays

As has been described for most organisms in which it has been studied, *Drosophila* show a complex and biphasic behavioral response upon exposure to ethanol vapor: an initial increase in locomotion is followed by incoordination, loss of postural control, and eventually sedation and immobility. Because current assays rely on continuous exposure to ethanol vapor under nonsteady state conditions, the level of ethanol absorbed by the flies is directly proportional to exposure time. Thus, low levels of accumulated ethanol stimulate locomotion and high levels depress it.

In flies, ethanol-induced changes in walking activity and pattern were first quantified using simple line-crossing assays (Singh and Heberlein, 2000; Bainton et al., 2000). When exposed to ethanol vapor in a chamber, flies show a transient increase in walking velocity; when exposed in narrow tubes, they show a temporary but dramatic increase in turning that is not explained simply by increased walking velocity (Singh and Heberlein, 2000). More recently, the development of automated assays has greatly enhanced the analysis efficiency and resolution of locomotor behaviors. The inebri-actometer (Fig. 1) measures simultaneously the activity of 128 flies, placed individually in narrow tubes (Parr et al., 2001). As flies move across the middle of each tube they break an infrared beam, which is recorded as motion. The kinetics and magnitude of locomotor activation and sedation measured with the inebri-actometer are essentially identical to those seen in the line-crossing assays. Finally, recent advances in computer and video technology have made feasible the automated and simultaneous tracking of several groups of freely walking flies (F. Wolf and U. Heberlein, in press; Fig. 2). In this system, two-dimensional traces of the movement of individual flies are established by following their position over time at 0.1-s intervals. Information about the velocity of movement, degree of turning, and position in the box can be extracted by specialized software. The high temporal and spatial resolution of this system led to the discovery of aspects of ethanol-induced locomotor behaviors that had been previously missed. For example, upon ethanol exposure, flies show an immediate and short-lived increase in locomotion; this is a startle response to the smell of ethanol. After a brief quiescent period, flies enter a sustained hyperactive phase that dissipates gradually as flies become sedated. The kinetics of onset and dissipation of this hyperactivity as well as the maximal velocities achieved are highly dose dependent.

The inebriometer, originally developed for selective breeding purposes (Cohan and Hoffman, 1986; Weber, 1988; Weber and Diggins, 1990), is an apparatus that measures the effect of ethanol (or any gas) on fly postural control (Fig. 3). It consists of a vertical cylinder fitted with a series of oblique mesh baffles (on which flies can stand and walk) that is perfused with ethanol vapor of defined concentrations. Groups of flies are introduced into the top of the cylinder, where they remain temporarily due to their natural propensity for negative geotaxis. As flies absorb and accumulate ethanol, they lose the ability to stand or walk properly and fall from one baffle to the next, eventually eluting from the bottom of the inebriometer. Because the level of ethanol absorbed by the flies is relatively linear over the time period of the assay, the mean elution time (MET) of the population is propor-

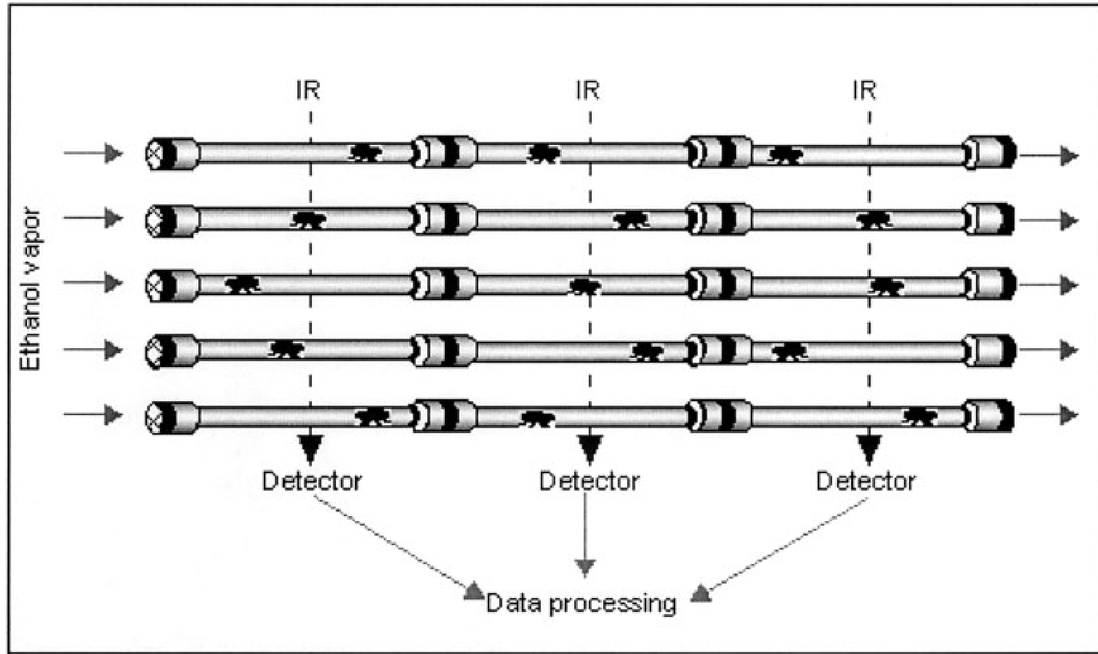

Figure 1 The inebri-actometer.

tional to the ethanol dose needed to cause loss of postural control, and thus is a measure of fly sensitivity to the acute intoxicating effects of ethanol.

In summary, ethanol exposure causes clear and measurable effects on *Drosophila* locomotion and postural control. In general, lower doses stimulate walking speed, while higher doses cause reduced movement, loss of postural control, and immobility. Interestingly, the ethanol concentrations that stimulate locomotion in flies are very similar to those causing the same effect in rodents and those generating disinhibition and euphoria in humans; the doses that cause incoordination and sedation are also comparable (Singh and Heberlein, 2000).

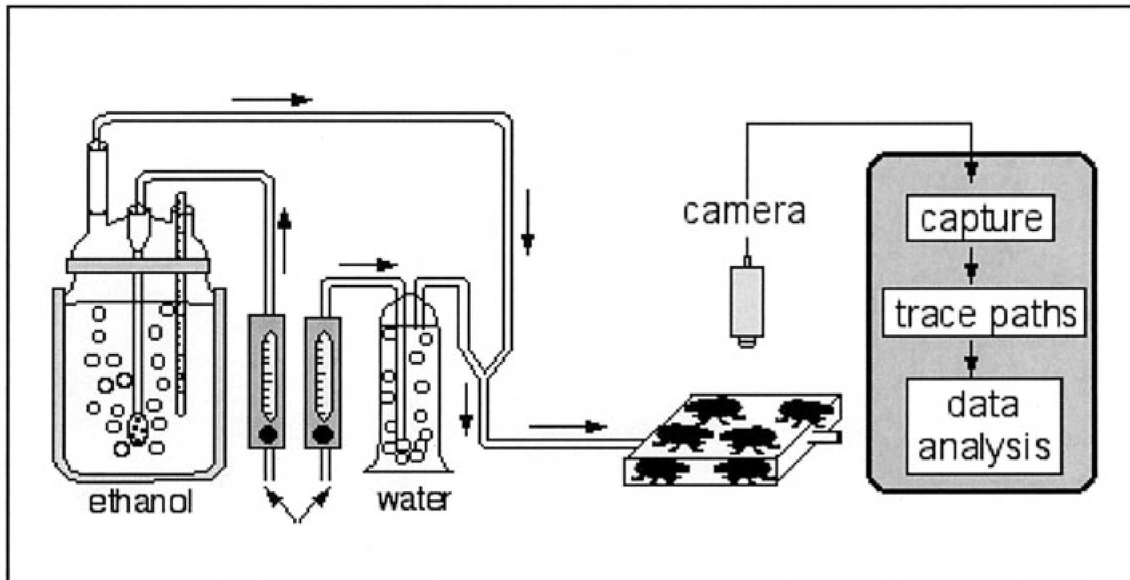

Figure 2 The locomotor-tracking system.

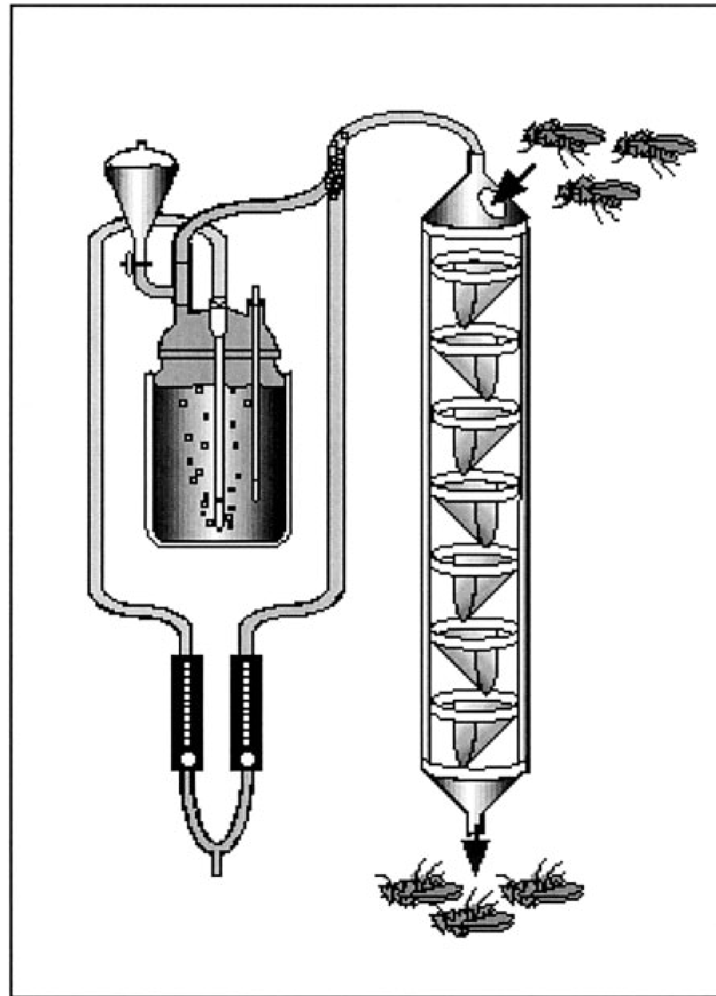

Figure 3 The inebriometer.

Genes That Regulate Acute Ethanol-Induced Behaviors in *Drosophila*

Genetic screens for single gene mutants with altered ethanol sensitivity have been carried out using the inebriometer (Moore et al., 1998; Singh and Heberlein, 2000). Several fly strains with chemically induced mutations that alter ethanol sensitivity in the inebriometer have been isolated (Singh and Heberlein, 2000). Among these, *barfly* and *tipsy* mutants show strongly reduced and increased ethanol sensitivity, respectively. Unfortunately, these mutants have been difficult to map genetically and have so far eluded molecular identification (D. Guarnieri, C. Singh, and U. Heberlein, unpublished). *cheapdate*, a transposon-induced mutant showing increased ethanol sensitivity, was found to disrupt the *amnesiac* gene, which encodes a PACAP-like neuropeptide (Feany and Quinn, 1995). In vertebrates, PACAPs signal through G-protein–coupled receptors that activate adenylate cyclase (AC) (reviewed in Vaudry et al., 2000). Consistent with a similar role for *amnesiac* are findings that mutations in the calcium/calmodulin-sensitive adenylyl cyclase *rutabaga* and the catalytic subunit of cAMP-dependent protein kinase (*pka-C1*) also cause increased ethanol sensitivity. In addition, the ethanol sensitivity defect of *amnesiac* and *rutabaga* can be reversed by pharmacologic activation of the cAMP pathway (Moore et al., 1998). On the other hand, flies with a mutation in *pka-RII,* one of the PKA regulatory subunits, show reduced sensitivity to ethanol in a climbing assay (Park et al., 2000). This mutation leads to an increase in the low basal activity of PKA and a strong reduction in cAMP-stimulated activity. While apparently contradictory, these results may reflect the complex biology of cAMP and PKA signaling. For example, modulation of the pathway in

different groups of cells, or in different subcellular compartments may result in different effects on behavior. Also consistent with this complexity is the observation that *amnesiac-rutabaga* double mutants are less sensitive to ethanol than either single mutant alone (Moore et al., 1998). A complex role for cAMP signaling and ethanol-induced behaviors is also observed in mice. For example, mice deficient for PKA-RIIβ show a phenotype similar to that seen with the fly PKA-RII mutants: a reduced sensitivity to the sedative effects of ethanol. PKA-RIIβ mutant mice also voluntarily consume more ethanol (Thiele et al., 2000). However, mice with reduced levels of Gαs, the adenylyl cyclase-stimulating G-protein, show increased ethanol sensitivity and reduced voluntary consumption (Wand et al., 2001). Taken together, these studies clearly link the cAMP signaling pathway to acute ethanol responsiveness in flies and mice. Determining exactly how, where, and when these effects take place in the nervous system should provide interesting new avenues for experimentation.

Role of Dopaminergic Systems in Acute Ethanol Responses in *Drosophila*

The locomotor-activating effects of drugs of abuse in mammals, including ethanol, have been proposed to model their positive reinforcing or rewarding properties (Wise and Bozarth, 1987). Consistent with this notion are observations that some of the neural circuits and neurochemical systems, such as the mesolimbic dopamine pathway, that are central to drug reinforcement also influence acute stimulant effects. Although this has been studied primarily for psychostimulants such as cocaine and methamphetamine, acute ethanol exposure also increases dopamine release and locomotor activity in rodents (Dar and Wooles, 1984; Imperato and Di Chiara, 1986). Genetic or pharmacologic manipulations that interfere with mesolimbic dopamine systems block not only the stimulant effects of ethanol, but also the rewarding properties (Phillips and Shen, 1996; Phillips et al., 1998; Cunningham et al., 2000; Risinger et al., 2000); however, other neurochemic systems and brain regions are also involved in drug reinforcement (reviewed in Wise, 2000).

A possible role for dopamine in ethanol-induced behavior in *Drosophila* was ascertained by using pharmacologic and genetic approaches. Reducing dopamine synthesis with the tyrosine hydroxylase inhibitor 3-iodotyrosine (Neckameyer, 1996) results in a significant reduction in ethanol-induced locomotor activity levels (Bainton et al., 2000). The timing of hyperactivity onset and sedation is, however, normal, suggesting a specific role for dopamine in the stimulant effects of ethanol. Genetic ablation of neural activity in dopaminergic and serotonergic neurons (using targeted expression of tetanus toxin) also reduced ethanol-induced locomotor activity levels (K. Woo and U. Heberlein, unpublished). Thus, as in mammals, dopaminergic (and perhaps serotonergic) neurotransmission contributes to ethanol stimulated locomotor activity in *Drosophila*; whether it is also involved in the rewarding properties of ethanol remains to be studied.

Ethanol Tolerance in *Drosophila*

In humans, chronic alcohol use leads to the development of tolerance, most simply defined as an acquired resistance to the inebriating effects of ethanol. Tolerance can develop to both the pleasurable and aversive effects of ethanol, potentially encouraging increased intake, the development of physical dependence, and addiction (reviewed in Tabakoff et al., 1986). Tolerance can be attained either by more efficient removal of alcohol from the body (or less efficient absorption) or by adaptations in neural function (reviewed in Fadda and Rossetti, 1998). The former is termed metabolic or pharmacokinetic tolerance; the latter is termed functional or pharmacodynamic tolerance and reflects changes in neurophysiology.

Drosophila develop ethanol tolerance, even after a single exposure to a moderate dose of ethanol, which is manifested as a delay in elution from the inebriometer and a delay in ethanol-induced sedation (Scholz et al., 2000). By definition this tolerance is functional, as there is no change in the rate of internal alcohol accumulation between the first and second exposure; it therefore takes higher ethanol doses to elicit the same behavioral response in previously exposed versus naïve flies. Because the ethanol from the first exposure is completely metabolized at the time of the second exposure (usually 4 h after the first), this tolerance paradigm most closely resembles rapid tolerance paradigms defined in rodents (Crabbe et al., 1979). Rapid tolerance has been shown to develop to the hypothermic, motor-impairing, and hypnotic effects of ethanol (reviewed in Lê and Mayer, 1996).

It is important to contrast this functional tolerance, observed after a single ethanol exposure, with previous definitions of ethanol tolerance in *Drosophila*. The latter has been used to describe the flies' ability to withstand the toxic effects of ethanol (reviewed in Geer et al., 1993). The assays usually involve growing flies continuously on culture medium containing relatively high concentrations (up to 10%) of ethanol, and by measuring the rate of survival to the adult

stage. This form of tolerance is thought to rely primarily on enzymatic or cellular functions that contribute to metabolism and/or elimination of ethanol (reviewed in Geer et al., 1993). For example, expression of the ethanol metabolizing enzyme alcohol dehydrogenase (ADH) is induced by ethanol in larvae (but not adults). This type of tolerance is therefore equivalent to what has been described in rodents as dispositional or metabolic tolerance (reviewed in Khanna and Israel, 1980).

Genetic ablation of neurotransmission (using targeted expression of tetanus toxin) revealed that a small group of neurons in the central complex of the fly brain is necessary for normal tolerance development (Scholz et al., 2000); these neurons are also involved in regulating the patterns of spontaneous locomotion in flies (Martin et al., 1999). It is likely that other brain regions are involved in tolerance development as well; systematic brain mapping studies have not yet been carried out. Finally, flies lacking tyramine-β-hydroxylase, an enzyme required for the synthesis of octopamine (Monastirioti et al., 1996), display reduced tolerance, implicating octopaminergic systems in the process (Scholz et al., 2000). Intact noradrenergic systems are required for ethanol tolerance development in mice (Tabakoff and Ritzmann, 1977; Lê et al., 1981). Because octopamine in invertebrates is thought to be functionally homologous to noradrenaline in vertebrates (reviewed in Evans, 1980; Roeder, 1999), these data demonstrate interesting similarities among the mechanisms of ethanol tolerance in flies and mammals. In addition, several quantitative aspects of tolerance, such as the extent of maximal tolerance and its duration (about 24 h), are also comparable in flies and rodents (Khanna et al., 1991, 1996). This opens the door for a genetic analysis of tolerance in *Drosophila*, an approach that will likely provide much needed molecular insight into the process in mammals, including humans.

Ethanol Preference in *Drosophila*

Flies are attracted to low concentrations of ethanol and avoid high concentrations (Parsons, 1979; Gelfand and McDonald, 1980; Depiereux et al., 1985). The preference of larvae for ethanol correlates with their levels of ADH activity: larvae with high ADH activity prefer ethanol-supplemented medium, whereas larvae lacking ADH avoid the medium containing 8% or more ethanol. Thus, *Drosophila* larvae show a preference for ethanol concentrations that mimic those found in their natural habitat of fermenting plant materials (reviewed in Dudley, 2000). Recently, ethanol preference has also been reported for adult flies (Cadieu et al., 1999). In this paradigm, flies are allowed to choose media with or without ethanol and their choice is quantified by measuring the duration of proboscis extension. Flies prefer ethanol-containing medium, and this preference is augmented by previous exposure to ethanol. On the other hand, previous exposure to ethanol in the presence of an ADH inhibitor, leads to avoidance of ethanol-containing medium. Thus, adult flies seem to "remember" the negative effects of high ethanol levels in their system; this is expressed as an avoidance of otherwise preferred ethanol concentrations.

Control of Ethanol Sensitivity versus Learning and Memory: Shared Mechanisms?

The long-lasting neural and behavioral changes that accompany addiction are thought to be similar to those involved in learning and memory (reviewed in Berke and Hyman, 2000; Hyman and Malenka, 2001). It is therefore perhaps not surprising that the first ethanol sensitivity mutant identified in flies, *cheapdate*, is a mutation in the memory gene *amnesiac* (Feany and Quinn, 1995; Moore et al., 1998). In addition, flies mutant for *rutabaga*, *pka-C1*, and the neural cell adhesion molecule *fasciclin II* show defects both in olfactory classical conditioning and ethanol sensitivity (Moore et al., 1998; Cheng et al., 2001). Although the extent of genetic overlap remains to be determined, it is not complete as several learning and memory mutants, including *dunce* (Dudai et al., 1976) and *volado* (Grotewiel et al., 1998), show apparently normal ethanol sensitivity when assayed in the inebriometer (Moore et al., 1998; U. Heberlein, unpublished observations). Whether these mutants have defects in ethanol tolerance or ethanol-induced locomotor behaviors has yet to be determined.

Despite the overlap in the genes regulating learning and memory and ethanol sensitivity in flies, the neuroanatomic loci that regulate these behaviors appear to be separable. Although multiple different lines of experimentation have shown that mushroom bodies (MBs) play a central role in olfactory classical conditioning (reviewed in Roman and Davis, 2001), these prominent brain structures appear to be dispensable for regulation of ethanol sensitivity (Moore et al., 1998; A. Rodan and U. Heberlein, in press). In addition, the MBs are not required in conditioning paradigms involving visual, tactile, or spatial cues (Wolf et al., 1998; Zars et al., 2000), suggesting that behavioral plasticity can be regulated by other, as yet unidentified brain regions. The brain regions that regulate other behavioral changes induced by ethanol,

such as loss of postural control, locomotor stimulation, and sedation, also remain to be defined.

Additional evidence for independent mechanisms underlying olfactory conditioning and ethanol sensitivity comes from studies of the temporal requirements for the neuropeptide gene *amnesiac* in these behaviors. Although heat shock-induced *amnesiac* overexpression in adult flies rescues the ethanol sensitivity defect of the *amnesiac* mutant flies (Moore et al., 1998), this protocol fails to rescue their memory defect (DeZazzo et al., 1999). However, restoration of normal olfactory memory can be achieved by providing *amnesiac* expression throughout development, either broadly (DeZazzo et al., 1999) or in a specific group of cells, such as the DPM neurons, whose many processes project to the neuropil containing the mushroom body axons (Waddell et al., 2000). Thus, *amnesiac* plays an acute role in the mature nervous system to regulate ethanol sensitivity, while its role in memory formation is at least in part developmental. Consistent with a developmental role for *amnesiac* in olfactory conditioning is the finding that mushroom body anatomy is somewhat abnormal in *amnesiac* mutants (Hitier et al., 1998).

Why would genes with roles in learning and memory, believed to function in nervous system plasticity, affect acute responsiveness to ethanol? The assays used to measure ethanol sensitivity are carried out over 20 to 30 min, providing ample time for neural and behavioral adaptations to take place. It is therefore possible that increased sensitivity to ethanol may be caused by a failure to adapt during the exposure period, whereas reduced sensitivity may reflect a preadapted (or tolerant) state or an accelerated adaptation during the exposure. Interestingly, an overlap between genes involved in learning and memory and those regulating ethanol sensitivity has also been observed in mice. For example, mice lacking PKCγ show impaired spatial learning (Abeliovich et al., 1993) and reduced ethanol sensitivity (Harris et al., 1995). Fyn-deficient mice, which are hypersensitive to the hypnotic effects of ethanol (Miyakawa et al., 1997), also show abnormal spatial learning (Grant et al., 1992).

Effects of Ethanol in *C. elegans*

Exposure of *C. elegans* to ethanol has been carried out by immersing worms into ethanol solutions of various concentrations. They respond first by increasing movement, a state that has been called “excited” (Morgan and Sedensky, 1995). This behavior proceeds to a progressive lack of coordination, followed by immobility and unresponsiveness to stimuli. The half-maximal concentration required to elicit behavioral change in wild-type worms is approximately 1000 mM ethanol. Although this concentration is high, the actual ethanol concentration within the worms has not been reported and is likely to be substantially lower, as the cuticle is a significant permeability barrier. Several mutants, isolated based on their altered sensitivity to volatile anesthetics (Morgan and Sedensky, 1994), also display altered sensitivity to the sedating effects of ethanol (Morgan and Sedensky, 1995). These studies suggest that the effects of ethanol and volatile anesthetics on *C. elegans* may be mediated by an overlapping set of gene products. A systematic genetic screen for mutations that specifically alter ethanol-induced behaviors has not been reported for *C. elegans.*

COCAINE

Cocaine is a plant alkaloid that has been used by humans for thousands of years as a local anesthetic and a powerful psychomotor stimulant. In rodents, acute cocaine administration also causes profound psychomotor alterations, consisting of increased spontaneous locomotion and expression of behavioral stereotopy, such as sniffing, rearing, and circling. Unlike ethanol, cocaine has a small number of known targets, namely the reuptake transporters for the catecholamines dopamine, serotonin, and norepinephrine (reviewed in Ritz et al., 1990; Amara and Sonders, 1998). Cocaine’s effect on the dopamine transporter (DAT) in the nucleus accumbens of the mesolimbic dopamine system results in increased synaptic dopamine levels, which in turn alters the timing and magnitude of signaling via dopamine receptors. This is believed to drive cocaine’s psychomotor stimulant and positive reinforcing properties becauses selective destruction of dopamine cells or pharmacologic inhibition of dopamine receptors prevents cocaine’s locomotor stimulant effects and also curtails self-administration (reviewed in Koob et al., 1998). In addition, mice lacking DAT are hyperactive and insensitive to the locomotor-activating effects of cocaine (Giros et al., 1996). These mice can still learn (albeit at a reduced rate) to self-administer cocaine, despite persistently high levels of extracellular dopamine (Rocha et al., 1998). It is likely that other neurotransmitter systems, particularly serotonin, or compensatory changes may drive the drug’s reinforcing properties in DAT’s absence. Consistent with this is the finding that cocaine’s rewarding effects are lost in mice lacking both DAT and the serotonin reuptake transporter SERT (Sora et al., 2001).

Acute Cocaine-Induced Behaviors in *Drosophila*

When exposed to volatilized free-base cocaine, flies show behaviors that are surprisingly similar to those elicited in mammals. Low cocaine doses induce intense grooming and reduced locomotion. Intermediate doses elicit severely aberrant patterns of locomotion: flies walk sideways, backwards, and in circles. Further increases in dose cause a series of uncoordinated and erratic hyperkinetic behaviors, and finally akinesia (from which flies recover) and death (McClung and Hirsh, 1998).

Cocaine-induced behaviors in *Drosophila* have been measured in three types of assays, all of which involve flash-volatilization of free-base cocaine from heated filaments (Fig. 4). After a brief exposure, flies are transferred to an assay chamber where they are observed for approximately 10 min. The first assay involves filming groups of flies and scoring their behavior on a scale of severity from 0 (normal behavior) to 7 (total akinesia); each fly is given a score according to the most severe behavior displayed during a particular time interval (McClung and Hirsh, 1998). In the second assay, small groups of flies are filmed and computer-generated locomotor traces are quantified for velocity and turning magnitude (Bainton et al., 2000). The final assay takes advantage of the flies' natural inclination for negative geotaxis, which is reduced upon cocaine exposure (Bainton et al., 2000). Although the behaviors induced by cocaine are complex and somewhat heterogeneous, all assays measure fairly robust and dose-dependent behavioral changes. However, further studies (e.g., with specific mutants) are needed to determine if these assays measure similar or different aspects of cocaine's effect on fly behavior.

Genetic and pharmacologic manipulations of dopaminergic and serotonergic systems in flies alter acute cocaine sensitivity. Dopamine is synthesized from tyrosine in a two-step enzymatic process catalyzed by tyrosine hydroxylase (TH) and dopa decarboxylase (DDC). TH activity can be blocked *in vivo* by feeding flies the competitive inhibitor 3-iodotyrosine (3IY), which leads to an approximately 90% reduction in dopamine levels (Neckameyer, 1996). Flies fed 3IY are less sensitive to the effects of cocaine; this resistance can be reversed by providing exogenous L-Dopa, the product of TH activity. Similar effects were seen with reserpine, a blocker of vesicular monoamine transporters that depletes dopamine from releasable synaptic vesicle pools (Bainton et al., 2000). On the other hand, genetic ablation of evoked synaptic release from dopaminergic and serotonergic neurons (using targeted expression of tetanus toxin in these neurons) causes increased sensitivity to cocaine (Li et al., 2000). Because this manipulation blocks function throughout development, it is likely to induce compensatory neuroadaptations—such as hypersensitivity of postsynaptic receptors—which may in turn lead to increased behavioral effects of the drug. Consistent with this explanation, hypersensitivity is associated with enhanced responsiveness of adult fly nerve cord preparations to a dopamine receptor agonist (Li et al., 2000). It is also possible that

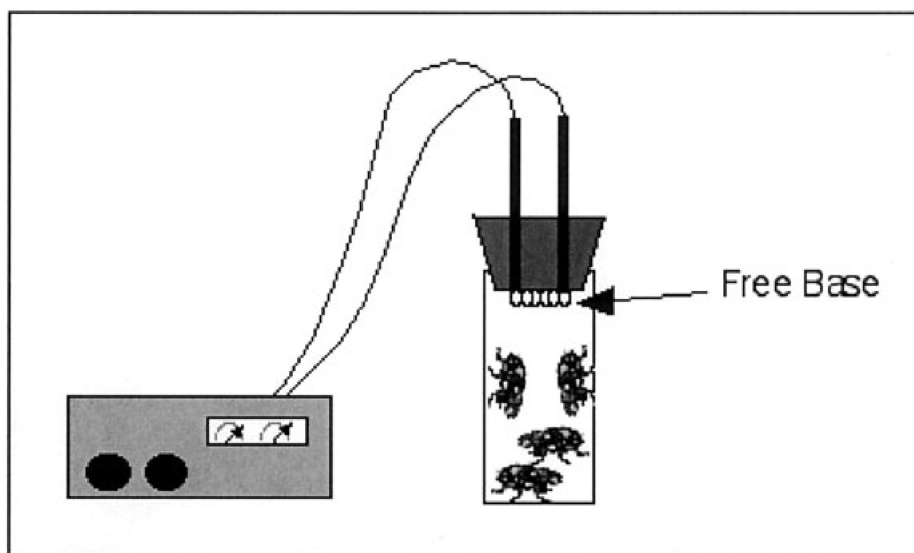

Figure 4 Cocaine volatization system.

the degree of inhibition or its neurochemical specificity determine the nature of the flies' response to cocaine. Regardless of the exact reason for this apparent discrepancy, the relative insensitivity of 3IY-treated flies suggests that cocaine acts by inhibiting dopamine reuptake in *Drosophila*, as is expected from its function in mammalian systems. Cocaine-sensitive serotonin and dopamine transporters have been identified in flies (Corey et al., 1994; Demchyshyn et al., 1994; Porzgen et al., 2001); analyses of their loss-of-function phenotypes should provide interesting additional clues about how cocaine alters *Drosophila* behavior. Cocaine-sensitive monoamine transporters have also been cloned from *C. elegans* (Jayanthi et al., 1998; Ranganathan et al., 2001).

Cocaine Sensitization in *Drosophila*

In mammals, repeated exposure to cocaine results in a progressive and long-lasting enhancement in the locomotor-stimulant effects elicited by subsequent drug challenges. This phenomenon, termed behavioral sensitization, is thought to underlie some aspects of drug addiction (reviewed in Robinson and Berridge, 1993, 2001). Flies show behavioral sensitization to cocaine, the extent of which depends on the number of exposures and their frequency (McClung and Hirsh, 1998). Interestingly, sensitization develops slowly (with maximal effect at 6 h after a single cocaine exposure) and lasts for a relatively long time (24–48 h after a single cocaine exposure).

The trace amine tyramine has been implicated as a positive modulator of cocaine sensitization (McClung and Hirsh, 1999). Tyramine is synthesized from tyrosine by tyrosine decarboxylase (TDC), and most tyramine is converted into octopamine by tyramine-β-hydroxylase (TβH). Two mutants with defects in this biosynthetic pathway have differential effects on cocaine sensitization. Flies lacking TβH, and therefore octopamine but not tyramine, show normal sensitization. However, flies with reduced levels of both tyramine and octopamine, due to a mutation in the *inactive* locus (Homyk, 1977), fail to sensitize; this defect can be reversed by feeding the mutant flies tyramine, but not other biogenic amines (McClung and Hirsh, 1999). Therefore, tyramine appears to play a crucial role in cocaine sensitization. Consistent with this is the finding that sensitized flies contain measurably higher levels of TDC activity and that the kinetics of induction of this enzymatic activity parallel that of sensitization (McClung and Hirsh, 1999). No role for tyramine in cocaine-induced behaviors has yet been described in mammals.

In addition to tyramine, cocaine sensitization requires the normal function of several genes that control circadian rhythms, such as *period, clock*, and *cycle* (Andretic et al., 1999). Two hallmarks of sensitization, induction of TDC activity and increased responsiveness of postsynaptic dopamine receptors, are defective in *period* mutants (Andretic et al., 1999). This provides possible mechanisms by which *period* (and other circadian genes) regulates cocaine sensitization. In addition, responsiveness of postsynaptic dopamine receptors, ascertained by applying the dopamine D2-receptor agonist quinpirole to the nerve cord of decapitated flies, is under circadian modulation, an effect that requires *period* function (Andretic and Hirsh, 2000). Interestingly, mutations in *period* protein binding partner *timeless* sensitize normally, implying that *period* function in circadian rhythms and cocaine sensitization involves different molecular mechanisms and spatial requirements (Andretic et al., 1999). Whether sensitization itself changes as a function of time of day and whether circadian genes regulate psychostimulant sensitization in mammals remains to be determined.

Chronic cocaine administration in rats leads to upregulation of several components of the cAMP pathway, including adenylyl cyclase and protein kinase A. This upregulation appears to be important, because animals administered modulators of the pathway into the nucleus accumbens (a basal forebrain region critically important for reward) show changes in cocaine-induced locomotor stimulation and sensitization (Miserendino and Nestler, 1995). Interestingly, mutant flies lacking the PKA-RII regulatory subunit, and therefore having reduced cAMP-stimulated PKA activity, show strong resistance to the acute effects of cocaine and a failure to sensitize (Park et al., 2000). In addition, ectopic expression of either stimulatory or inhibitory Gα subunits in dopaminergic and serotonergic neurons causes decreased and increased acute cocaine sensitivity, respectively, and a complete block of sensitization (Li et al., 2000). Because these manipulations affect presynaptic neurons, it is inferred that sensitization relies on G-protein signaling in these cells. However, a role for the postsynaptic neurons, through regulation of the receptors or other downstream signaling components, cannot be excluded at this time.

In summary, upon exposure to free-base cocaine, flies show a series of behavioral changes that are reminiscent of those seen in rodents. In addition, flies develop long-lasting behavioral sensitization to cocaine, an effect that requires the trace amine tyramine. Pharmacologic and genetic evidence suggests that these behavioral changes are due to cocaine's action on dopamine and/or serotonin systems. Interestingly,

behavioral sensitization to cocaine in flies also requires the function of genes, such as *period*, that regulate circadian rhythms. Although a role for mammalian *period* genes in cocaine induced behaviors has not been reported, methamphetamine (a drug that like cocaine acts on monoamine transporters) has been shown to alter the expression of mouse *Per1*, one of the three known *period* homologs (Nikaido et al., 2001). In addition, chronic cocaine treatment dysregulates circadian feeding patterns in the rat (Nikaido et al., 1999; Giorgetti and Zhdanova, 2000).

NICOTINE

About one-third of all adults abuse nicotine in the form of smoked tobacco, despite well-documented and severe health risks. Nicotine has a clear molecular target for action—it is an agonist for channel-forming nicotinic acetylcholine receptors (nAChR). The mechanisms that translate nicotine action on nAChRs into nicotine addiction, however, remain elusive. Nicotine addiction is likely tied to its reinforcing effects. For example, in rodents, nicotine reinforces self-administration and conditioned place preference, a measure of a drug's rewarding properties (Stolerman and Shoaib, 1991). Behaviorally, low doses of nicotine increase locomotor activity, and repeated exposure to low doses results in locomotor sensitization (Museo and Wise, 1990). Conversely, high doses of nicotine depress locomotion. Long-term nicotine exposure can cause tolerance to the locomotor depressant, hypothermic, and other effects of nicotine. As is the case for most drugs of abuse, nicotine acts on brain reward centers, including the mesolimbic dopamine system in rodents (Pontieri et al., 1996; Di Chiara, 2000). Moreover, mice lacking the beta-2 subunit of the nAChR do not self-administer nicotine and fail to show nicotine-induced dopamine release in the nucleus accumbens (Picciotto et al., 1997).

To date, 11 acetylcholine receptor subunits have been identified in mammalian neurons. Each receptor is a pentamer made up of subunits—usually two alpha and three beta—that have distinct, but overlapping expression patterns in subsets of neurons. Upon binding of acetylcholine, nAChRs undergo conformational changes to transiently open a cation-selective channel, resulting in depolarization of the neuron. Subsequently, the ion channel closes, and the receptor is temporarily refractory to agonist (Lester and Dani, 1995). Long-term nicotine exposure can cause upregulation of nAChRs (Pauly et al., 1996). It has been proposed that this unexpected increase in receptors is due to a homeostatic adaptation to nicotine-induced receptor desensitization and long-term inactivation. However, chronic nicotine treatment has been shown to both increase and decrease the efficacy of cholinergic systems and the effects may be receptor subtype dependent (reviewed in Dani and Heinemann, 1996). The molecular mechanisms of long-term inactivation and upregulation of nAChRs are poorly understood.

Effects of Nicotine on *C. elegans* Behavior

In invertebrates, nicotine action has been studied most extensively in the nematode *C. elegans*. Worms have 42 different predicted nAChR subunits (Bargmann, 1998). This large number of receptor subunits suggests that the simpler nervous system of the worm has evolved more complex signaling; GABA receptors are also abundantly represented in worms. Acetylcholine function has been studied most intensively at the body wall muscle neuromuscular junction, where acetylcholine is the major excitatory neurotransmitter, as well as in the egg-laying vulval muscles and the pharyngeal muscles. Bath application of acetylcholine causes pharyngeal and body muscle contraction, paralysis, and increased egg-laying, demonstrating that at least some of these putative nAChRs bind acetylcholine (Lewis et al., 1980). Additionally, blocking acetylcholinesterase activity with the insecticide aldicarb, which leads to an increase in synaptic acetylcholine levels and perdurance, has similar effects (Rand and Russell, 1984).

The genetics of cholinergic neurotransmission in *C. elegans* has been characterized largely based on the effects of the antihelminthic nAChR agonist levamisole. Like nicotine, levamisole causes body wall muscle contraction, paralysis, and stimulates egg laying. Forward genetic screens for levamisole resistance genes identified three nAChRs that comprise the levamisole receptors: the alpha type UNC-38 and the nonalpha types UNC-29 and LEV-1 (Lewis et al., 1980; Fleming et al., 1997). Mutations in any one of these three genes blocks the behavioral effects of levamisole, though UNC-29 can partially substitute for mutated LEV-1, suggesting that the nonalpha subunits may be partially exchangeable in forming functional channels with UNC-38 (Lewis et al., 1980). In an electrophysiologic preparation of the *C. elegans* neuromuscular junction, levamisole responses were found to be absent from *unc-29* or *unc-38* mutant muscles (Richmond and Jorgensen, 1999). Both mutants, however, can still respond to acetylcholine and to nicotine, indicating the presence of at least two types of nAChRs on body wall muscles. The nicotine-

sensitive acetylcholine receptor on body wall muscles has not been molecularly identified.

Nicotine Tolerance in *C. elegans*

As in vertebrates, the acute effects of nicotine change with chronic or repeated exposure. When worms are initially exposed to nicotine, they hypercontract and increase egg-laying. Both of these behaviors adapt upon prolonged nicotine exposure. Interestingly, nicotine adapted worms, when removed from nicotine, become uncoordinated, resembling levamisole receptor mutant worms (Lewis et al., 1980). Thus, *C. elegans* develop nicotine tolerance, and appear to become dependent on nicotine to maintain normal locomotor behavior after a prolonged exposure. Long-term nicotine exposure also affects vulval muscle control of egg laying, a process controlled by both cholinergic and serotonergic input (Waggoner et al., 2000; Duerr et al., 2001). Worms exposed to nicotine for 3 or more hours no longer lay eggs in response to levamisole, and this nicotine adaptation lasts up to 24 h. Nicotine-adapted vulval muscles are not refractory to stimulation as they remain responsive to serotonin.

The vulval muscles, unlike body wall muscles, do not respond to nicotine or levamisole when the nAChR UNC-29 is mutated, indicating that UNC-29 is likely the major transducer of nicotine-elicited signals for egg laying. In addition, UNC-29 expression is strongly downregulated in nicotine-adapted worms (Waggoner et al., 2000). Furthermore, the mechanism of downregulation is post-transcriptional, indicating that adaptation is an active process that requires an intracellular response. In vertebrates, chronic nicotine exposure can affect nAChR function, either directly by causing changes in receptor abundance, or through receptor extrinsic regulation of signaling (Dani and Heinemann, 1996). A role for PKC in acetylcholine signal transduction and receptor desensitization has been shown in vertebrate experiments (Huganir et al., 1986; Fenster et al., 1999). Interestingly, PKC deficient worms still respond to levamisole, but the levamisole response is not altered after prolonged nicotine exposure. Additionally, UNC-29 expression is unaffected by nicotine in PKC mutants. Thus, PKC mediates the adaptive response of vulval muscles that leads to nicotine tolerance. Interestingly, PKC deficient worms were also egg laying defective in response to exogenously applied serotonin, which normally stimulates vulval muscles directly (Waggoner et al., 2000). At the neuromuscular junction, serotonin acts presynaptically, potentially through a PKC-dependent mechanism, to downregulate acetylcholine release onto body wall muscles (Nurrish et al., 1999). As the vulval muscles receive both serotonergic and cholinergic input it will be interesting to determine if serotonin regulates acetylcholine adaptation through a PKC-dependent pathway in vulval muscles. Alternatively, PKC could more directly act in nAChR function. Thus, the mechanism by which *C. elegans* adapts to chronic nicotine exposure is by altering acetylcholine receptor protein levels through a PKC dependent mechanism. The challenge for nicotine research in worms now is to extend these findings to other nicotinic receptor-containing circuits and to determine if circuit properties also show adaptive responses.

Effects of Nicotine on *Drosophila* Behavior

Homology-based cloning and genome analysis has identified ten receptors with homology to nAChRs in *Drosophila* (reviewed in Gundelfinger, 1992; Littleton and Ganetzky, 2000). As is the case for insects in general, *Drosophila* do not use acetylcholine at the neuromuscular junction; their nAChRs are therefore nervous system specific. In fact, acetylcholine, rather than glutamate, is believed to be the primary excitatory neurotransmitter in flies. Mutations in nAChR subunits have, to our knowledge, not been reported. However, complete loss-of-function mutations in choline acetyl transferase (the enzyme that synthesizes acetylcholine) have been isolated but cause lethality. Temperature-sensitive alleles cause paralysis when shifted to the restrictive temperature as adults (Kitamoto et al., 1992). Similarly, mutations in acetylcholinesterase (the enzyme that hydrolyzes acetylcholine) are lethal, but mosaic animals with brains composed of wild-type and mutant cells show developmental and behavioral defects (Greenspan et al., 1980; Hall et al., 1980), and resistance to insecticides (Pralavorio and Fournier, 1992; Fournier et al., 1993). These severe and pleiotropic phenotypes are consistent with a prominent role of acetylcholine in the developing and adult nervous system of *Drosophila*.

When exposed to volatilized nicotine, adult flies show a series of behavioral responses that range from hyperactivity at low doses, to hypokinesis and akinesis at higher doses. These effects interfere with the flies' natural propensity for negative geotaxis, a fact that was used to quantify nicotine-induced behaviors (Bainton et al., 2000). As for cocaine and ethanol, dopaminergic systems appear to play a role in nicotine-induced behaviors: 3IY-induced reduction of dopamine leads to reduced nicotine responsiveness. Consistent with cocaine and nicotine exerting their effects, at least in part, through common mechanisms,

is the observation that the two drugs show pronounced synergy when coadministered (Bainton et al., 2000). This synergy was greatly diminished in 3IY-treated flies.

CONCLUSIONS AND FUTURE DIRECTIONS

Although the use of *Drosophila* and *C. elegans* to study the mechanisms involved in actions of drugs of abuse in the nervous system is relatively recent, some surprising similarities with mammalian systems have already begun to emerge at the behavioral, neurochemical, and molecular levels. The extent of this overlap will likely increase in the next few years as the invertebrate studies grow in breath and depth, and as molecules identified in these organisms are tested functionally in mammals. The main strength of invertebrate systems relies, of course, in their accessibility to forward genetic analysis by single gene mutagenesis. Screens for mutations that alter various drug-induced behaviors will likely provide novel and unsuspected molecules and mechanisms.

Reverse genetic analyses, made increasingly feasible by recent technological advances, will likely provide complimentary information. For example, double-stranded RNA-mediated gene expression interference (RNAi) (Fire et al., 1998), together with the existing whole-genome sequence information, allows the targeted inactivation of any gene or predicted gene. In *Drosophila*, the use of transgene-based RNAi overcomes the limitations of single embryo RNA injection, affording analysis of large populations of flies and functional testing of genes involved in adult nervous system function (Kalidas and Smith, 2002). In addition to transgenic approaches to RNAi, worms can be soaked in ds-RNAs or fed bacteria expressing these molecules, approaches that have been used for chromosome-wide systematic functional analyses (reviewed in Bargmann, 2001).

Genetic tools that allow temporal and spatial control of gene expression will help define the relevant brain regions and help differentiate a gene's role in developmental and/or acute functional events. In flies, these techniques include the GAL4/UAS system (Brand et al., 1994), which allows the expression of any gene in the nervous system in patterns dictated by available GAL4 lines. Temporal control over this binary expression system can now be achieved by hormonal regulation of GAL4 function (Osterwalder et al., 2001; Roman et al., 2001). Spatiotemporal control over gene expression can also be obtained by a tetracyclin-inducible system (Stebbins et al., 2001). Finally, the combined use of the GAL4/UAS system with a conditional dominant-negative *shibire* transgene, allows fast and reversible inhibition of synaptic transmission in targeted nervous system cells (Kitamoto, 2001). The latter system was used recently to show that the fly mushroom bodies are required during memory retrieval, but not acquisition or consolidation, in olfactory conditioning (Dubnau et al., 2001; McGuire et al., 2001). The combination of these powerful genetic tools, together with emerging trans-synaptic labeling techniques (Yoshihara et al., 1999), will likely lead to major advances in our understanding of the functional neuronal circuits underlying complex behaviors, such as those induced by drugs of abuse, in *Drosophila*. In *C. elegans*, gene expression can also be regulated spatially and temporally, primarily by the use of gene fusions with abundantly available cell-type specific promoters and heat-shock promoters, respectively. Because the connectivity of the entire nervous system is known and its wiring is relatively simple, worms are ideally suited for probing neural circuitry. For example, laser ablation of identified neurons has been used to dissect neural circuits in olfactory behavior (Bargmann et al., 1993).

Reliable, sensitive, and efficient assays to quantify acute drug responses and their adaptive changes are now well established. However, assays for drug preference and conditioned preference (measuring a drug's motivational properties) need to be developed. In conditioned place preference assays, the animal's choice for sensory cues (such as visual or tactile cues) that had been associated with drug exposure is measured. If the animal "likes" the drug's effect, it will choose drug-paired over nondrug-paired environmental cues. Ethanol is an important component of the natural environment of *Drosophila melanogaster*. Flies eat, court and mate, and lay their eggs on fermenting plant materials, which often contain high levels of ethanol and other alcohols (3% or more) (McKechnie and Morgan, 1982; Van Delden, 1982). It is therefore not surprising that fly larvae and adults are attracted to ethanol (as described above). In addition, it was shown recently that the preference of adult flies for ethanol is increased by previous exposure to ethanol (Cadieu et al., 1999). Although the commonly used olfactory conditioning paradigms in *Drosophila* use a negatively reinforcing stimulus, electric shock, flies can also learn and remember the association of a rewarding stimulus, sucrose, with specific odors (Tempel et al., 1983). *C. elegans* show a chemotactic response to many volatile odorants including alcohols (Mori, 1999). *C. elegans* also learn to avoid an attractive odor that had been previously paired with an aversive odor (Morrison et al., 1999), and learn to

prefer a temperature that had been associated with the presence of food (Gomez et al., 2001). Therefore, the development of assays for conditioned ethanol (or other drug) preference should be feasible, and will hopefully emerge in the near future. Having such assays in place will allow the definition of the brain circuits that mediate reward and provide the basis for genetic screens to decipher the underlying molecular mechanisms. In addition, a genetic, molecular, and neuroanatomical dissection of conditioned reward behaviors in invertebrates may provide further insights into the mechanisms underlying learning and memory, processes that, as discussed above, are believed to play a crucial role in drug addiction.

In this review we have focussed on invertebrate genetic models of drug abuse. Although invertebrate studies are still nascent, at least some surprising parallels with mammals, at the behavioral and molecular level, have emerged already. In the next few years, the combined use of forward and reverse genetic approaches should provide many novel insights into the mechanisms by which drugs of abuse alter behavior. If past performance is predictive, many of these mechanisms will likely be shared with mammals.

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