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Identification of Molecular and Neuroanatomical Substrates

Regulating Acute Cocaine Sensitivity in Drosophila melanogaster

by

Linus Tzu-Yen Tsai

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

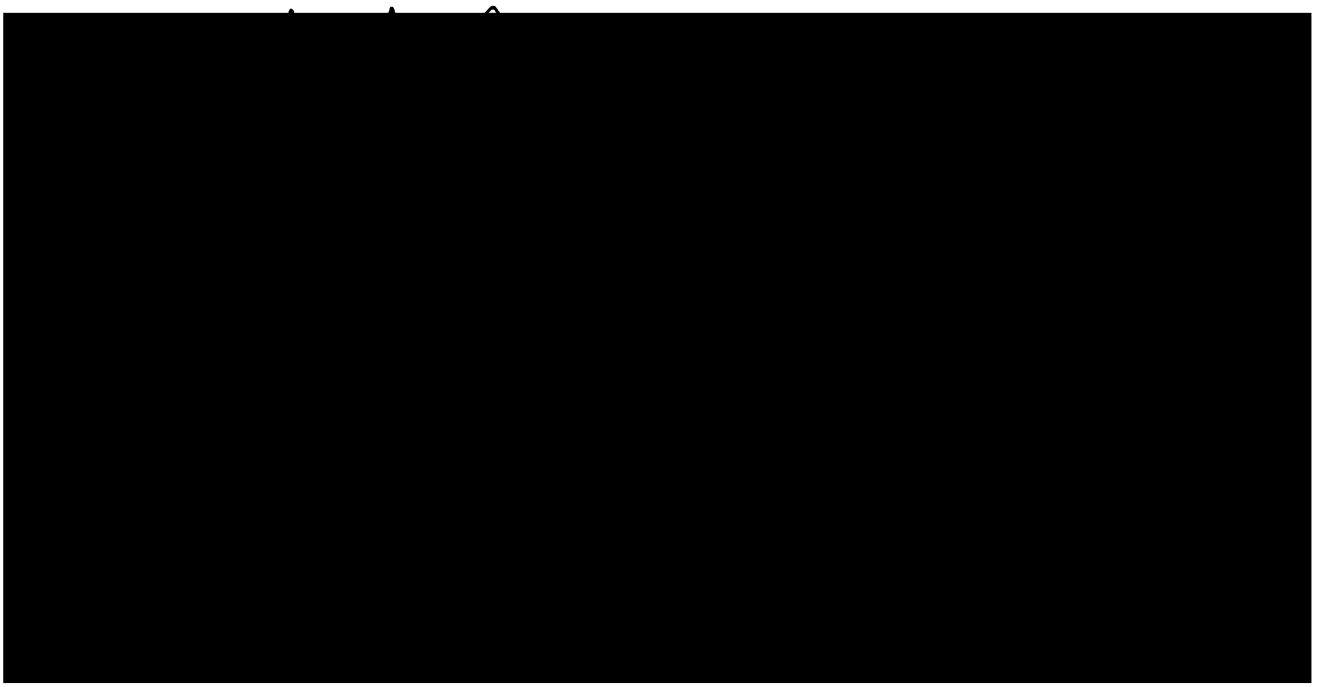
Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO



To my loved ones

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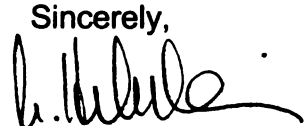
San Francisco, April 21st, 2004

To whom it concerns:

I hereby state that the work carried out by Linus Tsai for the purpose of his Ph. D. requirement is comparable to theses or dissertations normally awarded by the UCSF Graduate Program in Neuroscience. The work described in Chapter 1, which is published in the journal Current Biology, was carried out as a close collaboration between Linus Tsai and Roland Bainton; both authors contributed equally to this publication. The work described in Chapter 2, which has been submitted for publication, has been carried out by Linus Tsai in its majority; the work described in Figure 6 was carried out by our collaborator, Justin Blau.

Sincerely,



Ulrike Heberlein
Professor

Identification of molecular and neuroanatomical substrates regulating acute cocaine sensitivity in *Drosophila melanogaster*

Linus T.–Y. Tsai

Abstract

Susceptibility to drug addiction depends on a complex interaction between genetic and environmental factors. Studies of mammals have identified molecular substrates, neurochemical systems, and brain regions that mediate some of the actions of drugs of abuse. However, there still remains a fundamental lack of knowledge about what causes addiction and an even greater lack of insight into treatment options for patients afflicted by this disease.

The fruit fly *Drosophila melanogaster* has been a useful system for the genetic dissection of many developmental processes. Most of the genes and molecular pathways discovered in flies have structural or functional homologs in humans, revealing evolutionary conservation of developmental and physiological processes. More recently, *Drosophila* has been advanced as a model system for dissecting the mechanisms that regulate behavioral responses to drugs of abuse, including cocaine. Several of cocaine's most characteristic properties have been recapitulated in flies, including the induction of behaviors similar to those observed in mammals and the development of behavioral sensitization to repeated cocaine administration. We developed simple, high–throughput assays

to measure the behavioral effects of cocaine in flies. Paralleling mammalian findings, we show a common role for dopaminergic systems in mediating actions of cocaine and other drugs of abuse.

A genetic screen for mutants with altered cocaine sensitivity identified a role for the *Drosophila Lim–only* (*Lmo*) gene in the modulation of cocaine behaviors. *Lmo* mutants reveal a novel role for the *Drosophila* circadian pacemaker neurons (LNs) in modifying cocaine sensitivity. Reduced *Lmo* expression in the LNs increases cocaine sensitivity, while increased *Lmo* expression in the LNs reduces cocaine sensitivity. Furthermore, wild–type *Lmo* expression is necessary for robust circadian locomotor rhythms, suggesting a general role for *Lmo* in LN function. Genetic ablation or functional silencing of LNs reduces cocaine sensitivity, indicating that LN activity normally acts to increase cocaine sensitivity. Lastly, we show that the LNs' role in modulating cocaine actions is functionally and genetically separable from their role in modulating circadian rhythmicity. Together, these studies demonstrate *Drosophila* as a genetically tractable model organism in which to identify and characterize molecular and neurobiological substrates of drug action.

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Introduction

Drug addiction is a chronic relapsing disorder for which there are few effective treatments. In 2002, an estimated 22 million Americans aged 12 or older were classified with substance dependence or abuse (9.4 percent of the total population). This group does not even include those who abused tobacco, which has been estimated at 30% of the American population (Substance Abuse and Mental Health Services Administration, 2003). In 1995, addiction accounted for nearly 20% of what the federal government spent on entitlement programs (The National Center on Addiction and Substance Abuse at Columbia University, 1995). Drug addiction is an exceedingly complex psychiatric disorder defined behaviorally as either a loss of control over drug use or as compulsive drug–taking despite negative consequences. A better understanding of the neurobiological and molecular bases of addiction should aid in the development of more effective treatments for this disease. In that light, studies on the classic psychostimulant addictive drug, cocaine, have begun to provide insight into the mechanisms underlying addiction to drugs of abuse.

Cocaine is a naturally occurring plant alkaloid found in the leaves of the South American shrub, *Erythroxylon coca*. In pre–Columbian times, the Incas used the coca leaf for medicinal and recreational purposes, taking advantage of its stimulant properties to ward off fatigue and hunger and to enhance endurance and mood. Though initially banned by Spanish settlers, coca leaves were distributed to natives during work breaks in order to induce them to work longer

and harder in the mining of gold. Because coca leaves lost so much of their potency by the time they reached Europe, coca did not become widely used throughout the Europe until the active ingredient was isolated by Alfred Niemann in 1859. Commercial production began in the 1880's, and became popular among many notable scientists and physicians. The Italian neurologist Paolo Mantegazza extolled, "I would rather have a life span of ten years with coca than one of 1,000,000 centuries without coca" (Peterson, 1977). One of its early users and proponents, a young Sigmund Freud, wrote in a paper, "On Cocaine," of its ability to induce:

> "exhilaration and lasting euphoria, which in no way differs from the normal euphoria of the healthy person.... One senses an increase of self–control and feels more vigorous and more capable of work.... Long–lasting, intensive mental or physical work can be performed without fatigue; it is as though the need for food or sleep... were completely banished, [and] sleep can be just as easily omitted with no unpleasant consequences" (Freud, 1884).

Cocaine soon became widely available and was sold over the counter in many elixirs and formulations as a cure for ailments ranging from stomach upset and asthma to depression and alcohol or morphine addiction. Ironically, Coca–cola was first introduced in 1886 as a temperance drink "offering the virtues of coca without the vices of alcohol." Only in 1914 was non–medical cocaine use banned in the United States (Karch, 1998).

Historically, cocaine has been used as a recreational drug in its salt form, cocaine hydrochloride, by either nasal or IV administration. But recently, the smokeable free–base form, crack–cocaine, has become popular due to the more potent and rapid effects that result from its route of administration and increased

ability to cross the blood–brain barrier. Crack–cocaine delivers an intense, pleasurable euphoria that has been described by many as a "whole body orgasm." This short–lived high is often followed by a period of anxiety, depression, irritability and fatigue. Psychologically, long–term use almost always results in the development of an intense craving for the drug and can also lead to development of severe depression or paranoid psychosis. Of course, the biggest fallout of cocaine use may be the social consequences of addiction, with crime and violence often being a byproduct of acquiring the drug. A 2002 survey indicates about 1% of the U.S. population used cocaine with use of crack–cocaine at about 0.2% (Substance Abuse and Mental Health Services Administration, 2003).

Cocaine has widespread effects on multiple organ systems. However, its major effects, both acute and chronic, are mediated by the nervous system. Acutely, cocaine causes increases in blood pressure, body temperature, and heart and respiratory rates, accompanied by a euphoria, or "high." High doses can result in stroke, seizures, heart attacks or respiratory failure. Chronic use most commonly leads to severe craving, loss of appetitive behavior, and depression. Though the prevalence of cocaine addiction is not as high as those of nicotine and alcohol, cocaine has been widely studied because it is such a potent reinforcer and has one of the highest addictive liabilities of all drugs of abuse (Beers and Berkow, 2004). Additionally, it is of particular interest since its pharmacology and mechanism of action are well understood. Cocaine is a general inhibitor of plasma membrane monoamine transporters, thereby

increasing monoamine transmitter levels at the synapse. Much of the research on the neurobiological basis of its addictive properties has focused on its interaction with dopaminergic systems; however, more recent data has opened up the possibility of a role for serotonin and norepinephrine systems in development and maintenance of the addictive state.

Drosophila has long been a powerful genetic model organism, and its contributions to the understanding of developmental processes have been well–documented. In more recent years, groups have started to study this simple organism's rich behavioral repertoire. More traditional techniques, such as P–element mediated mutagenesis and transgenic expression, together with more recent advances in the sequencing of the *Drosophila* genome, gene array technology, and electrophysiological monitoring of neural activity, have made *Drosophila* an ideal model system for the study of the neural and genetic bases of these complex behaviors. Along these lines, my dissertation research uses this model organism to study how cocaine acts on the nervous system to generate acute behavioral responses, and to identify molecular and neural substrates that mediate these responses.

In this introduction, I will first review the literature on the study of cocaine in mammals, with a particular focus on the various methods that have been employed to study the genetic and neurobiological bases of its reinforcing qualities. In the second section, I will examine the contribution of *Drosophila* to the understanding of complex behaviors, with a focus on its use in studying behavioral responses to drugs of abuse. In the following chapters, I will first

present data that shows a role for dopamine in modulating acute responses to multiple drugs of abuse in *Drosophila*. Next, I will discuss the results of a screen for mutants with altered acute responses to cocaine, focusing on *Lmo* mutants and what they reveal about the neural circuits and molecular mechanisms that mediate cocaine responses. I will conclude with a quick evaluation of the benefits, risks and difficulties of such research and contemplate possible future directions based on the data presented.

Part I. Mechanisms of Action of Cocaine

Pharmacology of Cocaine

Cocaine and amphetamine are the most potent of a class of addictive drugs, the psychostimulants, which increase alertness, heighten arousal, and cause behavioral excitement. As an addictive drug, cocaine has two critical properties: it is reinforcing when administered acutely, and produces compulsive use when taken chronically. Cocaine produces its reinforcing effects pharmacologically by binding and inhibiting the Na^+/K^+–dependent plasma membrane transporters for dopamine (DA), serotonin (5–HT), and norepinephrine (NE) that are responsible for clearing the monoamine neurotransmitters from the synapse. Cocaine binds all three transporters at micromolar concentrations, and inhibits transport with similar potency (Johanson and Schuster, 1005). Additionally, at high doses, cocaine binds and blocks voltage–gated Na^+ channels (Matthews and Collins, 1983). As a result, cocaine is a potent local anesthetic, whose derivatives, lidocaine and procaine, are widely used in medical and dental practice.

Because selective Na^+ channel blockers are not addictive and are not reinforcing in animal models, this pharmacologic action of cocaine, as it pertains to addiction, has been disregarded. Similarly, antidepressant drugs that block the serotonin transporter (SERT) and/or the norepinephrine transporter (NET), but not the dopamine transporter (DAT), do not exhibit many of the abuse–associated qualities of cocaine. First, they do not produce reinforcement

in animal models, nor do they produce euphoria, craving, or abuse despite long–term use (Tzschentke, 1998). Specific DAT inhibitors such as methylphenidate, on the other hand, are reinforcing. This has led to the prevailing dopamine hypothesis of cocaine reinforcement, which suggests that action of cocaine on DAT, and the resulting potentiation of dopaminergic transmission is responsible for the reinforcing qualities of cocaine (Kuhar et al., 1991). This theory has been expanded to include a general role for dopamine in reinforcement and addiction to all drugs of abuse (Di Chiara, 1995).

Human Cocaine Studies

Due to the many legal and ethical limitations of doing studies with a highly addictive controlled substance, studies of cocaine's actions on naïve individuals are rare, and have not provided the information that such studies of nicotine and alcohol have. However, studies of cocaine abusers have contributed to the understanding of the genetic and environmental bases of drug addiction, as well as to the understanding of the neurochemical, neuroanatomical, and molecular bases of addiction.

Genetic Risk Factors

Twin and family studies have long established a genetic contribution to the development of addiction to alcohol. Multiple studies have estimated that genetic factors account for 40–60% of the risk of alcohol addiction, with the remaining risk related to environmental factors (Prescott and Kendler, 1999; Schuckit,

2000). Simlarly, family studies have also indicated that cocaine abuse liability aggregates in families (Bierut et al., 1998; Merikangas et al., 1998). Twin and adoption studies have indicated that genetic factors are responsible for a great part of this increased risk. Multiple groups have estimated the genetic heritability of cocaine dependence at 70–80% for both men and women (Kendler and Prescott, 1998; van den Bree et al., 1998; Kendler et al., 2000). These rates of cocaine abuse heritability are in line with other common medical disorders such as adult onset diabetes and hypertension. Complex disorders such as these have genetic bases, but no single genetic locus can explain the risk. In these diseases, polymorphisms in multiple genes are likely to contribute to total risk in complex combinations.

Studies on the co–morbidity of cocaine and other substance abuse in familial studies have shown the presence of both common and substance–specific factors in familial transmission of abuse liability (Bierut et al., 1998). Twin studies have reinforced this data, and indicated three general principles. First, most of the genetic factors that influence illicit use and abuse are common to all classes of drugs tested. Second, substance–specific genetic factors only have an influence on drug use, but not drug abuse. Lastly, shared environmental risk factors also impact drug use more than they do drug abuse (Kendler et al., 2003). These results indicate that addiction to drugs of abuse share common genetic bases, and likely a common underlying neurobiology. Secondarily, they suggest that studying the common substrates and mechanisms

of drug action will yield greater insight into the process of addiction than studying those that are drug–specific.

One such molecular substrate shared my multiple drugs of abuse may be the D_2 dopamine receptor gene (DRD2). Variations in the DRD2 have been associated with increased abuse risk for multiple substance abuse disorders including alcoholism, cocaine, nicotine, and opioid dependence, and obesity (Noble, 2000). As in studies of alcoholics, allelic prevalence of the *Taq*I A minor (A1) allele was significantly higher in cocaine–dependent subjects as compared to controls (Noble et al., 1993; Persico et al., 1996). Interestingly, quantitative trait locus (QTL) analyses of recombinant mouse strains have localized QTLs for alcohol drinking preference to the DRD2 chromosomal region (Phillips et al., 1994).

Imaging Studies

Imaging studies on the brains of drug–addicted subjects have also revealed insight into the mechanisms underlying cocaine action and cocaine addiction. In particular, imaging studies have provided information on cocaine pharmacokinetics and pharmacologic mechanism of action. PET scan studies using [^{11}C]cocaine have shown a correlation between DAT occupancy by cocaine and the "high" perceived with cocaine use (Volkow et al., 1997a). Similar studies have also reinforced the importance of route of administration in the "high" achieved by cocaine use (Volkow et al., 1997a; Volkow et al., 2000). Additionally, PET studies using methylphenidate, a selective DAT inhibitor, and

[^{11}C]raclopride, which can measure drug–induced changes in dopamine, have shown a stong correlation between increased DA levels and the reinforcing "high" (Volkow et al., 1999b). These data provide further evidence that it is cocaine's action on DAT that is responsible for its reinforcing effects.

Imaging studies have also provided information about the changes in brain circuitry and function that underlie the behavioral changes of an addicted individual. PET and autopsy studies have consistently showed decreases in dopamine D2 receptors in cocaine addicts, as well as decreases in activity in areas of the brain to which midbrain dopaminergic neurons project (Volkow et al., 1993; Thompson et al., 1997). These neurons have been strongly implicated as a reward or motivation center that may be dysregulated in the addicted state (see below). Other PET studies have shown decreases in D2 receptor density and decreased glucose metabolism within the striatum of drug–naive individuals with the DRD2 A1 allele (Pohjalainen et al., 1998).

PET studies on cocaine addicts have also shown a decrease in DA release during cocaine administration (Volkow et al., 1997b). This finding, coupled with observed decreases in D2 receptors in addicts, has led to the proposal that addiction is a reward deficiency syndrome (Blum et al., 2000; Comings and Blum, 2000). In this theory, addiction results from a decrease in activity of the dopaminergic striatal reward neurons. Understimulation of these reward circuits puts subjects at a greater risk for seeking drugs that can compensate for this dopaminergic deficit. In support of this hypothesis, individuals with low D2 receptor levels found the DAT–specific inhibitor

methylphenidate pleasant, while individuals with high dopamine receptor availability found it unpleasant (Volkow et al., 1999a).

Mammalian Models of Cocaine Addiction

Animal models for addiction have been developed in order to avoid many of the ethical, financial, and technological limitations of human research. Briefly, I will review some of the major animal models for human drug abuse, as well as some of their more important contributions towards the understanding of addiction. Although ideally, an animal model would be isomorphic to the human disease, the multifactorial bases of addiction makes this ideal impossible to achieve. However, many individual aspects of drug use and abuse have been successfully modeled, and findings based on these models have found relevant analogies to the human condition.

Behavioral Paradigms

1. Acute Effects

The acute effects of cocaine administration in animals are remarkably similar in humans and rodents. Cocaine induces increased heart rate and body temperature, as well as many measurable psychomotor behaviors. At low doses, rodents show dramatic increases in locomotor activation and rearing/climbing behaviors. At moderate doses, an increase in stereotypical behaviors such as licking, sniffing, head bobbing, circling and grooming can be observed. And at high doses, cocaine can cause seizures and sudden death. Cocaine's ability to

produce locomotor activation at low doses is shared with almost all other drugs of abuse. This shared property, together with further evidence presented below, has led to the psychomotor stimulant theory of addiction, which proposes a connection between stimulant properties of drugs and their abuse liability (Wise and Bozarth, 1987). Based on this hypothesis, many groups have used acute locomotor responses to cocaine as a fast, simple readout for assessing its reinforcing or addictive properties.

2. Reinforcement

In order to model cocaine's hedonic and/or reinforcing properties, three main behavioral paradigms have been developed: self–administration, enhancement of brain self–stimulation reward, and conditioned place preference. Many animal species can readily learn to self–administer cocaine intravenously by pressing a lever in an operant learning paradigm. Even freebase cocaine self–administration via smoking can be demonstrated in monkeys (Carroll et al., 1990). When given unlimited access to cocaine, Rhesus monkeys ignored food and administered drug to the point of death within 5 days (Johanson et al., 1976). A variation on the self–administration paradigm is the use of progressive ratio schedules to determine the "break point" of drug responding. In this paradigm, an increasing number of operant responses (lever–presses) are needed to obtain further drug delivery. Animals eventually stop responding when the response requirement is too great for the amount of reinforcement the drug gives. This "breakpoint" is directly related to an animal's motivation to self–administer drug

and serves as a good measure of its reinforcing properties.

Another model for drug reinforcement is the enhancement of brain stimulation reward. In this behavioral paradigm, animals are allowed to administer intracranial self–stimulation (ICSS), electric shocks to the brain that can be pleasurable to the animal at high enough stimulation frequencies. Cocaine enhances brain self–stimulation as measured by a shift in the frequency-response curve for ICSS. That is, at ICSS frequencies to which animals normally don't respond (i.e. not pleasurable enough a stimulus), animals will respond if administered cocaine. This has been interpreted to show cocaine enhances the rewarding effects properties of brain stimulation, and reliably predicts addictive liability of various drugs (Wise et al., 1992).

Lastly, reinforcing properties of cocaine have also been demonstrated in conditioned place preference (CPP) paradigms. Most CPP studies are run as a classical–conditioning paradigm, with the motivational or reinforcing properties of the drug serve as an unconditioned stimulus (US) that is paired with a previously neutral set of cues, the conditioned stimuli (CS). After training, the CS acquire secondary motivational properties such that they elicit either approach (reinforcing) or withdrawal (aversive) behaviors when subsequently presented a choice between a chamber containing the CS and another containing neutral stimuli. By computing the amount of time spent in each chamber, a measure of the reinforcing properties of drugs can be determined. Recent studies have introduced operant conditioning to this paradigm through the administeration the drug during training only when the animal entered the conditioning chamber

(Crowder and Hutto, 1992). These studies showed that this procedure is even more potent than classic CPP in producing place preference.

3. Models for Chronic Cocaine Use

Chronic cocaine administration can result in alterations in responses to cocaine. In humans, habitual users report tolerance to cocaine's euphoric effects with increased use. At the same time, addicts report an increase in craving upon chronic cocaine use. Animal models can recapitulate these plastic changes in drug responding. Depending upon the pattern of administration, animals can either become tolerant or sensitized to cocaine's effects, usually measured as changes in cocaine–induced locomotor activity or stereotypy. In general, continuous administration (usually via infusion pumps) causes tolerance, while intermittent administration results in sensitization. Sensitization and tolerance can also be demonstrated for cocaine self–administration (Schenk and Partridge, 1997). Because locomotor sensitization has been neuroanatomically localized to the motivational circuits which also underlie cocaine's reinforcing properties (see below), it has been suggested that sensitization of this circuit underlies increases in drug–motivated behaviors such as craving (Robinson and Berridge, 1993).

Although cocaine withdrawal is not thought to be a critical part of the addictive process, withdrawal–induced anhedonia has been modeled by increased ICSS thresholds in brain reward paradigms upon cessation of cocaine administration (Markou and Koob, 1991). Lastly, the self–administration paradigm has been used as a model for craving and relapse. After a period of

extinction in which operant responses do not provide drug, animals are tested under various conditions to determine what factors can reinstate responding. These studies have shown that reinstatement of drug seeking can be elicited by acute priming injections of drugs, drug-associated cues, and environmental stressors. Craving and relapse are elicited in recovering addicts by similar cues (Shaham et al., 2003).

Neuroanatomical Circuits Underlying Cocaine Behaviors

Another important contribution of animal models of drug use and abuse has been the identification of the brain circuitry that underlies drug–related behaviors. The basic techniques that have been used to uncover these circuits are modifications of the self–administration, ICSS, and CPP paradigms, such that the drug or electrical stimulation is spatially controlled. Importantly, because the stimulation is not presented to the animal's sensory world, their unsensed, motivating properties are revealed (Wise, 2002).

Olds and Milner were the first to show that rats would work for direct electrical stimulation of specific regions of the brain, establishing the concept of reward centers of the brain (Olds and Milner, 1954). Further research showed that rats would seek out environments in which they received brain stimulation, suggesting these areas of the brain were also motivational centers (Coons et al., 1965). Subsequent studies have localized the neuroanatomical pathways that mediate reinforcement of brain stimulation and natural rewards to the mesolimbic dopaminergic system and its inputs and outputs. Lastly, abundant evidence has

been collected via localized administration of drugs that this system also mediates the reinforcing properties of cocaine and other drugs of abuse (Wise, 1984).

The mesolimbic dopamine system consists of dopaminergic projections from the midbrain ventral tegmental area (VTA) to the nucleus accumbens (NA). Psychostimulant drugs are self–administered into the NA shell, including amphetamine, cocaine, and the specific DAT blocker nomifensine (Hoebel et al., 1983; Carlezon et al., 1995). These dopaminergic cells receive multiple inputs, including glutamatergic input from the medial pre–frontal cortex (mPFC), GABAergic input from the ventral pallidum (VP) and NA, and cholinergic input from the pedunculopontine tegmental and latero–dorsal pontine nucei. Various drugs that interact with these inputs to increase mesolimbic dopamine neuron activity are self–administered into the VTA. Cholinergic drugs (e.g. nicotine) are self–administered and produce CPP in the VTA (Yeomans et al., 1985; Ikemoto and Wise, 2002). $GABA_A$ antagonists, which block inhibitory GABAergic input, are self–administered into VTA, as are opioid agonists, which inhibit the GABAergic neurons presynaptically (Bozarth and Wise, 1981; Ikemoto et al., 1997a; Ikemoto et al., 1997b). Lastly, blockade of glutamate receptors in the VTA blocks the rewarding effects of glutamatergic input that can be generated by electrical stimulation, D1 receptor activation, or NMDA receptor activation in the mPFC (You et al., 1998; McBride et al., 1999; Wise, 2002).

The mesolimbic dopamine neurons synapse onto the spines of the GABAergic medium spiny neurons of the NA, of which there are 3 main

subtypes: D1 receptor–expressing neurons that also express dynorphin and substance P and project to the substantia nigra; D2 receptor–expressing neurons that also express enkephalin and project primarily to the VP; and neurons that co–express both D1 and D2 receptors as well as enkephalin and substance P, and whose projections are unknown (Gerfen, 1992; Surmeier et al., 1996). It is likely that the D1–containing projections to the substantia nigra mediate the locomotor–activating effects of cocaine and other drugs of abuse, as direct D1 antagonist administration into the NA blocks locomotor activation. Glutamatergic inputs from other cortical areas also synapse onto the medium spiny neurons, and NMDA antagonists are self–administered into the NA (Carlezon and Wise, 1996). Lastly, excitatory cholinergic and inhibitory inputs make connections with these neurons, and both cholinergic agents and opiates are self–administered directly in to the NA (Goeders et al., 1984; Ikemoto et al., 1998). Decreases in medium spiny neuron output, which may occur thorugh increases in inhibitory mesolimbic dopamine neuron input, direct inhibitory opiate action, or through the blockade of excitatory cholinergic or glutamatergic inputs, is a common effect of self–administered drugs, and likely mediates their reinforcing properties (Wise, 2002). Studies to determine the neuroanatomical substrates of sensitization and reinstatement have also implicated the same neuronal circuits in these more plastic behaviors as well (Pierce and Kalivas, 1997; Shaham *et al.*, 2003).

Genetics of Cocaine–Related Behaviors

1. Genetic Approaches to the Study of Cocaine

One of the major benefits of using animal models is the potential for utilizing genetic tools to gain an increased understanding of diseases. Strains that have been selectively bred for differences in cocaine responses demonstrate that, as in humans, cocaine–related behaviors are heritable (Marley et al., 1998). Schechter showed that rats could be selectively bred to be either cocaine–preferring (CP) or cocaine–nonpreferring (CNP) using a simple CPP task (Schechter, 1992). Interestingly, he found that cocaine–induced locomotor activation was also significantly decreased in CNP rats, suggesting a common genetic basis for the reinforcing and locomotor–activating effects of cocaine, in support of the psychomotor stimulant theory of drug abuse.

Inbred rodent strains also show differential responses to cocaine (Downing et al., 2003a). Recombinant inbred lines have been made from these strains in order to define QTLs for locomotor sensitization to cocaine as well as for cocaine–induced locomotor activation and seizures (Miner and Marley, 1995b; Miner and Marley, 1995a; Phillips et al., 1998). As expected, these studies showed that genetic bases of cocaine responses are polygenic. Phillips *et al.* showed both common and unique QTLs for sensitization and locomotor activation (Phillips et al., 1998). Though QTL mapping has not identified any single cocaine behavior genes, multiple studies show a large segment of chromosome 15 to be involved in locomotor activation (Jones et al., 1999; Boyle

and Gill, 2001). This chromosomal region is particularly interesting because QTLs for locomotor activation of other drugs, including phencyclidine and ethanol, have mapped to this area, and it has also has been linked to dopamine receptor DRD2 densities (Downing et al., 2003b).

Narrowing QTL intervals is made difficult and expensive due to the small effect of any individual gene in these polygenic behaviors and the number of mice needed to define these intervals. As of yet, no novel candidate genes have been identified in cocaine QTL studies. Another approach to studying diseases with polygenic inheritance is to try to identify all possible sources of genetic variation that contribute to the disease state. As a result, many researchers have turned to transgenics and targeted deletions of candidate genes in order to identify genes and molecules that mediate drug responses. In this vein, knockouts have been used to great effect to address longstanding questions about cocaine's pharmacologic site of action, what neurotransmitter pathways are involved in its reinforcement, and the relationship between cocaine's locomotor activating and reinforcing effects.

2. Monoamine Transporter Knockouts

The dopamine hypothesis of cocaine reward arose from data showing that selective DAT, but not SERT or NET inhibitors are rewarding. Furthermore, lesions of dopamine neurons and studies of dopamine receptor inhibitors in brain stimulation and self–administration paradigms have implicated dopamine circuits in reward mechanisms (De Wit and Wise, 1977; Roberts et al., 1980; Gallistel et

al., 1982; Roberts and Koob, 1982). In order to confirm this hypothesis, targeted deletions of the dopamine transporter were produced and have provided some surprising results that call the dopamine hypothesis into question (Uhl et al., 2002; Gainetdinov and Caron, 2003).

DAT knockout mice showed no cocaine–induced locomotor activation (Giros et al., 1996). Together with data that shows normal cocaine–induced locomotion in SERT and NET knockouts, these data demonstrate conclusively that DAT mediates cocaine–induced changes in locomotor activity (Xu et al., 2000; Sora et al., 2001). Somewhat surprisingly, however, cocaine remained rewarding to these mice. DAT knockouts show intact cocaine CPP and self–administration (Rocha et al., 1998; Sora et al., 1998). These results go against the dopamine hypothesis of cocaine reward; furthermore, they provide evidence for a disassociation of cocaine–induced hyperactivity and its rewarding properties, refuting the psychostimulant theory of addiction.

Based on these data, hypotheses arose that other monoamine transporters might also be involved. However, NET and SERT knockouts showed either enhanced or unchanged cocaine CPP (Sora et al., 1998; Xu et al., 2000). Only when both DAT and SERT were removed was cocaine–induced CPP blocked (Sora et al., 2001). These results suggest that multiple sites for cocaine's rewarding actions exist, providing a redundancy that is only unmasked in DAT knockouts.

It is likely that in DAT knockouts, compensatory developmental changes occur that allow other monoamine transporters to mediate reward. In support of

this, selective inhibitors for both SERT and NET produced strong place preference in DAT knockout, but nor wild–type mice (Hall et al., 2002). A remaining question is whether the rewarding properties of cocaine are still mediated by dopamine in these mice. Both cocaine and amphetamine elevated dopamine levels in the nucleus accumbens (NA) in DAT knockouts, as did a specific NET inhibitor, indicating a role for NET in clearing dopamine in the NA (Carboni et al., 2001). Together, these monoamine transporter knockouts support a major, if not complete, role for DAT and dopamine in mediating cocaine reward. However, the contributions of cocaine action on NET and SERT in reward under more normal circumstances remain to be determined. Tissue–specific and inducible knockouts that prevent large–scale developmental compensations may help clarify their roles.

3. DA Receptor Knockouts

Both CPP and self–administration studies have given mixed results with regards to which dopamine receptor subtype(s) might be the substrates for the observed dopaminergic reinforcement. On the whole, most D2 agonists can elicit CPP, while D1 agonists do not. However, CPP studies also indicate that D1 antagonists rather than D2 antagonists attenuate cocaine–induced place preference (Tzschentke, 1998). Together these results are paradoxical, and indicate either an incomplete understanding of the receptor pharmacology (either of specific D1 or D2 receptor isoforms or action on non–dopaminergic receptors)

or alternatively that different receptor subtypes may be involved in different aspects of reinforcement, perhaps in different areas of the brain.

Targeted deletions of all five dopamine receptor subtypes have been generated and tested for cocaine's reinforcing and locomotor–activating effects. Cocaine–induced hyperactivity was eliminated in D1 receptor knockouts; however, cocaine CPP was retained in these knockouts (Lecklin et al., 1995; Miner et al., 1995). Together with behavioral data from D1 receptor inhibitors, these results indicate that dopaminergic action through the D1 receptor underlies cocaine's locomotor activating properties, but not its rewarding properties.

D2 receptor knockouts show increased self–administration of high, but not low doses of cocaine (Caine et al., 2002). Self–administration of high doses of cocaine occurs with regular interinjection intervals under fixed ratio schedules; and as dose increases, interinjection intervals also increase, suggesting a regulation of optimal drug levels (Katz, 1989). The increased self–administration of high–dose cocaine in D2 receptor knockouts indicates a deficit in the ability of these mice to regulate cocaine intake in this manner. The increased responding perhaps reflects an attempt for the animal to compensate for a decrease in cocaine's reinforcing properties. These results dovetail with recent studies in rhesus monkeys which showed that lower D2 levels predict higher cocaine self–administration rates (Morgan et al., 2002). It will be interesting to see how these mice behave in other models reinforcement, especially in a progressive ratio self–administration paradigm.

Together, these dopamine receptor knockouts provide further evidence against the psychomotor stimulant theory of cocaine reward, while supporting the view of addiction as a reward deficiency syndrome. In particular, it adds to growing evidence for a role for D2 receptors in regulating cocaine's reinforcing properties and provides a mechanism for how the changes in D2 receptor expression levels in cocaine users and DRD2A1 allele carriers might underlie the increased risk of drug reinstatement or addiction.

4. Drawbacks of Targeted Gene Deletions

As the above results show, targeted gene deletions can be useful for understanding the pharmacologic action of drugs and the neural circuits with which they interact. One major drawback of this method is the choice of candidate genes examined generally originates from extant knowledge about how drugs work, and is therefore constrained to the study of already well–characterized processes with expected outcomes. Another drawback in behavioral studies is that the gene is deleted throughout the brain, complicating interpretation of the data for a specific brain region or behavioral circuit for which there are very complicated inputs and outputs. As an example, though studies of dopamine receptor knockouts have focused on action of dopamine on the nucleus accumbens output cells, dopamine receptors are expressed in the VTA and on many of the neuronal inputs into the mesolimbic dopaminergic system as well. Another drawback is that the developmental compensations/adaptations that result from loss of a gene often make interpretation of behavioral data

complicated. For instance, there are multiple examples showing that the developmental compensation that occurs in mice with receptor knockouts results in different behavioral phenotypes compared to wild–type mice administered locally–applied, specific receptor antagonists (Castanon et al., 2000). Compensation may be an especially important caveat to take into account in the study of the nervous system, an organ with unique properties of plasticity.

Ideally, genetic models of addiction would reflect the genetic changes that occur in human addicts. Along these lines, determining candidate polymorphisms from QTLs and using these genetic variations as bases for genetic knock–ins should be a long–term goal. In general, the gene expression changes of candidate genes identified in human addicts or in rodents bred for changes in cocaine responses, have been subtle. As has been observed for dopamine receptor and transporter densities in mice associated with cocaine QTLs, small changes in expression have been correlated with marked changes in cocaine response (Jones et al., 1999; Janowsky et al., 2001). A better way to model these findings would be the generation of mice with more subtle changes in gene expression. Both conditional and tissue–specific knockouts or gene replacements with attenuated expression levels should help accomplish this goal.

5. Identifying Novel Molecular Substrates for Cocaine Addiction

Forward genetic screening in mice, especially for complex behaviors, requires vast amounts of resources, and has rarely been undertaken successfully. One experimental method that has been more recently used to

identify novel genes involved in drug behavior are gene expression profiles. Screens for transcripts that were elevated upon cocaine administration using differential display or subtractive hybridization techniques have isolated such genes as the one encoding the neuropeptide CART (cocaine and amphetamine–regulated transcript), which has been subsequently shown to induce hyperlocomotion and CPP when administered to the VTA (Jaworski et al., 2003). Similarly identified, rGβ1, a G protein β subunit that is induced in the nucleus accumbens by chronic cocaine administration, has been shown to be involved in cocaine sensitization (Wang et al., 1997).

More recent studies have used gene chip microarray technology to detect expression changes for a larger set of genes. Using this technology, Nestler and colleagues have used an inducible bitransgenic ΔFosB mouse to study the effects of ΔFosB on gene regulation in the nucleus accumbens. ΔFosB had previously been shown to be induced within the NA by chronic exposure to multiple drugs of abuse; furthermore, overexpression of ΔFosB using these mice enhanced cocaine's rewarding properties (Kelz et al., 1999; Nestler et al., 2001). So far, the signaling molecule Cdk-5 and the transcription factor NF-KB have been identified as genes involved in modulating long–term changes that may underlie cocaine addiction (Ang et al., 2001; Bibb et al., 2001).

Various studies have estimated the number of drug–regulated genes to be 1-5%, so it will be important to develop techniques and strategies to identify which of these many genes contribute meaningfully to the addictive process (Nestler, 2000; Toda et al., 2002). In this vein, Caron and colleagues used 3

genetic models (DAT, NET, and VMAT knockouts) and one pharmacologic model (cocaine–sensitized mice) of cocaine supersensitivity and looked for shared expression changes (Yao et al., 2004). Six genes were identified, including an adenylate cyclase and a regulator of nitric oxide signaling, which are involved in signaling processes previously implicated in the drug responses (Moore et al., 1998; Nestler, 2001; Collins and Kantak, 2002; Gholami et al., 2002). In addition, a novel role for the synaptic scaffolding protein PSD–95 was identified, and subsequent behavioral experiments with PSD–95 knockout mice showed altered cocaine–induced plasticity within reward circuits as well as altered cocaine–induced locomotor activation. Examination of PSD-95's role in cocaine reinforcement paradigms awaits.

These gene–profiling studies have mostly focused on genes that are involved in establishing the addicted state. Identification of these genes will undoubtedly provide markers for addiction susceptibility, but it is also important that they provide information on potential therapeutic targets for treating addiction. Along these lines, research should also focus on uncovering genes that may be involved in drug reinstatement behaviors, since most patients do not seek treatment until their reward pathways are already altered to the addicted state. More recently, animal models of reinstatement have been developed, though they are more difficult and time–consuming to enact. However, the potential for providing pharmacologic targets provides a strong impetus for attacking these obstacles.

Part II. *Drosophila* as a Model System for Drug Studies

There is no doubt that *Drosophila* has been a useful model system in which to perform forward genetic screens. *Drosophila* has been successfully used to study processes as diverse as the role of *homeobox* genes in development and differentiation to more recent characterizations of genes involved in complex behaviors such as learning and memory. Throughout these studies, the *Drosophila* model system has been especially useful in identifying novel genes and molecules involved in these processes. For instance, forward genetic screens in *Drosophila* first identified all of the major molecular components of the circadian clock. Importantly, subsequent studies in mammalian models have shown conserved functions for these genes in regulation of mammalian circadian rhythms.

Advantages and Disadvantages of *Drosophila*

The advantages of using *Drosophila* center around the ability to perform rapid, high–throughput assays and the potential for discovery of novel genes and pathways involved in behaviors. Its short generation time and wealth of genetic and molecular tools allow for the relatively easy forward genetic identification of genes and their subsequent manipulation. Specifically, numerous visible markers, balancer chromosomes, and tagged transposable elements facilitate the creation and molecular characterization of mutants. Additionally, transgenic techniques, including the binary GAL4/UAS expression system and stable RNA interference (RNAi) constructs, permit temporal and spatial control of gene

expession (Rorth, 1996; Lee and Carthew, 2003). These techniques will be discussed in detail as they pertain to individual experiments described in the rest of the introduction and subsequent chapters. Additionally, the completion of the *Drosophila* genome sequence and its ongoing annotation provides rapid access to gene function as well as many additional molecular tools.

Important for the study of drug responses, flies have a complex behavioral repertoire, including foraging behaviors, intricate courtship rituals, rest/activity rhythms, and motivated movement (Sokolowski, 2001). Behavioral plasticity is also quite evident: they learn and develop long-term memories in associative learning paradigms, develop tolerance and sensitization to various drugs of abuse, and entrain multiple aspects of behavior to light and temperature. Via genetic screens, the molecular mechanisms of many aspects of these behaviors have begun to be elucidated in *Drosophila*. By and large, the genes and biochemical pathways identified via *Drosophila* mutants have led to the discovery of homologous genes and pathways in mammals with similar functions.

Drosophila also provides some more specific advantages with regards to the study of complex behaviors that have been largely overlooked. First, controlling genetic background becomes increasingly important when studying quantitative traits under polygenic influences. As in mice, even wild–type flies vary in many morphological and behavioral characteristics (Gerlai, 1996; Sokolowski, 2001). Interactions between varying genetic backgrounds and mutations that underlie a phenotype of interest have previously shown that specific genetic backgrounds can enhance or completely eliminate phenotype

(de Belle and Heisenberg, 1996). In flies, standard techniques to place mutants and wild–type controls into the same genetic background allow for relatively fast elimination of genetic background differences. This is an especially important issue to take into consideration when using strains generated in different laboratories, as most often, inter–laboratory genetic backgrounds are not comparable.

Lastly, one potential benefit of using *Drosophila* to study behaviors is the use of P element transpositional mutagenesis to generate mutants. Other than the well–documented techniques to include sequences within the P element that can act as enhancer traps or inducible promoters, P elements offer the benefit of creating hypomorphic mutations, which are more likely than gene deletions to reflect the subtle gene regulation changes that underlie natural variation in behavioral responses to drugs. In this manner, studying these hypomorphic mutations should complement studies of complete gene deletions, which can often be easily generated via these same P element insertions.

Ethanol Studies

Acute effects

Drosophila has recently been advanced as a model system to examine the genetic and molecular bases for the actions of drugs of abuse (Wolf and Heberlein, 2003). There are striking similarities between the behavioral responses of flies and mammals to drugs like alcohol and cocaine, and recent studies have determined that at least some of the molecular substrates for the

action of these drugs are shared as well. Because the mechanisms of action of ethanol are so poorly understood (ethanol has the capacity to alter functions of many membrane proteins, but no single receptor has been identified), phenotype–driven screens in *Drosophila* may be especially useful in identifying important regulators of ethanol responses (Peoples et al., 1996; Bellen, 1998).

As do humans, *Drosophila* show a biphasic behavioral response to ethanol administration (Moore et al., 1998; Bainton et al., 2000). Initially, ethanol induces a marked increase in locomotor activity, analogous to the disinhibition and euphoria reported in humans, which correlates with low levels of ethanol within the fly. As ethanol levels increase in the fly, hyperlocomotion is followed by incoordination, loss of postural control, and finally sedation. Interestingly, the ethanol concentrations that cause increased locomotor activity and incoordination/sedation in flies are very similar to those which cause parallel behaviors in rodents and humans (Singh and Heberlein, 2000; Wolf et al., 2002).

Simple behavioral assays have been developed to evaluate these two broad phases of ethanol response. Ethanol–induced changes in locomotor activity were first quantified manually using simple line–crossing assays, which showed increases in activity as well as increases in ethanol–induced turning (Singh and Heberlein, 2000). This assay has been automated to increase throughput: the inebri–actometer measures the beam crossings of 128 individual flies in small tubes simultaneously (Parr et al., 2001). Recently, an automated assay to measure more detailed locomotor responses has been developed (Wolf et al., 2002). In this assay, small groups flies are allowed to freely move in a

small box or tube. Fly position and body axis are recorded each 1/10th of a second, and subsequent data extraction can provide simple measures such as fly velocity and degree of turning, as well as more complicated measures such as percentage of time spent at certain speeds (which may reflect different behavioral states) or in certain areas of the testing arena. Wolf *et al.* utilized this assay to show that the flies move in bouts of activity, with variable periods of inactivity separating these bouts. Upon ethanol exposure, flies increased the duration of their bouts (a measure of the probability of bout termination) as well as the amount of time spent in states of fast locomotion, movement of greater than 20 mm/s; meanwhile, bout frequency, a measure of the probability of bout initiation, was unaltered by ethanol administration (Wolf et al., 2002). It is likely that these locomotor endophenotypes may be controlled by different neuronal circuits and/or by different signaling pathways. A similar analysis of spontaneous walking showed that the protocerebral bridge neurons of the central complex are involved in maintenance, but not initiation of locomotor movement (Martin et al., 1999). An analysis of these more specific measures should thus allow for better categorization of mutants into meaningful functional subgroups; ideally, mutant analyses will also provide insight into the neuroanatomical or molecular pathways that control these phenotypes.

Locomotor assays can also give insight into the sedative phase of ethanol response, which is generally observed as the dissipation of locomotor hyperactivity. Another assay that primarily measures later phases of ethanol–induced behaviors is the inebriometer, an apparatus which was first

developed to selectively breed flies for increased or decreased sensitivity to alcohol (Cohan and Hoffmann, 1986; Weber, 1988). In this assay, vaporized ethanol is administered into a column fitted with mesh baffles upon which the flies can walk and hang. Flies are inserted into the top of the column, where they remain initially due to their innate propensity to negatively geotax. As flies become more inebriated and lose their ability to retain postural control, they elute from the column and are counted in 3–minute time bins (Moore et al., 1998). From this data, a mean elution time can be calculated, which corresponds to the mean ethanol concentration needed to cause loss of postural control.

Genetic screens for mutants that show altered responses to the acute effects of ethanol have been performed using the inebriometer (Moore et al., 1998; Singh and Heberlein, 2000). Similar screens using the locomotor tracking assay are also currently underway (Wolf and Heberlein, unpublished data). The mutant *cheapdate*, which has increased sensitivity to the intoxicating effects of ethanol, was isolated using the inebriometer, and has been shown to result from disruption of the *amnesiac* (*amn*) gene (Moore et al., 1998). *amnesiac* encodes a PACAP–like neuropeptide that has also been implicated in olfactory associative learning (Quinn et al., 1979; Feany and Quinn, 1995). It has been proposed that the amnesiac neuropeptide increases adenylate cyclase (AC) activity post–synaptically by activating G protein coupled receptors, and that the inability to stimulate adenylate cyclase leads to the increased sensitivity of *cheapdate* mutants (Moore et al., 1998). Consistent with this proposal, mutants in the calcium/calmodulin–sensitive adenylate cyclase *rutabaga* (*rut*), as well as

mutants in the catalytic subunit of cAMP–dependent protein kinase (DCO) also caused increased ethanol sensitivity. Furthermore, a pharmacologic agent that stimulates the cAMP pathway reversed *amn* and *rut* phenotypes, while a PKA inhibitor caused increased sensitivity of wild–type flies. *amn* and *rut* flies also showed precocious ethanol–induced hyperactivity when analyzed in the locomotor tracking system.

An analysis of the bout structure of this precocious hyperactivity showed that *amn* flies had increased fast locomotion and activity bout length, while *rut* flies had increased bout frequency and activity bout length. These results first suggest cAMP signaling is likely involved in many aspects of ethanol–related behavioral responses. Furthermore, they suggest that *amn* and *rut* both share and have unique involvements in regulating aspects of this behavior. These findings help to explain the finding that flies with a mutation in a regulatory subunit for PKA (*pka–RII*), causing decreased cAMP–induced PKA activity, were resistant to the sedating effects of alcohol (Park et al., 2000). Perhaps *pka–RII*, and *amn* and *rut* act in different cells to regulate ethanol responses in opposite ways. The cAMP pathway has been implicated in both acute and chronic responses to ethanol in other addiction models as well. Acute exposure to ethanol potentiates receptor–mediated increases in cAMP in cell culture systems, and mice that overexpress an adenylyl cyclase show resistance to ethanol's sedating effects and a greater propensity for developing tolerance to ethanol (Gordon et al., 1992; Tabakoff et al., 2001). Moreover, cAMP metabolism has

also been implicated in mediating the reinforcing effects of drugs of abuse (Diamond and Gordon, 1997; Nestler and Aghajanian, 1997).

Other genes implicated in regulation of acute responses of *Drosophila* to ethanol have been identified via reverse genetics. Based on the fact that the first two alcohol mutants, *rutabaga* and *amnesiac*, were also learning or memory mutants, *fasciclin II* (*fasII*) mutants, which have deficits in olfactory learning, were tested for their response to ethanol in the inebriometer (Cheng *et al.*, 2001). These flies also showed increased sensitivity to ethanol, which suggests shared mechanisms may underlie learning and drug response. However, expression of *fasII* from a heatshock transgene failed to restore wild–type responses, even though they could rescue learning phenotypes. These results suggest a more complicated relationship between learning and ethanol phenotypes. Lastly, metabotropic $GABA_B$ receptors and GABAergic systems have been implicated in acute responses to ethanol in rodent models (Dar, 1996). Based on these studies, Dzitoyeva et al showed that administration of pharmacologic $GABA_B$ receptor antagonists reduced the sedative effects of ethanol (Dzitoyeva et al., 2003). Furthermore they induced reductions of $GABA_B$ receptor expression by injected dsRNA and observed a similar resistance to ethanol.

Ethanol Tolerance

In humans, chronic alcohol use leads to the development of both pharmacokinetic (metabolic) and pharmacodynamic (functional) tolerance; the latter reflects neural adaptations to chronic alcohol administration. *Drosophila*

have been shown to develop a functional tolerance to a single dose of ethanol. Tolerance is manifested as an increased mean elution time in the inebriometer or as a longer–lasting hyperactive phase in the locomotor tracking system (Scholz et al., 2000). As ethanol from the first exposure is completely metabolized at the time of the second exposure, this tolerance paradigm most closely resembles rapid tolerance paradigms observed in rodents (Crabbe et al., 1979). Based on a previously identified role for $GABA_B$ receptors in development of ethanol tolerance, Dzitoyeva *et al.* used both pharmacology and RNAi–mediated techniques to confirm a similar role for $GABA_B$ receptors in development of tolerance to ethanol in flies (Zaleski et al., 2001; Dzitoyeva et al., 2003). Preliminary work from genetic screens has also uncovered a role for cellular stress–response pathways in mediating certain aspects of tolerance (Scholz and Heberlein, unpublished data). Additionally, development and characterization of longer–term forms of tolerance are being actively pursued (Berger and Heberlein, unpublished data).

Ethanol Preference

Ethanol preference has been demonstrated in larvae, and more recently in adult flies (Parsons, 1980; Cadieu et al., 1999). Larvae are attracted to low concentrations of ethanol, but are repelled by high concentrations. Larval studies showed that the activity of the alcohol–metabolizing enzyme alcohol dehydrogenase (ADH) correlated with the preference larvae showed for ethanol (Gelfand and McDonald, 1980; Depiereux et al., 1985). In adult

ethanol–preference assays, flies are given a choice between food with or without ethanol, and their preference is measured as the duration of proboscis extension on each food. Adult flies have been shown to prefer ethanol–containing media, and furthermore, previous exposure to ethanol increases preference in later trials. In light of recent data that shows a central role for octopamine in mediating the reinforcing properties of sugar in both bees and flies, it will be interesting to determine whether ethanol preference behaviors are mediated by similar circuits (Schwaerzel et al., 2003).

Cocaine studies

Although the pharmacologic receptor(s) for cocaine have been identified and are well–characterized, the downstream effects of cocaine action remain nebulous. Multiple cocaine–sensitive monoamine transporters have been identified in flies, though no mutants in these genes exist to date (Corey et al., 1994; Demchyshyn et al., 1994; Porzgen et al., 2001). The presence of homologous transporters with similar responses to cocaine, however, illustrates the potential for discovering functionally homologous downstream regulators of cocaine responses in *Drosophila*.

Acute Responses

When exposed to volatilized free–base cocaine, flies show many behavioral responses that are similar to those of rodents (McClung and Hirsh, 1998). Low doses elicit intense grooming and a reduction in locomotion.

Moderate doses induce highly stereotyped patterns of locomotion including repetitive circling, and sideways and backwards motion. Higher doses induce severely uncoordinated, hyperkinetic behaviors such as erratic flight and spinning. Usually these hyperkinetic behaviors progress to complete akinesia, from which the flies can recover over a five– to ten–minute period.

The first attempts to quantify this behavior used a behavioral scoring system based on the observed behaviors (McClung and Hirsh, 1998). Flies were filmed and each fly was given a score of 0–7 based on the most severe behavioral response observed over a five minute interval. Cocaine effect was then calculated as a percentage of flies that had behavioral scores ≥ 5 (hyperkinetic movements). Later studies quantified cocaine's effect on disrupting startle–induced negative geotaxis, as assayed in a glass cylinder dubbed the crackometer (Bainton et al., 2000). Flies normally are motivated to run to the top of the cylinder when startled, but cocaine interferes with this behavior in a dose–dependent manner. An evaluation of the crackometer and behavioral scoring methods indicates that both methods are comparable, as flies that do not negatively geotax in response to startle would generally be assessed a behavioral score of 5 or greater. Lastly, a more detailed analysis of cocaine–induced behaviors has been performed using a locomotor tracking assay similar to that described above for tracking ethanol–induced changes in activity (Bainton et al., 2000). Measures of locomotor speed and turning in response to cocaine have been quantified in this manner. Unfortunately, the broad range of cocaine–induced behaviors and the steep U–shaped

dose–response curves for these tracking measures has made it difficult to apply this assay more generally as an assessment of cocaine sensitivity (Tsai and Heberlein, unpublished data). Genetic screens for cocaine mutants with altered acute responses to cocaine have been identified in the crackometer, and mutants in the *Lmo* gene, a negative regulator of LIM–homeodomain transcription factor activity, will be described in chapter two.

A role for cAMP signaling in acute responses to cocaine has also been uncovered in *Drosophila*. Mutants with reduced PKA–RII regulatory subunit expression have reduced cAMP–activated PKA activity levels and show marked resistance to the acute effects of cocaine (Park et al., 2000). However, targeted expression of either a stimulatory ($G\alpha_s$) or inhibitory ($G\alpha_i$) $G\alpha$ subunit in dopamine and sertonin neurons resulted in reduced ($G\alpha_s$) or increased ($G\alpha_i$) acute sensitivity to cocaine (Li et al., 2000). These results are somewhat contradictory, since expression of $G\alpha_i$ and PKA–RII regulatory subunit mutations, which both should result in decreased cAMP–activated PKA activity, showed opposite phenotypes. However, Park *et al.* also showed that in PKA–RII mutants, basal PKA activity was significantly increased (Park et al., 2000). Perhaps, basal PKA activity, rather than cAMP–stimulated PKA activity is a more important factor for regulation of acute cocaine responses. Alternatively, cAMP pathways may function in a more complicated manner via multiple neurons or pathways to regulate cocaine responses, as has been observed in acute ethanol responses (Rodan et al., 2002).

Cocaine Sensitization

In mammals, an important property of drugs of abuse, and especially cocaine, is its ability to induce enhanced behavioral responses to repeated administration. This behavioral sensitization has been proposed to underlie important aspects of the addictive process including craving (Robinson and Berridge, 1993). Flies can also develop a behavioral sensitization to repeated administration of cocaine, even after a single dose (McClung and Hirsh, 1998).

This paradigm has been effectively utilized to uncover a novel role for circadian genes in the regulation of cocaine sensitization (Andretic et al., 1999). Andretic *et al.* screened through existing *Drosophila* behavioral mutants with the hypothesis that learning mutants might affect the plastic process of sensitization. Instead they found that multiple genes involved in regulating circadian rhythmicity showed cocaine sensitization abnormalities. Alleles for *period* (per), *clock*, *cycle*, and *doubletime*, which are central components of the molecular clock, all showed reduced sensitization induced by cocaine. Another component of the clock, *timeless* (*tim*), however, showed normal cocaine sensitization. These data not only demonstrate a novel role for circadian genes in non–circadian functions, but also suggest a novel *tim*–independent molecular role for *per*. These data spurred cocaine sensitization studies on mice with deletions of the two mouse *per* homologs. While both *mPer1* and *mPer2* mutants show normal acute responses to cocaine, *mPer1* mice fail to sensitize or develop conditioned place preference to cocaine; *mPer2* mutants, on the other hand show increased sensitization and enhanced conditioned place preference (Abarca et al., 2002).

Together, these results demonstrate the powerful potential of screens in *Drosophila* to identify novel molecules involved in responses to drugs of abuse.

Circuits Underlying Responses to Drugs

Acute Responses to Drugs

In mammals, a common property of drugs of abuse is the stimulation of mesolimbic dopaminergic circuits (Wise and Bozarth, 1985; Di Chiara, 1995; Schultz, 1997). As will be described in chapter one, pharmacologic depletions of dopamine implicate dopaminergic systems in mediating acute responses to multiple drugs of abuse, including ethanol, cocaine, and nicotine (Bainton et al., 2000). Further, G protein signaling within dopaminergic and serotonergic cells has been implicated in acute cocaine responses via the targeted overexpression of stimulatory or inhibitory Gα subunits (Li *et al.*, 2000). In the *Drosophila* adult brain, six clusters of dopamine neurons make broad projections throughout the brain, in particular to regions of the mushroom bodies, central complex, and subesophageal ganglion (SEG) (Budnik and White, 1988; Nassel and Elekes, 1992). High levels of dopamine receptor expression have also been observed in the mushroom bodies (Han *et al.*, 1996; Kim *et al.*, 2003). However, flies with pharmacologic ablations of the mushroom bodies showed normal responses to ethanol in the inebriometer and locomotor tracking assays, suggesting dopaminergic projections to non–mushroom body sites regulate drug responses (Rodan *et al.*, 2002; Wolf and Heberlein, unpublished observations).

Rodan *et al.* defined regions of the brain in which cAMP signaling

regulates ethanol responses (Rodan *et al.*, 2002). In this study, PKA inhibitor transgene expression was driven by a collection of GAL4 lines which expressed in various patterns. Though not likely the only neurons involved, neurons of the Pars Intercerebralis (PI) and ventral SEG were found to mediate resistance to the incoordinating effects of ethanol, but not to its locomotor activating effects. Both these sets of neurosecretory neurons send projections towards the esophagus and to the hypocerebral ganglion–corpus cardiacum complex, a neuroendocrine organ (Shiga *et al.*, 2000). The presence of dopaminergic projections into the SEG suggests a possible interaction between these sets of neurons.

Ethanol Tolerance

By testing a collection of *Drosophila* mutants with structural abnormalities in specific parts of the brain, Scholz *et al.* showed that the neurons of the central complex of the fly brain are necessary for the normal development of rapid ethanol tolerance (Scholz et al., 2000). These results were confirmed by disrupting neurotransmission of these neurons using targeted expression of a tetanus toxin transgene. These central complex neurons had previously been shown to be involved in regulating bouts of locomotion (Martin et al., 1999). The mushroom body complex, a region of the brain previously implicated as a mediator of olfactory learning and memory, does not seem to be involved in development of ethanol tolerance. Lastly, flies with mutations in tyramine–β–hydroxylase, an enzyme required for octopamine synthesis, display reduced tolerance to ethanol (Scholz et al., 2000). This finding is especially

interesting in light of rodent data showing a role for noradrenergic systems in development of tolerance (Le et al., 1981). As octopamine is the invertebrate functional homolog for vertebrate norepinephrine (Roeder, 1999), these results indicate that conserved mechanisms mediate tolerance in these two different systems. These similarities also suggest great promise that identification of *Drosophila* mutants with altered ethanol tolerance will provide meaningful insight into the genetic and molecular mechanisms underlying functional tolerance in humans.

Cocaine Sensitization

A role for dopamine and/or serotonin systems has also been defined for the development of cocaine sensitization in addition to its role in acute cocaine sensitivity (Li *et al.*, 2000). Overexpression of stimulatory or inhibitory Gα subunits in dopamine and serotonin neurons blocks cocaine sensitization, as does synaptic silencing using targeted expression of a tetanus toxin transgene. The trace amine tyramine has also been implicated as a key regulator of cocaine sensitization(McClung and Hirsh, 1999). *Inactive* mutants, which have markedly reduced tyramine levels, fail to sensitize to cocaine. Furthermore, *inactive* mutant sensitization is restored to type by tyramine feeding, but not by feeding other biogenic amines. A functional role for tyramine and other trace amines in mammals has been hotly contested. However, the recent cloning of a family of trace amine G protein–coupled receptors has solidified an unknown, but functional role for tyramine (Borowsky *et al.*, 2001). Tyramine levels are elevated

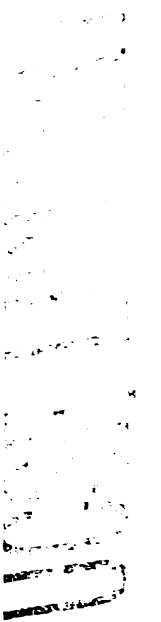
in the brain and its dysregulation has been implicated in processes such as Parkinson's disease, depression, and schizophrenia (Premont *et al.*, 2001). The identification of *Drosophila* tyramine–containing neurons and their projection patterns remains a future endeavor; however a functional tyramine receptor has been cloned which mediates neuromodulatory effects of tyramine at the neuromuscular junction (Nagaya *et al.*, 2002).

Conclusions

Although many genetic factors contribute to the risk of addiction to drugs of abuse, only the DRD2 locus has been associated with an increased risk in humans. In rodent models, unbiased approaches to identify genes responsible for strain differences in drug responses have not yet been successful. Although mouse knockouts have provided an understanding of the pharmacological actions of drugs of abuse, they have not provided great insight into risk factors for addiction or potential targets for treatment of addiction. Furthermore, reverse genetic approaches have not identified novel molecules or pathways that might mediate important aspects of addiction.

Drosophila provides a well–established genetic system for the study of drug responses. Flies exhibit a wealth of complex behaviors, including both acute and chronic responses to alcohol and cocaine, which are similar to those of mammals. Early work from this less than decade–old field of study has shown that these drug behaviors are mediated by many of the same molecules and neurotransmitter systems previously implicated in mammalian drug responses.

Furthermore, novel molecules and systems identified in *Drosophila* have already informed and found parallels in mammalian models. In the remainder of this dissertation, I will first describe efforts to use this system to define neurotransmitter systems that mediate acute drug responses; then I will discuss the identification of a novel gene that regulates cocaine responses and the characterization of a set of neurons that mediates its effects.



References

Abarca, C., U. Albrecht and R. Spanagel (2002). Cocaine sensitization and reward are under the influence of circadian genes and rhythm. Proc Natl Acad Sci U S A *99*(13): 9026-30.

Andretic, R., S. Chaney and J. Hirsh (1999). Requirement of circadian genes for cocaine sensitization in Drosophila. Science *285*(5430): 1066-8.

Ang, E., J. Chen, P. Zagouras, H. Magna, J. Holland, E. Schaeffer and E. J. Nestler (2001). Induction of nuclear factor-kappaB in nucleus accumbens by chronic cocaine administration. J Neurochem *79*(1): 221-4.

Bainton, R. J., L. T. Tsai, C. M. Singh, M. S. Moore, W. S. Neckameyer and U. Heberlein (2000). Dopamine modulates acute responses to cocaine, nicotine and ethanol in Drosophila. Curr Biol *10*(4): 187-94.

Beers, M. H. and R. Berkow, Eds. (2004). The Merck manual of diagnosis and therapy. Rahway, N.J.,, Merck Sharp & Dohme Research Laboratories [etc.].

Bellen, H. J. (1998). The fruit fly: a model organism to study the genetics of alcohol abuse and addiction? Cell *93*(6): 909-12.

Bibb, J. A., J. Chen, J. R. Taylor, P. Svenningsson, A. Nishi, G. L. Snyder, Z. Yan, Z. K. Sagawa, C. C. Ouimet, A. C. Nairn, E. J. Nestler and P. Greengard (2001). Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. Nature *410*(6826): 376-80.

Bierut, L. J., S. H. Dinwiddie, H. Begleiter, R. R. Crowe, V. Hesselbrock, J. I. Nurnberger, Jr., B. Porjesz, M. A. Schuckit and T. Reich (1998). Familial transmission of substance dependence: alcohol, marijuana, cocaine, and

habitual smoking: a report from the Collaborative Study on the Genetics of Alcoholism. Arch Gen Psychiatry *55*(11): 982-8.

Blum, K., E. R. Braverman, J. M. Holder, J. F. Lubar, V. J. Monastra, D. Miller, J. O. Lubar, T. J. Chen and D. E. Comings (2000). Reward deficiency syndrome: a biogenetic model for the diagnosis and treatment of impulsive, addictive, and compulsive behaviors. J Psychoactive Drugs *32 Suppl*: i-iv, 1-112.

Borowsky, B., N. Adham, K. A. Jones, R. Raddatz, R. Artymyshyn, K. L. Ogozalek, M. M. Durkin, P. P. Lakhlani, J. A. Bonini, S. Pathirana, N. Boyle, X. Pu, E. Kouranova, H. Lichtblau, F. Y. Ochoa, T. A. Branchek and C. Gerald (2001). Trace amines: identification of a family of mammalian G protein-coupled receptors. Proc Natl Acad Sci U S A *98*(16): 8966-71.

Boyle, A. E. and K. Gill (2001). Sensitivity of AXB/BXA recombinant inbred lines of mice to the locomotor activating effects of cocaine: a quantitative trait loci analysis. Pharmacogenetics *11*(3): 255-64.

Bozarth, M. A. and R. A. Wise (1981). Intracranial self-administration of morphine into the ventral tegmental area in rats. Life Sci *28*(5): 551-5.

Budnik, V. and K. White (1988). Catecholamine-containing neurons in Drosophila melanogaster: distribution and development. J Comp Neurol *268*(3): 400-13.

Cadieu, N., J. Cadieu, L. El Ghadraoui, A. Grimal and Y. Lamboeuf (1999). Conditioning to ethanol in the fruit fly-a study using an inhibitor of ADH. J Insect Physiol *45*(6): 579-586.

Caine, S. B., S. S. Negus, N. K. Mello, S. Patel, L. Bristow, J. Kulagowski, D. Vallone, A. Saiardi and E. Borrelli (2002). Role of dopamine D2-like receptors in

cocaine self-administration: studies with D2 receptor mutant mice and novel D2 receptor antagonists. J Neurosci *22*(7): 2977-88.

Carboni, E., C. Spielewoy, C. Vacca, M. Nosten-Bertrand, B. Giros and G. Di Chiara (2001). Cocaine and amphetamine increase extracellular dopamine in the nucleus accumbens of mice lacking the dopamine transporter gene. J Neurosci *21*(9): RC141: 1-4.

Carlezon, W. A., Jr., D. P. Devine and R. A. Wise (1995). Habit-forming actions of nomifensine in nucleus accumbens. Psychopharmacology (Berl) *122*(2): 194-7.

Carlezon, W. A., Jr. and R. A. Wise (1996). Microinjections of phencyclidine (PCP) and related drugs into nucleus accumbens shell potentiate medial forebrain bundle brain stimulation reward. Psychopharmacology (Berl) *128*(4): 413-20.

Carroll, M. E., K. L. Krattiger, D. Gieske and D. A. Sadoff (1990). Cocaine-base smoking in rhesus monkeys: reinforcing and physiological effects. Psychopharmacology (Berl) *102*(4): 443-50.

Castanon, N., K. Scearce-Levie, J. J. Lucas, B. Rocha and R. Hen (2000). Modulation of the effects of cocaine by 5-HT1B receptors: a comparison of knockouts and antagonists. Pharmacol Biochem Behav *67*(3): 559-66.

Cheng, Y., K. Endo, K. Wu, A. R. Rodan, U. Heberlein and R. L. Davis (2001). Drosophila fasciclinII is required for the formation of odor memories and for normal sensitivity to alcohol. Cell *105*(6): 757-68.

Cohan, F. M. and A. A. Hoffmann (1986). Genetic divergence under uniform selection. II. Different responses to selection for knockdown resistance to ethanol

among Drosophila melanogaster populations and their replicate lines. Genetics *114*(1): 145-64.

Collins, S. L. and K. M. Kantak (2002). Neuronal nitric oxide synthase inhibition decreases cocaine self-administration behavior in rats. Psychopharmacology (Berl) *159*(4): 361-9.

Comings, D. E. and K. Blum (2000). Reward deficiency syndrome: genetic aspects of behavioral disorders. Prog Brain Res *126*: 325-41.

Coons, E. E., M. Levak and N. E. Miller (1965). Lateral hypothalamus: learning of food-seeking response motivated by electrical stimulation. Science *150*(701): 1320-1.

Corey, J. L., M. W. Quick, N. Davidson, H. A. Lester and J. Guastella (1994). A cocaine-sensitive Drosophila serotonin transporter: cloning, expression, and electrophysiological characterization. Proc Natl Acad Sci U S A *91*(3): 1188-92.

Crabbe, J. C., H. Rigter, J. Uijlen and C. Strijbos (1979). Rapid development of tolerance to the hypothermic effect of ethanol in mice. J Pharmacol Exp Ther *208*(1): 128-33.

Crowder, W. F. and C. W. Hutto, Jr. (1992). Operant place conditioning measures examined using morphine reinforcement. Pharmacol Biochem Behav *41*(4): 825-35.

Dar, M. S. (1996). Mouse cerebellar GABAB participation in the expression of acute ethanol-induced ataxia and in its modulation by the cerebellar adenosinergic A1 system. Brain Res Bull *41*(1): 53-9.

de Belle, J. S. and M. Heisenberg (1996). Expression of Drosophila mushroom body mutations in alternative genetic backgrounds: a case study of the mushroom body miniature gene (mbm). Proc Natl Acad Sci U S A *93*(18): 9875-80.

De Wit, H. and R. A. Wise (1977). Blockade of cocaine reinforcement in rats with the dopamine receptor blocker pimozide, but not with the noradrenergic blockers phentolamine or phenoxybenzamine. Can J Psychol *31*(4): 195-203.

Demchyshyn, L. L., Z. B. Pristupa, K. S. Sugamori, E. L. Barker, R. D. Blakely, W. J. Wolfgang, M. A. Forte and H. B. Niznik (1994). Cloning, expression, and localization of a chloride-facilitated, cocaine-sensitive serotonin transporter from Drosophila melanogaster. Proc Natl Acad Sci U S A *91*(11): 5158-62.

Depiereux, E., N. Hougouto, J. Lechien, M. Libion-Mannaert, M. C. Lietaert, E. Feytmans and A. Elens (1985). Larval behavioral response to environmental ethanol in relation to alcohol dehydrogenase activity level in Drosophila melanogaster. Behav Genet *15*(2): 181-8.

Di Chiara, G. (1995). The role of dopamine in drug abuse viewed from the perspective of its role in motivation. Drug Alcohol Depend *38*(2): 95-137.

Diamond, I. and A. S. Gordon (1997). Cellular and molecular neuroscience of alcoholism. Physiol Rev *77*(1): 1-20.

Downing, C., K. Rodd-Henricks, R. J. Marley and B. C. Dudek (2003a). Genetic variation in the psychomotor stimulant properties of cocaine in Mus musculus. Psychopharmacology (Berl) *167*(2): 159-66.

Downing, C., K. K. Rodd-Henricks, L. Flaherty and B. C. Dudek (2003b). Genetic analysis of the psychomotor stimulant effect of ethanol. Genes Brain Behav *2*(3): 140-51.

Dzitoyeva, S., N. Dimitrijevic and H. Manev (2003). Gamma-aminobutyric acid B receptor 1 mediates behavior-impairing actions of alcohol in Drosophila: adult RNA interference and pharmacological evidence. Proc Natl Acad Sci U S A *100*(9): 5485-90.

Feany, M. B. and W. G. Quinn (1995). A neuropeptide gene defined by the Drosophila memory mutant amnesiac. Science *268*(5212): 869-73.

Freud, S. (1884). Über Coca. Centralblatt für die gesammte Therapie *2*(7): 289-314. Translated by Steven A. Edminster; additions to the translation by Frederick C. Redlich. in Cocaine Papers, 1975, edited by R. Byck, New York, NY.

Gainetdinov, R. R. and M. G. Caron (2003). Monoamine transporters: from genes to behavior. Annu Rev Pharmacol Toxicol *43*: 261-84.

Gallistel, C. R., M. Boytim, Y. Gomita and L. Klebanoff (1982). Does pimozide block the reinforcing effect of brain stimulation? Pharmacol Biochem Behav *17*(4): 769-81.

Gelfand, L. J. and J. F. McDonald (1980). Relationship between ADH activity and behavioral response to environmental alcohol in Drosophila. Behav Genet *10*(3): 237-49.

Gerfen, C. R. (1992). The neostriatal mosaic: multiple levels of compartmental organization. Trends Neurosci *15*(4): 133-9.

Gerlai, R. (1996). Gene-targeting studies of mammalian behavior: is it the mutation or the background genotype? Trends Neurosci *19*(5): 177-81.

Gholami, A., A. Haeri-Rohani, H. Sahraie and M. R. Zarrindast (2002). Nitric oxide mediation of morphine-induced place preference in the nucleus accumbens of rat. Eur J Pharmacol *449*(3): 269-77.

Giros, B., M. Jaber, S. R. Jones, R. M. Wightman and M. G. Caron (1996). Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature *379*(6566): 606-12.

Goeders, N. E., J. D. Lane and J. E. Smith (1984). Self-administration of methionine enkephalin into the nucleus accumbens. Pharmacol Biochem Behav *20*(3): 451-5.

Gordon, A. S., D. Mochly–Rosen and I. Diamond (1992). Alcoholism: a possible G–protein disorder. G Proteins *8*: 191-216.

Hall, F. S., X. F. Li, I. Sora, F. Xu, M. Caron, K. P. Lesch, D. L. Murphy and G. R. Uhl (2002). Cocaine mechanisms: enhanced cocaine, fluoxetine and nisoxetine place preferences following monoamine transporter deletions. Neuroscience *115*(1): 153-61.

Han, K. A., N. S. Millar, M. S. Grotewiel and R. L. Davis (1996). DAMB, a novel dopamine receptor expressed specifically in Drosophila mushroom bodies. Neuron *16*(6): 1127-35.

Hoebel, B. G., A. P. Monaco, L. Hernandez, E. F. Aulisi, B. G. Stanley and L. Lenard (1983). Self-injection of amphetamine directly into the brain. Psychopharmacology (Berl) *81*(2): 158-63.

Ikemoto, S., B. S. Glazier, J. M. Murphy and W. J. McBride (1998). Rats self-administer carbachol directly into the nucleus accumbens. Physiol Behav *63*(5): 811-4.

Ikemoto, S., R. R. Kohl and W. J. McBride (1997a). GABA(A) receptor blockade in the anterior ventral tegmental area increases extracellular levels of dopamine in the nucleus accumbens of rats. J Neurochem *69*(1): 137-43.

Ikemoto, S., J. M. Murphy and W. J. McBride (1997b). Self-infusion of GABA(A) antagonists directly into the ventral tegmental area and adjacent regions. Behav Neurosci *111*(2): 369-80.

Ikemoto, S. and R. A. Wise (2002). Rewarding effects of the cholinergic agents carbachol and neostigmine in the posterior ventral tegmental area. J Neurosci *22*(22): 9895-904.

Janowsky, A., C. Mah, R. A. Johnson, C. L. Cunningham, T. J. Phillips, J. C. Crabbe, A. J. Eshleman and J. K. Belknap (2001). Mapping genes that regulate density of dopamine transporters and correlated behaviors in recombinant inbred mice. J Pharmacol Exp Ther *298*(2): 634-43.

Jaworski, J. N., A. Vicentic, R. G. Hunter, H. L. Kimmel and M. J. Kuhar (2003). CART peptides are modulators of mesolimbic dopamine and psychostimulants. Life Sci *73*(6): 741-7.

Johanson, C. E., R. L. Balster and K. Bonese (1976). Self-administration of psychomotor stimulant drugs: the effects of unlimited access. Pharmacol Biochem Behav *4*(1): 45-51.

Johanson, C. E. and C. R. Schuster (1005). Cocaine. Psychopharmacology: The Fourth Generation of Progress. D. J. Kupfer. New York, Raven Press, Ltd.: 1685-1697.

Jones, B. C., L. M. Tarantino, L. A. Rodriguez, C. L. Reed, G. E. McClearn, R. Plomin and V. G. Erwin (1999). Quantitative-trait loci analysis of cocaine-related behaviours and neurochemistry. Pharmacogenetics *9*(5): 607-17.

Karch, S. B. (1998). A Brief History of Cocaine. Boca Raton, Fla., CRC Press.

Katz, J. L. (1989). Drugs as reinforcers: pharmacological and behavioural factors. The neuropharmacological basis of reward. J. M. Lieberman and S. J. Cooper. Oxford, Oxford University Press.

Kelz, M. B., J. Chen, W. A. Carlezon, Jr., K. Whisler, L. Gilden, A. M. Beckmann, C. Steffen, Y. J. Zhang, L. Marotti, D. W. Self, T. Tkatch, G. Baranauskas, D. J. Surmeier, R. L. Neve, R. S. Duman, M. R. Picciotto and E. J. Nestler (1999). Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. Nature *401*(6750): 272-6.

Kendler, K. S., K. C. Jacobson, C. A. Prescott and M. C. Neale (2003). Specificity of genetic and environmental risk factors for use and abuse/dependence of cannabis, cocaine, hallucinogens, sedatives, stimulants, and opiates in male twins. Am J Psychiatry *160*(4): 687-95.

Kendler, K. S., L. M. Karkowski, M. C. Neale and C. A. Prescott (2000). Illicit psychoactive substance use, heavy use, abuse, and dependence in a US population-based sample of male twins. Arch Gen Psychiatry *57*(3): 261-9.

Kendler, K. S. and C. A. Prescott (1998). Cocaine use, abuse and dependence in a population-based sample of female twins. Br J Psychiatry *173*: 345-50.

Kim, Y. C., H. G. Lee, C. S. Seong and K. A. Han (2003). Expression of a D1 dopamine receptor dDA1/DmDOP1 in the central nervous system of Drosophila melanogaster. Gene Expr Patterns *3*(2): 237-45.

Kuhar, M. J., M. C. Ritz and J. W. Boja (1991). The dopamine hypothesis of the reinforcing properties of cocaine. Trends Neurosci *14*(7): 299-302.

Le, A. D., J. M. Khanna, H. Kalant and A. E. LeBlanc (1981). The effect of lesions in the dorsal, median and magnus raphe nuclei on the development of tolerance to ethanol. J Pharmacol Exp Ther *218*(2): 525-9.

Lecklin, A., L. Tuomisto and E. MacDonald (1995). Metoprine, an inhibitor of histamine N-methyltransferase but not catechol-O-methyltransferase, suppresses feeding in sated and in food deprived rats. Methods Find Exp Clin Pharmacol *17*(1): 47-52.

Lee, Y. S. and R. W. Carthew (2003). Making a better RNAi vector for Drosophila: use of intron spacers. Methods *30*(4): 322-9.

Li, H., S. Chaney, I. J. Roberts, M. Forte and J. Hirsh (2000). Ectopic G-protein expression in dopamine and serotonin neurons blocks cocaine sensitization in Drosophila melanogaster. Curr Biol *10*(4): 211-4.

Markou, A. and G. F. Koob (1991). Postcocaine anhedonia. An animal model of cocaine withdrawal. Neuropsychopharmacology *4*(1): 17-26.

Marley, R. J., D. M. Arros, K. K. Henricks, M. E. Marley and L. L. Miner (1998). Sensitivity to cocaine and amphetamine among mice selectively bred for differential cocaine sensitivity. Psychopharmacology (Berl) *140*(1): 42-51.

Martin, J. R., T. Raabe and M. Heisenberg (1999). Central complex substructures are required for the maintenance of locomotor activity in Drosophila melanogaster. J Comp Physiol [A] *185*(3): 277-88.

Matthews, J. C. and A. Collins (1983). Interactions of cocaine and cocaine congeners with sodium channels. Biochem Pharmacol *32*(3): 455-60.

McBride, W. J., J. M. Murphy and S. Ikemoto (1999). Localization of brain reinforcement mechanisms: intracranial self-administration and intracranial place-conditioning studies. Behav Brain Res *101*(2): 129-52.

McClung, C. and J. Hirsh (1998). Stereotypic behavioral responses to free-base cocaine and the development of behavioral sensitization in Drosophila. Curr Biol *8*(2): 109-12.

McClung, C. and J. Hirsh (1999). The trace amine tyramine is essential for sensitization to cocaine in Drosophila. Curr Biol *9*(16): 853-60.

Merikangas, K. R., M. Stolar, D. E. Stevens, J. Goulet, M. A. Preisig, B. Fenton, H. Zhang, S. S. O'Malley and B. J. Rounsaville (1998). Familial transmission of substance use disorders. Arch Gen Psychiatry *55*(11): 973-9.

Miner, L. L., J. Drago, P. M. Chamberlain, D. Donovan and G. R. Uhl (1995). Retained cocaine conditioned place preference in D1 receptor deficient mice. Neuroreport *6*(17): 2314-6.

Miner, L. L. and R. J. Marley (1995a). Chromosomal mapping of loci influencing sensitivity to cocaine-induced seizures in BXD recombinant inbred strains of mice. Psychopharmacology (Berl) *117*(1): 62-6.

Miner, L. L. and R. J. Marley (1995b). Chromosomal mapping of the psychomotor stimulant effects of cocaine in BXD recombinant inbred mice. Psychopharmacology (Berl) *122*(3): 209-14.

Moore, M. S., J. DeZazzo, A. Y. Luk, T. Tully, C. M. Singh and U. Heberlein (1998). Ethanol intoxication in Drosophila: Genetic and pharmacological evidence for regulation by the cAMP signaling pathway. Cell *93*(6): 997-1007.

Morgan, D., K. A. Grant, H. D. Gage, R. H. Mach, J. R. Kaplan, O. Prioleau, S. H. Nader, N. Buchheimer, R. L. Ehrenkaufer and M. A. Nader (2002). Social dominance in monkeys: dopamine D2 receptors and cocaine self-administration. Nat Neurosci *5*(2): 169-74.

Nagaya, Y., M. Kutsukake, S. I. Chigusa and A. Komatsu (2002). A trace amine, tyramine, functions as a neuromodulator in Drosophila melanogaster. Neurosci Lett *329*(3): 324-8.

Nassel, D. R. and K. Elekes (1992). Aminergic neurons in the brain of blowflies and Drosophila: dopamine- and tyrosine hydroxylase-immunoreactive neurons and their relationship with putative histaminergic neurons. Cell Tissue Res *267*(1): 147-67.

Nestler, E. J. (2000). Genes and addiction. Nat Genet *26*(3): 277-81.

Nestler, E. J. (2001). Molecular basis of long-term plasticity underlying addiction. Nat Rev Neurosci *2*(2): 119-28.

Nestler, E. J. and G. K. Aghajanian (1997). Molecular and cellular basis of addiction. Science *278*(5335): 58-63.

Nestler, E. J., M. Barrot and D. W. Self (2001). DeltaFosB: a sustained molecular switch for addiction. Proc Natl Acad Sci U S A *98*(20): 11042-6.

Noble, E. P. (2000). Addiction and its reward process through polymorphisms of the D2 dopamine receptor gene: a review. Eur Psychiatry *15*(2): 79-89.

Noble, E. P., K. Blum, M. E. Khalsa, T. Ritchie, A. Montgomery, R. C. Wood, R. J. Fitch, T. Ozkaragoz, P. J. Sheridan, M. D. Anglin and et al. (1993). Allelic association of the D2 dopamine receptor gene with cocaine dependence. Drug Alcohol Depend *33*(3): 271-85.

Olds, J. and P. Milner (1954). Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. J Comp Physiol Psychol *47*(6): 419-27.

Park, S. K., S. A. Sedore, C. Cronmiller and J. Hirsh (2000). Type II cAMP-dependent protein kinase-deficient Drosophila are viable but show developmental, circadian, and drug response phenotypes. J Biol Chem *275*(27): 20588-96.

Parr, J., A. Large, X. Wang, S. C. Fowler, K. L. Ratzlaff and D. M. Ruden (2001). The inebri-actometer: a device for measuring the locomotor activity of Drosophila exposed to ethanol vapor. J Neurosci Methods *107*(1-2): 93-9.

Parsons, P. A. (1980). Larval responses to environmental ethanol in Drosophila melanogaster: variation within and among populations. Behav Genet *10*(2): 183-90.

Peoples, R. W., C. Li and F. F. Weight (1996). Lipid vs protein theories of alcohol action in the nervous system. Annu Rev Pharmacol Toxicol *36*: 185-201.

Persico, A. M., G. Bird, F. H. Gabbay and G. R. Uhl (1996). D2 dopamine receptor gene TaqI A1 and B1 restriction fragment length polymorphisms: enhanced frequencies in psychostimulant-preferring polysubstance abusers. Biol Psychiatry *40*(8): 776-84.

Phillips, T. J., J. C. Crabbe, P. Metten and J. K. Belknap (1994). Localization of genes affecting alcohol drinking in mice. Alcohol Clin Exp Res *18*(4): 931-41.

Phillips, T. J., M. G. Huson and C. S. McKinnon (1998). Localization of genes mediating acute and sensitized locomotor responses to cocaine in BXD/Ty recombinant inbred mice. J Neurosci *18*(8): 3023-34.

Pierce, R. C. and P. W. Kalivas (1997). A circuitry model of the expression of behavioral sensitization to amphetamine-like psychostimulants. Brain Res Brain Res Rev *25*(2): 192-216.

Pohjalainen, T., J. O. Rinne, K. Nagren, P. Lehikoinen, K. Anttila, E. K. Syvalahti and J. Hietala (1998). The A1 allele of the human D2 dopamine receptor gene predicts low D2 receptor availability in healthy volunteers. Mol Psychiatry *3*(3): 256-60.

Porzgen, P., S. K. Park, J. Hirsh, M. S. Sonders and S. G. Amara (2001). The antidepressant-sensitive dopamine transporter in Drosophila melanogaster: a primordial carrier for catecholamines. Mol Pharmacol *59*(1): 83-95.

Premont, R. T., R. R. Gainetdinov and M. G. Caron (2001). Following the trace of elusive amines. Proc Natl Acad Sci U S A *98*(17): 9474-5.

Prescott, C. A. and K. S. Kendler (1999). Genetic and environmental contributions to alcohol abuse and dependence in a population-based sample of male twins. Am J Psychiatry *156*(1): 34-40.

Quinn, W. G., P. P. Sziber and R. Booker (1979). The Drosophila memory mutant amnesiac. Nature *277*(5693): 212-4.

Roberts, D. C. and G. F. Koob (1982). Disruption of cocaine self-administration following 6-hydroxydopamine lesions of the ventral tegmental area in rats. Pharmacol Biochem Behav *17*(5): 901-4.

Roberts, D. C., G. F. Koob, P. Klonoff and H. C. Fibiger (1980). Extinction and recovery of cocaine self-administration following 6-hydroxydopamine lesions of the nucleus accumbens. Pharmacol Biochem Behav *12*(5): 781-7.

Robinson, T. E. and K. C. Berridge (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain Res Brain Res Rev *18*(3): 247-91.

Rocha, B. A., F. Fumagalli, R. R. Gainetdinov, S. R. Jones, R. Ator, B. Giros, G. W. Miller and M. G. Caron (1998). Cocaine self-administration in dopamine-transporter knockout mice. Nat Neurosci *1*(2): 132-7.

Rodan, A. R., J. A. Kiger, Jr. and U. Heberlein (2002). Functional dissection of neuroanatomical loci regulating ethanol sensitivity in Drosophila. J Neurosci *22*(21): 9490-501.

Roeder, T. (1999). Octopamine in invertebrates. Prog Neurobiol *59*(5): 533-61.

Rorth, P. (1996). A modular misexpression screen in Drosophila detecting tissue-specific phenotypes. Proc Natl Acad Sci U S A *93*(22): 12418-22.

Schechter, M. D. (1992). Rats bred for differences in preference to cocaine: other behavioral measurements. Pharmacol Biochem Behav *43*(4): 1015-21.

Schenk, S. and B. Partridge (1997). Sensitization and tolerance in psychostimulant self-administration. Pharmacol Biochem Behav *57*(3): 543-50.

Scholz, H., J. Ramond, C. M. Singh and U. Heberlein (2000). Functional ethanol tolerance in Drosophila. Neuron *28*(1): 261-71.

Schuckit, M. A. (2000). Genetics of the risk for alcoholism. Am J Addict *9*(2): 103-12.

Schultz, W. (1997). Dopamine neurons and their role in reward mechanisms. Curr Opin Neurobiol *7*(2): 191-7.

Schwaerzel, M., M. Monastirioti, H. Scholz, F. Friggi-Grelin, S. Birman and M. Heisenberg (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. J Neurosci *23*(33): 10495-502.

Shaham, Y., U. Shalev, L. Lu, H. De Wit and J. Stewart (2003). The reinstatement model of drug relapse: history, methodology and major findings. Psychopharmacology (Berl) *168*(1-2): 3-20.

Shiga, S., I. Toyoda and H. Numata (2000). Neurons projecting to the retrocerebral complex of the adult blow fly, Protophormia terraenovae. Cell Tissue Res *299*(3): 427-39.

Singh, C. M. and U. Heberlein (2000). Genetic control of acute ethanol-induced behaviors in Drosophila. Alcohol Clin Exp Res *24*(8): 1127-36.

Sokolowski, M. B. (2001). Drosophila: genetics meets behaviour. Nat Rev Genet *2*(11): 879-90.

Sora, I., F. S. Hall, A. M. Andrews, M. Itokawa, X. F. Li, H. B. Wei, C. Wichems, K. P. Lesch, D. L. Murphy and G. R. Uhl (2001). Molecular mechanisms of cocaine reward: combined dopamine and serotonin transporter knockouts eliminate cocaine place preference. Proc Natl Acad Sci U S A *98*(9): 5300-5.

Sora, I., C. Wichems, N. Takahashi, X. F. Li, Z. Zeng, R. Revay, K. P. Lesch, D. L. Murphy and G. R. Uhl (1998). Cocaine reward models: conditioned place preference can be established in dopamine- and in serotonin-transporter knockout mice. Proc Natl Acad Sci U S A *95*(13): 7699-704.

Substance Abuse and Mental Health Services Administration (2003). Overview of Findings from the 2002 National Survey on Drug Use and Health. (Series H-21, DHHS Publication No. SMA 03-3774). Rockville, MD.

Surmeier, D. J., W. J. Song and Z. Yan (1996). Coordinated expression of dopamine receptors in neostriatal medium spiny neurons. J Neurosci *16*(20): 6579-91.

Tabakoff, B., E. Nelson, M. Yoshimura, K. Hellevuo and P. L. Hoffman (2001). Phosphorylation cascades control the actions of ethanol on cell cAMP signalling. J Biomed Sci *8*(1): 44-51.

The National Center on Addiction and Substance Abuse at Columbia University (1995). Substance Abuse and Federal Entitlement Programs. New York, NY.

Thompson, J., N. Thomas, A. Singleton, M. Piggott, S. Lloyd, E. K. Perry, C. M. Morris, R. H. Perry, I. N. Ferrier and J. A. Court (1997). D2 dopamine receptor gene (DRD2) Taq1 A polymorphism: reduced dopamine D2 receptor binding in the human striatum associated with the A1 allele. Pharmacogenetics *7*(6): 479-84.

Toda, S., J. F. McGinty and P. W. Kalivas (2002). Repeated cocaine administration alters the expression of genes in corticolimbic circuitry after a 3-week withdrawal: a DNA macroarray study. J Neurochem *82*(5): 1290-9.

Tzschentke, T. M. (1998). Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. Prog Neurobiol *56*(6): 613-72.

Uhl, G. R., F. S. Hall and I. Sora (2002). Cocaine, reward, movement and monoamine transporters. Mol Psychiatry *7*(1): 21-6.

van den Bree, M. B., E. O. Johnson, M. C. Neale and R. W. Pickens (1998). Genetic and environmental influences on drug use and abuse/dependence in male and female twins. Drug Alcohol Depend *52*(3): 231-41.

Volkow, N. D., J. S. Fowler, G. J. Wang, R. Hitzemann, J. Logan, D. J. Schlyer, S. L. Dewey and A. P. Wolf (1993). Decreased dopamine D2 receptor availability is associated with reduced frontal metabolism in cocaine abusers. Synapse *14*(2): 169-77.

Volkow, N. D., G. J. Wang, M. W. Fischman, R. Foltin, J. S. Fowler, D. Franceschi, M. Franceschi, J. Logan, S. J. Gatley, C. Wong, Y. S. Ding, R. Hitzemann and N. Pappas (2000). Effects of route of administration on cocaine induced dopamine transporter blockade in the human brain. Life Sci *67*(12): 1507-15.

Volkow, N. D., G. J. Wang, M. W. Fischman, R. W. Foltin, J. S. Fowler, N. N. Abumrad, S. Vitkun, J. Logan, S. J. Gatley, N. Pappas, R. Hitzemann and C. E. Shea (1997a). Relationship between subjective effects of cocaine and dopamine transporter occupancy. Nature *386*(6627): 827-30.

Volkow, N. D., G. J. Wang, J. S. Fowler, J. Logan, S. J. Gatley, A. Gifford, R. Hitzemann, Y. S. Ding and N. Pappas (1999a). Prediction of reinforcing responses to psychostimulants in humans by brain dopamine D2 receptor levels. Am J Psychiatry *156*(9): 1440-3.

Volkow, N. D., G. J. Wang, J. S. Fowler, J. Logan, S. J. Gatley, R. Hitzemann, A. D. Chen, S. L. Dewey and N. Pappas (1997b). Decreased striatal dopaminergic responsiveness in detoxified cocaine-dependent subjects. Nature *386*(6627): 830-3.

Volkow, N. D., G. J. Wang, J. S. Fowler, J. Logan, S. J. Gatley, C. Wong, R. Hitzemann and N. R. Pappas (1999b). Reinforcing effects of psychostimulants in humans are associated with increases in brain dopamine and occupancy of D(2) receptors. J Pharmacol Exp Ther *291*(1): 409-15.

Wang, X. B., M. Funada, Y. Imai, R. S. Revay, H. Ujike, D. J. Vandenbergh and G. R. Uhl (1997). rGbeta1: a psychostimulant-regulated gene essential for establishing cocaine sensitization. J Neurosci *17*(15): 5993-6000.

Weber, K. (1988). An apparatus for measurement of resistance to gas-phase reagents. Drosophila Information Services *67*: 91-93.

Wise, R. A. (1984). Neural mechanisms of the reinforcing action of cocaine. NIDA Res Monogr *50*: 15-33.

Wise, R. A. (2002). Brain reward circuitry: insights from unsensed incentives. Neuron *36*(2): 229-40.

Wise, R. A., P. Bauco, W. A. Carlezon, Jr. and W. Trojniar (1992). Self-stimulation and drug reward mechanisms. Ann N Y Acad Sci *654*: 192-8.

Wise, R. A. and M. A. Bozarth (1985). Brain mechanisms of drug reward and euphoria. Psychiatr Med *3*(4): 445-60.

Wise, R. A. and M. A. Bozarth (1987). A psychomotor stimulant theory of addiction. Psychol Rev *94*(4): 469-92.

Wolf, F. W. and U. Heberlein (2003). Invertebrate models of drug abuse. J Neurobiol *54*(1): 161-78.

Wolf, F. W., A. R. Rodan, L. T. Tsai and U. Heberlein (2002). High-resolution analysis of ethanol-induced locomotor stimulation in Drosophila. J Neurosci *22*(24): 11035-44.

Xu, F., R. R. Gainetdinov, W. C. Wetsel, S. R. Jones, L. M. Bohn, G. W. Miller, Y. M. Wang and M. G. Caron (2000). Mice lacking the norepinephrine transporter are supersensitive to psychostimulants. Nat Neurosci *3*(5): 465-71.

Yao, W. D., R. R. Gainetdinov, M. I. Arbuckle, T. D. Sotnikova, M. Cyr, J. M. Beaulieu, G. E. Torres, S. G. Grant and M. G. Caron (2004). Identification of PSD-95 as a regulator of dopamine-mediated synaptic and behavioral plasticity. Neuron *41*(4): 625-38.

Yeomans, J. S., O. Kofman and V. McFarlane (1985). Cholinergic involvement in lateral hypothalamic rewarding brain stimulation. Brain Res *329*(1-2): 19-26.

You, Z. B., T. M. Tzschentke, E. Brodin and R. A. Wise (1998). Electrical stimulation of the prefrontal cortex increases cholecystokinin, glutamate, and dopamine release in the nucleus accumbens: an in vivo microdialysis study in freely moving rats. J Neurosci *18*(16): 6492-500.

Zaleski, M. J., J. R. Nunes Filho, T. Lemos and G. S. Morato (2001). GABA(B) receptors play a role in the development of tolerance to ethanol in mice. Psychopharmacology (Berl) *153*(4): 415-24.

Chapter 1

Dopamine Modulates Acute Responses to Cocaine, Nicotine, and Ethanol in *Drosophila*

Linus T.-Y. Tsai*, Roland J. Bainton*, Carol M. Singh, Monica S. Moore, Wendi S. Neckameyer, and Ulrike Heberlein

* These authors contributed equally to this work.

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Abstract

Background: A common property of drugs of abuse in mammals is their ability to facilitate the release of the neurotransmitter/neuromodulator dopamine in specific brain regions involved in reward and motivation. This increase in synaptic dopamine levels is believed to act as a positive reinforcer and to mediate some of the acute responses to drugs. The mechanisms by which dopamine regulates acute drug responses and addiction remain unknown.

Results: We present evidence that dopamine plays a role in the responses of *Drosophila* to cocaine, nicotine, and ethanol. We use a startle-induced negative geotaxis assay and a locomotor tracking system to measure the effect of psychostimulants on fly behavior. Using these assays, we show that acute responses to cocaine and nicotine are blunted by pharmacologically-induced reductions in dopamine levels. Co-administration of cocaine and nicotine shows a high degree of synergy, consistent with an action through convergent pathways. In addition, we find that dopamine is involved in the acute locomotor-activating of ethanol, but not its sedating effects.

Conclusions: We show that in *Drosophila*, as in mammals, dopaminergic pathways play a role in modulating specific behavioral responses to cocaine, nicotine, and ethanol. We suggest that *Drosophila* can therefore be used as a genetically tractable model system in which to study the mechanisms underlying behavioral responses to multiple drugs of abuse.

Background

Although psychostimulants, opiates, and ethanol all have different primary effects and modes of action in the central nervous system (CNS), current theories suggest that their positive reinforcing properties are mediated in part by an elevation of extracellular dopamine in the nucleus accumbens (Kuhar *et al.*, 1991; Di Chiara, 1995; Wise, 1996; Koob *et al.*, 1998). The associated acute locomotor-stimulating effects of these drugs have been proposed to model reinforcement (Wise and Bozarth, 1987). Selective destruction of dopamine neurons or pharmacological inhibition of dopaminergic systems prevents the stimulatory effects of most drugs of abuse; these manipulations also curtail drug self-administration (Wise, 1996; Koob *et al.*, 1998). For example, dopamine D1 and D2 receptor antagonists block locomotor hyperactivity and self-administration of cocaine in rodents (Bergman *et al.*, 1990; Britton *et al.*, 1991; Cabib *et al.*, 1991; Hubner and Moreton, 1991; Caine and Koob, 1994; Tella, 1994). In addition, mice carrying a deletion of the dopamine D1 receptor or the dopamine transporter (DAT) are hyperactive and insensitive to the locomotor-activating effects of cocaine (Xu *et al.*, 1994; Giros *et al.*, 1996). However, DAT mutant mice can still be trained to self-administer cocaine and establish cocaine-conditioned place preference (Rocha *et al.*, 1998; Sora *et al.*, 1998). These results suggest that other neurotransmitter systems are also involved in the rewarding effects of cocaine.

Drosophila, with its accessibility to genetic and molecular analysis, is an attractive model system to investigate the molecular mechanisms regulating behaviors induced by drugs of abuse. Here we show that responses elicited by

cocaine, nicotine, and ethanol in flies are similar to those observed in mammals, and that dopaminergic systems play a role in the manifestation of these behaviors.

Results and Discussion

Cocaine and nicotine-induced behaviors

Upon exposure to volatilized free-base cocaine, adult *Drosophila* demonstrated dose-dependent behavioral responses. Low doses (50-75 μg) induced primarily grooming and hyperactivity. Moderate doses (100-150 μg) led to hypokinesis and stereotypic locomotion often manifested as circling. High doses (200-400 μg) induced spasmodic activity, tremor, and finally, akinesia. These behaviors are qualitatively very similar to those described by McClung and Hirsh (McClung and Hirsh, 1998). However, our drug responses were approximately two-fold weaker; i. e., behaviors elicited by 200 μg cocaine in our laboratory are similar to those described for 100 μg by McClung and Hirsh (McClung and Hirsh, 1998). The reasons for this discrepancy are not clear.

Although the behaviors elicited were distinct at different cocaine doses, there was substantial individual variation among flies in a single experiment. In addition, individual flies often displayed a diversity of behaviors during any one-minute observation period, making unambiguous scoring and quantitative analyses difficult. To avoid these complications we developed a simple climbing assay, based on the observation that cocaine exposure interfered with the fly's normal propensity to negatively geotax in response to mechanical stimulation (see Materials and methods). Briefly, cocaine- or mock-exposed flies were introduced

into a 1-foot-long narrow cylinder and knocked to the bottom. Mock-treated flies responded by quickly climbing to the top of the cylinder. This response was reduced in a dose-dependent manner upon cocaine exposure. The proportion of flies that failed to climb, thus remaining at or near the bottom of the cylinder, was averaged over three consecutive trials, providing a quantitative and reproducible measure of the effect of the drug (Fig. 1A, closed circles). The (+) isomer of cocaine, which has reduced biologic activity in mammals, also showed reduced efficacy when tested in our climbing assay (Spealman *et al.*, 1983) (Fig. 1A, open circles). Flies recovered normal behavior in 15-20 minutes after exposure to moderate cocaine doses.

An overlapping, but not identical set of behaviors occurred when flies were exposed to volatilized nicotine. These behaviors included a very rapid onset of hyperactivity and spasmodic movements, leading to grooming, hypokinesis, and to akinesia in the most affected individuals. Repetitive locomotor behaviors were less obvious than in flies exposed to cocaine. Nonetheless, nicotine exposure impaired the flies' ability to negatively geotax in a dose-dependent manner (Fig. 1B). Flies recovered quickly, in approximately 5 minutes, after exposure to moderate nicotine doses (~6 μg). Nicotine's actions are likely to be CNS-specific since nicotinic acetylcholine receptors in insects are confined to the nervous system, where they appear to play a major excitatory role (Restifo and White, 1990; Gundelfinger, 1992).

In summary, a simple startle-induced climbing assay allows us to measure cocaine and nicotine induced behaviors. The assay is quantitative, reproducible,

and shows specificity, as indicated by (+) cocaine's reduced efficacy. It differs from direct observation of drug-exposed flies in that flies are subjected to mechanical stimulation in an environment conducive to negative geotaxis. Thus, the effect of the drug is measured by its ability to interfere with a normally robust fly behavior.

Role of dopamine in cocaine and nicotine responses

In mammals, cocaine inhibits the reuptake of monoaminergic neurotransmitters from the synaptic cleft through its interaction with plasma membrane monoamine transporters, including the transporters for dopamine (DAT), norepinephrine, and serotonin (Ritz *et al.*, 1990). While the direct target of cocaine's action is not known in flies, a cocaine-sensitive serotonin transporter has been characterized; a cocaine-sensitive dopamine transporter has not been identified to date (Corey *et al.*, 1994; Demchyshyn *et al.*, 1994).

To explore a role for dopamine in acute responses to cocaine and nicotine, we tested flies with severely reduced dopamine levels. This was achieved by feeding flies 3-Iodotyrosine (3IY), a competitive antagonist of tyrosine hydroxylase (TH) (Neckameyer, 1996; see Materials and methods). The conversion of tyrosine to L-Dopa by TH is an obligate and rate-limiting step in the synthesis of dopamine. 3IY treatment reduces global dopamine levels approximately 10-fold without affecting serotonin levels (Table I). Unlike mammals, *Drosophila* does not synthesize norepinephrine from dopamine; therefore, 3IY treatment should specifically deplete dopamine (Restifo and White, 1990). Complete loss of dopamine is incompatible with viability. Loss of function mutations in the

Drosophila pale locus, encoding TH, lead to embryonic lethality (Neckameyer and White, 1993). In addition, prolonged 3IY treatment of larvae and adults causes akinesia and eventually, death (Neckameyer, 1996; our observations) (our observations). Thus, our behavioral assays were carried out under conditions of partial dopamine loss, conditions that permit normal baseline behaviors. For example, 3IY-treated flies displayed normal locomotion and geotaxis and performed normally in mock-exposure experiments (Neckameyer, 1998; see below and Materials and methods). However, they showed a significant reduction, approximately 35%, in their sensitivity to the effects of cocaine and nicotine (Fig. 2A). This effect was attributable to a decrease in dopamine levels, since L-Dopa treatment restored normal sensitivity to 3IY-treated flies (Fig. 2A) under conditions that partially restored dopamine levels (Table I). L-Dopa treatment alone at this dose did not affect drug sensitivity (Fig. 2A). Thus, a reduction in dopamine levels that did not affect basal locomotion or geotaxis resulted in diminished sensitivity to cocaine and nicotine, suggesting that dopamine mediates some of the acute effects of these drugs in *Drosophila*.

To obtain a more detailed view of the effect of dopamine depletion on cocaine-induced behaviors, we documented the patterns of locomotion of control and 3IY-treated flies with a locomotor tracking system (see Materials and methods). Groups of 5 flies were exposed to cocaine, transferred to a viewing chamber, and filmed for several minutes. The patterns of locomotion of control and 3IY-treated flies during the 2^{nd} and 4^{th} minute after exposure are shown in Figure 3; the two time intervals were chosen to illustrate the gradual increase in drug effect

observed during the first 5 minutes of exposure. The response to mock-exposure (0µg cocaine) was similar in control and 3IY-treated flies (compare Fig. 3A, B with 3G, H). However, flies differed markedly in their response to cocaine. Control flies exposed to 100 µg cocaine showed reduced locomotion and some circling in the 1-2 minute interval (Fig. 3C); cocaine's effect is stronger in the 3-4 minute period when circling behavior is predominant (Fig. 3D). 3IY-treated flies exposed to the same dose of cocaine were less affected (Fig. 3I, J); they showed reduced locomotion relative to mock-treated flies, but little circling, even in the 3-4 minute interval. These effects are comparable to those obtained in control flies exposed to 50 µg cocaine (data not shown). Upon exposure to 200 µg cocaine, control flies showed spasmodic movements (observed as zig-zag patterns of movement) and severe hypokinesis (Fig. 3E, F). In contrast, spasmodic movements were rare in 3IY-treated flies exposed to 200 µg cocaine; these flies circled and were still quite mobile (Fig. 3K, L). To quantify the effect of dopamine depletion on cocaine responses more accurately, we compared the velocity of movement and the degree of turning of control and 3IY-treated flies (see Materials and Methods). As described above, exposure to moderate cocaine doses (100-200 µg) is associated with reduced locomotion and increased circling (Table II). 3IY-treated flies were found to move significantly faster than controls upon exposure to 200 µg cocaine a dose that strongly reduced locomotion in control flies. The degree of circling was significantly reduced in 3IY-treated flies compared to controls exposed to 100 µg cocaine (Table II). While it is not possible to accurately quantify the overall effect of cocaine using the locomotor tracking system, we estimate that 3IY-

treated flies exposed to 200 µg cocaine display behaviors that are similar to those induced by 100 µg cocaine in untreated flies. This approximately 50% reduction in cocaine's effect is comparable to the 35% reduction measured in the climbing assay (Fig. 2A).

In *Drosophila*, dopamine biosynthetic enzymes are expressed not only in the nervous system but also in the epidermis, where they participate in cuticle formation (Budnik and White, 1987; Wright, 1987; Birman *et al.*, 1994). Therefore, HPLC measurements of dopamine levels in fly extracts (Table I) reflect the combined levels in cuticle and CNS. While these data clearly establish that 3IY and L-Dopa are ingested and have the predicted consequences, they do not allow us to quantify dopamine levels in the CNS. Unfortunately, dopamine levels in dissected brain preparations were too low for reliable measurement. With the purpose of reducing dopamine transmission specifically in the nervous system we treated flies with reserpine . In mammals, reserpine efficiently depletes synaptic dopamine and other monoamines by inhibiting the vesicular monoamine transporter-2 (VMAT-2) thus preventing it from concentrating these neurotransmitters in synaptic vesicles (Liu and Edwards, 1997). Consistent with the notion that reserpine would not affect cuticle dopamine levels, flies treated with reserpine show only a small but significant reduction in global dopamine, but surprisingly, no changes in global serotonin levels (Table I). Like 3IY-treated flies, these flies showed a dose-dependent reduction in their response to cocaine (Fig. 2B) while performing normally in mock-exposure experiments. A maximal reduction in sensitivity of 30% was achieved with 30 µM reserpine; higher doses

reduced fly fitness causing an apparent increase in cocaine sensitivity (data not shown). These data also suggest that *Drosophila* have a VMAT that functions similarly to the mammalian transporter; sequences with strong homology to the mammalian reserpine-sensitive VMAT-2 have recently been identified in the *Drosophila* genome (our observations). Why serotonin levels are not altered by reserpine treatment is unclear.

In summary, we show that two mechanistically-distinct pharmacological manipulations that reduce available dopamine render flies more resistant to the effects of cocaine. Whereas inhibition of dopamine biosynthesis with 3IY is known to alter CNS and cuticular dopamine levels, inhibition of VMAT by reserpine, and the resulting degradation of dopamine in synaptic terminals, should be CNS-specific(Neckameyer, 1996). This suggests that the observed behavioral effects are caused by reduced levels of CNS dopamine rather than loss in the cuticle. In addition, we expect that compromised cuticle formation would, if anything, increase drug sensitivity, which is not the case. The monoamines tyramine and octopamine do not appear to be involved in regulating cocaine sensitivity, as mutants that interfere with their synthesis show normal cocaine responses (McClung and Hirsh, 1999; our observations). We cannot completely exclude a role for serotonin in regulating cocaine responsiveness. While we find that serotonin levels appear normal in extracts from 3IY-treated flies (Table I), it is possible that amine levels vary locally. However, serotonin-specific immunohistochemical analysis of brains from 3IY-treated adult flies also failed to reveal detectable changes (data not shown).

Cocaine-nicotine behavioral synergy

Genetic or pharmacological manipulations that affect a common process often display dose-dependent synergistic interactions. Because our pharmacological depletion data suggested that both cocaine and nicotine exert their effects in part by modulating dopamine release, we tested for behavioral interactions between the two drugs. Simultaneous co-administration of low doses of cocaine and nicotine resulted in pronounced synergy in our behavioral assay (Fig. 4). For example, only 1% and 2% of flies failed to climb when exposed to 1.5 μg nicotine or 50 μg cocaine, respectively (Fig. 4A); a 59% climbing failure was achieved upon coadministration, an approximately 20-fold increase over an additive response. This synergy was greatly diminished in 3IY-treated flies (Fig. 4B), consistent with the hypothesis that these drugs act in part through a common dopaminergic pathway. That the response was not completely abrogated by 3IY is not surprising in that this treatment did not fully deplete dopamine levels. In addition, these drugs may also synergize through non-dopaminergic pathways. These experiments do not establish whether cocaine and nicotine act on the same cells in the fly's nervous system. However, if cocaine and nicotine acted completely independently, their combined administration would be expected to have additive effects.

In mammals, cocaine inhibits dopamine reuptake into dopaminergic terminals in the nucleus accumbens, whereas nicotine is believed to activate acetylcholine receptors, some of which are located presynaptically on dopaminergic neurons that project to the nucleus accumbens. Consistent with a

convergent action of these drugs on nucleus accumbens dopamine regulation, cocaine and nicotine show an additive effect on dopamine release in the nucleus accumbens of freely moving rats (Zernig *et al.*, 1997). In addition, self-administration of cocaine and nicotine has been shown to activate common regions in the mesocorticolimbic dopamine system in rats, as measured by induction of Fos-related protein expression (Pich *et al.*, 1997). Whether additive changes in dopamine levels in the nucleus accumbens lead to synergistic effects on rodent behavior has, to our knowledge, not been tested.

Role of dopamine in acute ethanol responses

Dopamine's involvement in acute drug responses can also be demonstrated with ethanol. When placed in a small chamber or in narrow tubes, flies displayed a basal level of locomotion (Fig. 5A, B) (see Materials and methods). Upon exposure to ethanol vapor, they immediately increased their locomotor activity. After 7-10 minutes of hyperlocomotion, the flies became gradually less coordinated and increasingly sedated. After approximately 15 minutes, they stopped righting themselves. Flies treated with 3IY also showed an increase in locomotor activity upon ethanol exposure, but this effect was significantly reduced during the first 5 minutes of exposure (Fig. 5A, B). The effect of 3IY was reversed by simultaneously feeding L-Dopa. The duration of the locomotor activating effects of ethanol and consequently the exposure time leading to sedation was not obviously changed by alterations in dopamine levels. Ethanol absorption was not modified by 3IY and/or L-Dopa treatment (Fig. 5C) arguing that

dopamine depletion did not alter ethanol pharmacokinetics, but specifically reduced the flies' response to ethanol-induced hyperactivity. Similarly, acute ethanol exposure increases dopamine release and locomotor activity in rodents (Dar and Wooles, 1984; Imperato and Di Chiara, 1986); the latter can be blocked by administration of dopamine receptor antagonists (Phillips and Shen, 1996).

Conclusions

In summary, we show that upon acute exposure to cocaine, nicotine, and ethanol, *Drosophila* displays a series of motor behaviors that are similar to those observed in mammals. In addition, we present evidence that, as in mammals, dopaminergic systems are involved in the manifestation of specific drug-induced behaviors. Recent studies using dopamine receptor antagonists in a decapitated preparation of *Drosophila* have implicated dopaminergic systems in locomotion and grooming and the responses to cocaine and cocaethylene (Yellman *et al.*, 1997; Torres and Horowitz, 1998). Taken together, these studies show not only that behaviors induced by acute drug exposure are surprisingly similar in flies and mammals, but also that at least some of the neurotransmitter systems that mediate these behaviors are conserved. We suggest that *Drosophila* can therefore be used as a genetically tractable model system in which to study the mechanisms underlying behavioral responses to multiple drugs of abuse.

Materials and Methods

Drosophila culture and behavioral assays

Adult male *Drosophila melanogaster* of the Canton-S wild type strain were used for all experiments. These flies were grown on cornmeal molasses food at 25°C and 70% relative humidity. Cocaine (free base, Sigma), (+) cocaine (NIDA), and nicotine (Sigma) solutions were prepared by dissolution in ethanol (cocaine) or water (nicotine). Drugs were delivered as described by McClung and Hirsh (McClung and Hirsh, 1998). Flies are exposed for 1 minute, then placed into glass columns (23.5 cm long, 2.5 cm diameter) lined with nylon mesh (Monofilament Cloth 250 microns). Columns are knocked on a soft surface to force flies to the bottom. After 1 minute, flies remaining within 1 cm of the bottom are counted; this procedure is performed 3 times at 1-minute intervals. Mock-treated flies respond by rapidly climbing back to the top of the column, presumably as a response to startle and due to their normal motivation to negatively geotaxis. Drug-treated flies show reduced climbing as they carry out the various abnormal locomotor behaviors described in the text. Drug-treated flies show little or no recovery during the 3 minutes of the assay. The drug effect score is the average (over 3 minutes) of the number of flies that remain near the bottom and is expressed as % of the total number of flies. These scores distribute normally with respect of the mean and were analyzed statistically using Student's paired t-test.

Drug feeding protocols

Pharmacological treatment with 3IY (Sigma) and L-Dopa (Sigma) was carried out as described by Neckameyer (Neckameyer, 1996). 3IY and L-Dopa were dissolved in an aqueous 5% sucrose, 2% yeast solution and a saturating amount (1.2 ml) was added to glass vials lined with 3MM paper. Newly-eclosed males were kept in these vials for 40-48 hours at 25°C and 90% relative humidity before drug exposure. Reserpine (Sigma) was prepared as above and flies were treated for 24 hours. Upon mock exposure flies treated with 3IY and/or L-Dopa displayed behaviors indistinguishable from vehicle-treated flies.

Ethanol-induced behaviors

Adult male flies were exposed to 3IY and/or L-Dopa for 48 hours as described above. Flies were introduced 4 at a time into a 6 x 6 x 1.5 cm acrylic exposure chamber and allowed to rest for 1 minute. They were then videotaped during exposure to a constant flow of air for 5 minutes followed by ethanol vapor (approximately 15 mM) for 25 minutes as described before (Moore *et al.*, 1998). Exposure chambers were covered with a grid of orthogonal lines spaced 1.5 cm apart. Locomotor activity of each individual fly was quantified manually as the number of lines crossed in 1-minute intervals. Basal locomotion without ethanol was measured in narrow perforated plastic tubes (3 mm diameter, 9 cm length) and expressed as the number of lines (at 1 cm intervals) crossed per minute. The flies' wings were clipped 24 hours prior to testing to avoid flight during the ethanol exposure.

Locomotor tracking system

Five adult male flies were exposed to volatilized cocaine for 15 sec and placed in a thin 3 cm X 4 cm observation chamber. They were videotaped at a capture rate of 15 frames/sec beginning 30s after exposure. Traces were generated using the Dynamic Image Analysis System (Solltech Inc.). Paths joining contiguous traces were graphically represented over one minute intervals as dots (center of trace) connected by lines (path traveled between frames). From these paths, the velocity of movement (mm/sec) and the degree of turning/circling (°/mm) were calculated and subjected to pair-wise statistical comparisons using the Wilcoxon-Mann-Whitney test (velocity and turning of cocaine-exposed flies do not distribute normally). Flies that failed to move during the 1-minute period were eliminated from the calculations of turning.

References

Bergman, J., J. B. Kamien and R. D. Spealman (1990). Antagonism of cocaine self-administration by selective dopamine D(1) and D(2) antagonists. Behav Pharmacol *1*(4): 355-363.

Birman, S., B. Morgan, M. Anzivino and J. Hirsh (1994). A novel and major isoform of tyrosine hydroxylase in Drosophila is generated by alternative RNA processing. J Biol Chem *269*(42): 26559-67.

Britton, D. R., P. Curzon, R. G. Mackenzie, J. W. Kebabian, J. E. Williams and D. Kerkman (1991). Evidence for involvement of both D1 and D2 receptors in

maintaining cocaine self-administration. Pharmacol Biochem Behav *39*(4): 911-5.

Budnik, V. and K. White (1987). Genetic dissection of dopamine and serotonin synthesis in the nervous system of Drosophila melanogaster. J Neurogenet *4*(6): 309-14.

Cabib, S., C. Castellano, V. Cestari, U. Filibeck and S. Puglisi-Allegra (1991). D1 and D2 receptor antagonists differently affect cocaine-induced locomotor hyperactivity in the mouse. Psychopharmacology (Berl) *105*(3): 335-9.

Caine, S. B. and G. F. Koob (1994). Effects of dopamine D-1 and D-2 antagonists on cocaine self-administration under different schedules of reinforcement in the rat. J Pharmacol Exp Ther *270*(1): 209-18.

Corey, J. L., M. W. Quick, N. Davidson, H. A. Lester and J. Guastella (1994). A cocaine-sensitive Drosophila serotonin transporter: cloning, expression, and electrophysiological characterization. Proc Natl Acad Sci U S A *91*(3): 1188-92.

Dar, M. S. and W. R. Wooles (1984). Striatal and hypothalamic neurotransmitter changes during ethanol withdrawal in mice. Alcohol *1*(6): 453-8.

Demchyshyn, L. L., Z. B. Pristupa, K. S. Sugamori, E. L. Barker, R. D. Blakely, W. J. Wolfgang, M. A. Forte and H. B. Niznik (1994). Cloning, expression, and localization of a chloride-facilitated, cocaine-sensitive serotonin transporter from Drosophila melanogaster. Proc Natl Acad Sci U S A *91*(11): 5158-62.

Di Chiara, G. (1995). The role of dopamine in drug abuse viewed from the perspective of its role in motivation. Drug Alcohol Depend *38*(2): 95-137.

Giros, B., M. Jaber, S. R. Jones, R. M. Wightman and M. G. Caron (1996). Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature *379*(6566): 606-12.

Gundelfinger, E. D. (1992). How complex is the nicotinic receptor system of insects? Trends Neurosci *15*(6): 206-11.

Hubner, C. B. and J. E. Moreton (1991). Effects of selective D1 and D2 dopamine antagonists on cocaine self-administration in the rat. Psychopharmacology (Berl) *105*(2): 151-6.

Imperato, A. and G. Di Chiara (1986). Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. J Pharmacol Exp Ther *239*(1): 219-28.

Koob, G. F., P. P. Sanna and F. E. Bloom (1998). Neuroscience of addiction. Neuron *21*(3): 467-76.

Kuhar, M. J., M. C. Ritz and J. W. Boja (1991). The dopamine hypothesis of the reinforcing properties of cocaine. Trends Neurosci *14*(7): 299-302.

Liu, Y. and R. H. Edwards (1997). The role of vesicular transport proteins in synaptic transmission and neural degeneration. Annu Rev Neurosci *20*: 125-56.

McClung, C. and J. Hirsh (1998). Stereotypic behavioral responses to free-base cocaine and the development of behavioral sensitization in Drosophila. Curr Biol *8*(2): 109-12.

McClung, C. and J. Hirsh (1999). The trace amine tyramine is essential for sensitization to cocaine in Drosophila. Curr Biol *9*(16): 853-60.

Moore, M. S., J. DeZazzo, A. Y. Luk, T. Tully, C. M. Singh and U. Heberlein (1998). Ethanol intoxication in Drosophila: Genetic and pharmacological evidence for regulation by the cAMP signaling pathway. Cell *93*(6): 997-1007.

Neckameyer, W. S. (1996). Multiple roles for dopamine in Drosophila development. Dev Biol *176*(2): 209-19.

Neckameyer, W. S. (1998). Dopamine modulates female sexual receptivity in Drosophila melanogaster. J Neurogenet *12*(2): 101-14.

Neckameyer, W. S. and K. White (1993). Drosophila tyrosine hydroxylase is encoded by the pale locus. J Neurogenet *8*(4): 189-99.

Phillips, T. J. and E. H. Shen (1996). Neurochemical bases of locomotion and ethanol stimulant effects. Int Rev Neurobiol *39*: 243-82.

Pich, E. M., S. R. Pagliusi, M. Tessari, D. Talabot-Ayer, R. Hooft van Huijsduijnen and C. Chiamulera (1997). Common neural substrates for the addictive properties of nicotine and cocaine. Science *275*(5296): 83-6.

Restifo, L. L. and K. White (1990). Molecular and genetic approaches to neurotransmitter and neuromodulator systems in Drosophila. Adv. Insect Physiol. *22*: 115-219.

Ritz, M. C., E. J. Cone and M. J. Kuhar (1990). Cocaine inhibition of ligand binding at dopamine, norepinephrine and serotonin transporters: a structure-activity study. Life Sci *46*(9): 635-45.

Rocha, B. A., F. Fumagalli, R. R. Gainetdinov, S. R. Jones, R. Ator, B. Giros, G. W. Miller and M. G. Caron (1998). Cocaine self-administration in dopamine-transporter knockout mice. Nat Neurosci *1*(2): 132-7.

Sora, I., C. Wichems, N. Takahashi, X. F. Li, Z. Zeng, R. Revay, K. P. Lesch, D. L. Murphy and G. R. Uhl (1998). Cocaine reward models: conditioned place preference can be established in dopamine- and in serotonin-transporter knockout mice. Proc Natl Acad Sci U S A *95*(13): 7699-704.

Spealman, R. D., R. T. Kelleher and S. R. Goldberg (1983). Stereoselective behavioral effects of cocaine and a phenyltropane analog. J Pharmacol Exp Ther *225*(3): 509-14.

Tella, S. R. (1994). Differential blockade of chronic versus acute effects of intravenous cocaine by dopamine receptor antagonists. Pharmacol Biochem Behav *48*(1): 151-9.

Torres, G. and J. M. Horowitz (1998). Activating properties of cocaine and cocaethylene in a behavioral preparation of Drosophila melanogaster. Synapse *29*(2): 148-61.

Wise, R. A. (1996). Neurobiology of addiction. Curr Opin Neurobiol *6*(2): 243-51.

Wise, R. A. and M. A. Bozarth (1987). A psychomotor stimulant theory of addiction. Psychol Rev *94*(4): 469-92.

Wright, T. R. (1987). The genetics of biogenic amine metabolism, sclerotization, and melanization in Drosophila melanogaster. Adv Genet *24*: 127-222.

Xu, M., X. T. Hu, D. C. Cooper, R. Moratalla, A. M. Graybiel, F. J. White and S. Tonegawa (1994). Elimination of cocaine-induced hyperactivity and dopamine-mediated neurophysiological effects in dopamine D1 receptor mutant mice. Cell *79*(6): 945-55.

Yellman, C., H. Tao, B. He and J. Hirsh (1997). Conserved and sexually dimorphic behavioral responses to biogenic amines in decapitated Drosophila. Proc Natl Acad Sci U S A *94*(8): 4131-6.

Zernig, G., I. A. O'Laughlin and H. C. Fibiger (1997). Nicotine and heroin augment cocaine-induced dopamine overflow in nucleus accumbens. Eur J Pharmacol *337*(1): 1-10.

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Figure Legends

Figure 1

Cocaine and nicotine cause dose-dependent changes in *Drosophila* geotaxis. The indicated doses of cocaine (**A,** filled circles) and nicotine (**B**) were delivered by flash-volatilization to wild type male flies. Approximately 20 flies per assay were transferred to a vertical cylinder to quantify negative geotaxis and startle responses. Empty circles in (**A**) correspond to (+) cocaine exposures. Error bars in all figures correspond to SEM (n=9-10 except for the highest and lowest doses, where n=3-4).

Figure 2

Pharmacological reductions of dopamine levels reduce *Drosophila* sensitivity to cocaine and nicotine. (**A**) Adult wild type flies were fed a yeast/sucrose solution for 48 hours with or without 3-iodo-tyrosine (3IY, 10 mg/ml) and/or L-Dopa (1 mg/ml) as indicated. The responses to cocaine and nicotine were measured as described in Materials and methods. 3IY-induced dopamine depletion caused a significant reduction in the sensitivity to cocaine and nicotine that was reversed by simultaneous administration of L-Dopa. L-Dopa alone had no significant effect. Asterisks indicate $P<0.001$ (Students paired t-test) relative to control, yeast/sucrose-fed flies (n=20 and 17 for 200 µg and 400 µg cocaine, respectively; n=13 for 6 µg nicotine). All other comparisons showed no significant differences. (**B**) Adult flies were fed the indicated doses of reserpine mixed with yeast/sucrose

for 24 hours prior to behavioral testing. Reserpine caused a dose-dependent reduction in cocaine sensitivity ($P<0.003$ for 30 μM reserpine, n=12).

Figure 3

Cocaine-induced locomotor patterns of control and dopamine-depleted flies. Flies were exposed to 0, 100, or 200 μg cocaine as indicated. Panels **A-F** and **G-L** correspond to vehicle-treated control (-3IY) and 3IY-treated (+3IY) flies, respectively. The drug effect increased gradually during the first 5 minutes after exposure; the locomotor patterns during the 2nd (1-2) and 4th (3-4) minute are shown to illustrate this effect. 3IY-treatment had no obvious effect on mock-treated flies (0 μg cocaine). However, the effect of cocaine was reduced in 3IY-treated flies at all doses and times tested. See Table II for quantification of these data.

Figure 4

Drosophila responds synergistically to co-administration of low doses of cocaine and nicotine. (**A**) The behavioral response of wild type flies to the indicated low doses of nicotine, cocaine, or both was measured in the negative geotaxis assay as described in Materials and methods (n = 9-15). (**B**) This synergistic response is significantly reduced ($P<0.001$, n=9) in flies in which dopamine levels had been reduced by 3IY treatment.

Figure 5

Pharmacological reduction of dopamine levels decrease *Drosophila* sensitivity to ethanol-induced hyperactivity. (**A, B**) Adult flies treated with 3IY and/or L-Dopa were tested for locomotor activity in the absence and presence of ethanol vapor (see Materials and methods). Average locomotion is shown at 1-minute-intervals in (**A**) and averaged over 5-minute-intervals in (**B**). The black bar at the bottom of panel **A** corresponds to the period of ethanol exposure (from 0-20 minutes). 3IY-treated flies show a significant reduction in ethanol-induced locomotor activation in the first 5 minutes of exposure (P=0.0012, n=19). This reduction was reversed by co-administration of L-Dopa (flies fed 3IY and L-Dopa are not different from controls in this time period). Drug treatments did not alter basal locomotion (**B**) measured in narrow tubes (see Materials and methods); this method was used because locomotion in the ethanol-exposure chambers in the absence of ethanol was too low for proper quantification (see panel **A** prior to ethanol exposure). (**C**) Treatment with 3IY and/or L-Dopa did not alter ethanol absorption. The ethanol concentration was measured in extracts prepared from flies exposed to ethanol for 10 and 20 minutes using an alcohol dehydrogenase-coupled spectrophotometric assay (Moore *et al.*, 1998). Treatment groups in panel **C** are as defined in panel **B.**

Table I

Treatment of *Drosophila* with 3IY or reserpine reduces dopamine but not serotonin levels. 25 adult male flies were quick-frozen in liquid nitrogen and homogenized in 200 µl 0.1 M perchloric acid. Monoamine levels were measured using high pressure liquid chromatography as described by Neckameyer (Neckameyer, 1996). This procedure does not allow the quantification of local changes in monoamine levels. We suspect that the more dramatic reduction of dopamine in 3IY-treated flies is caused by the added depletion in the cuticle. Asterisks denote $P<0.0015$.

Table II

Treatment with 3IY reduces the sensitivity of flies to the locomotor-depressing and circling-inducing effects of cocaine. The median velocity (mm/sec) and median turning (°/mm) of flies exposed to various doses of cocaine is shown for the 2nd (1-2 min) and 4th (3-4 min) minute after exposure. Pairwise comparisons between 3IY- and vehicle-treated flies were carried out using the Wilcoxon-Mann-Withney U test. Asterisks denote statistically significant differences ($P<0.05$). All pairwise comparisons between mock- and cocaine-treated flies showed highly significant differences ($P\leq0.0063$).

Figure 1

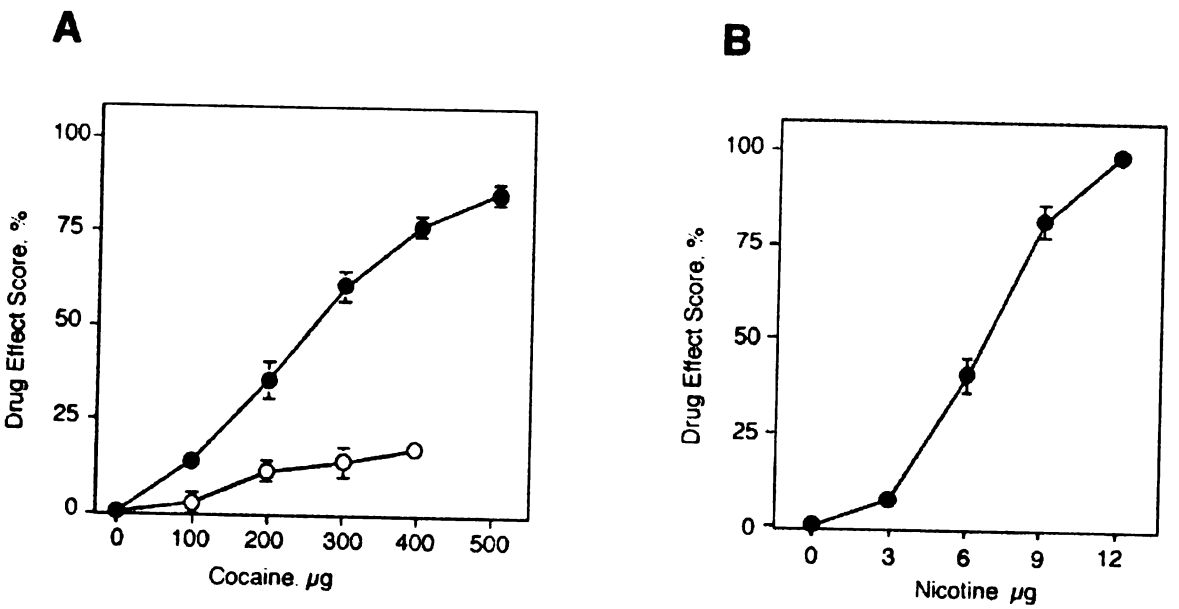
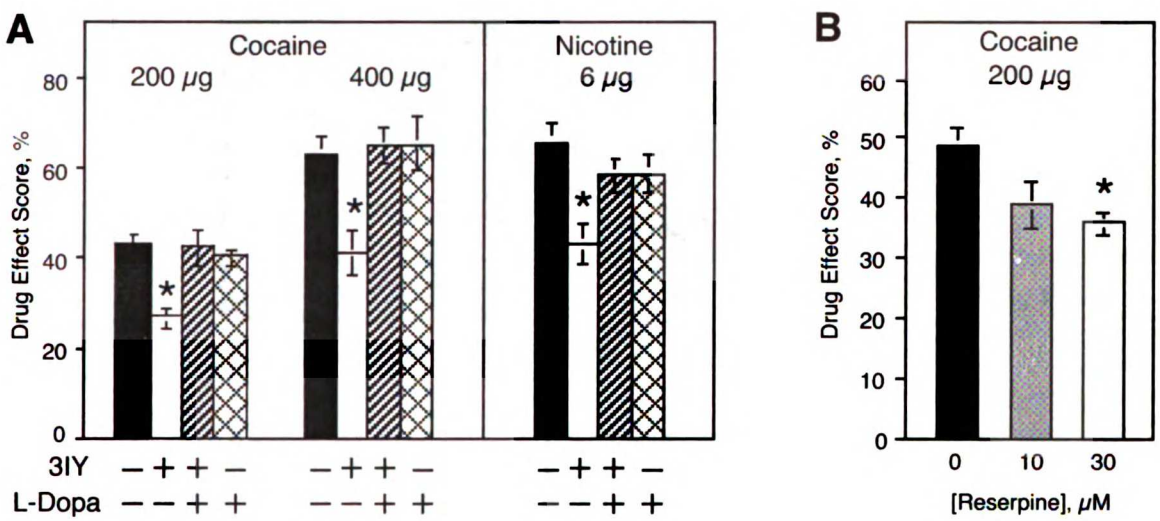

A
100
75
50
25
0
Drug Effect Score, %
0
100
200
300
400
500
Cocaine, μg
B
100
75
50
25
0
Drug Effect Score, %
0
3
6
9
12
Nicotine μg

Figure 2


A
Cocaine
200 μg
400 μg
Nicotine
6 μg
Drug Effect Score, %
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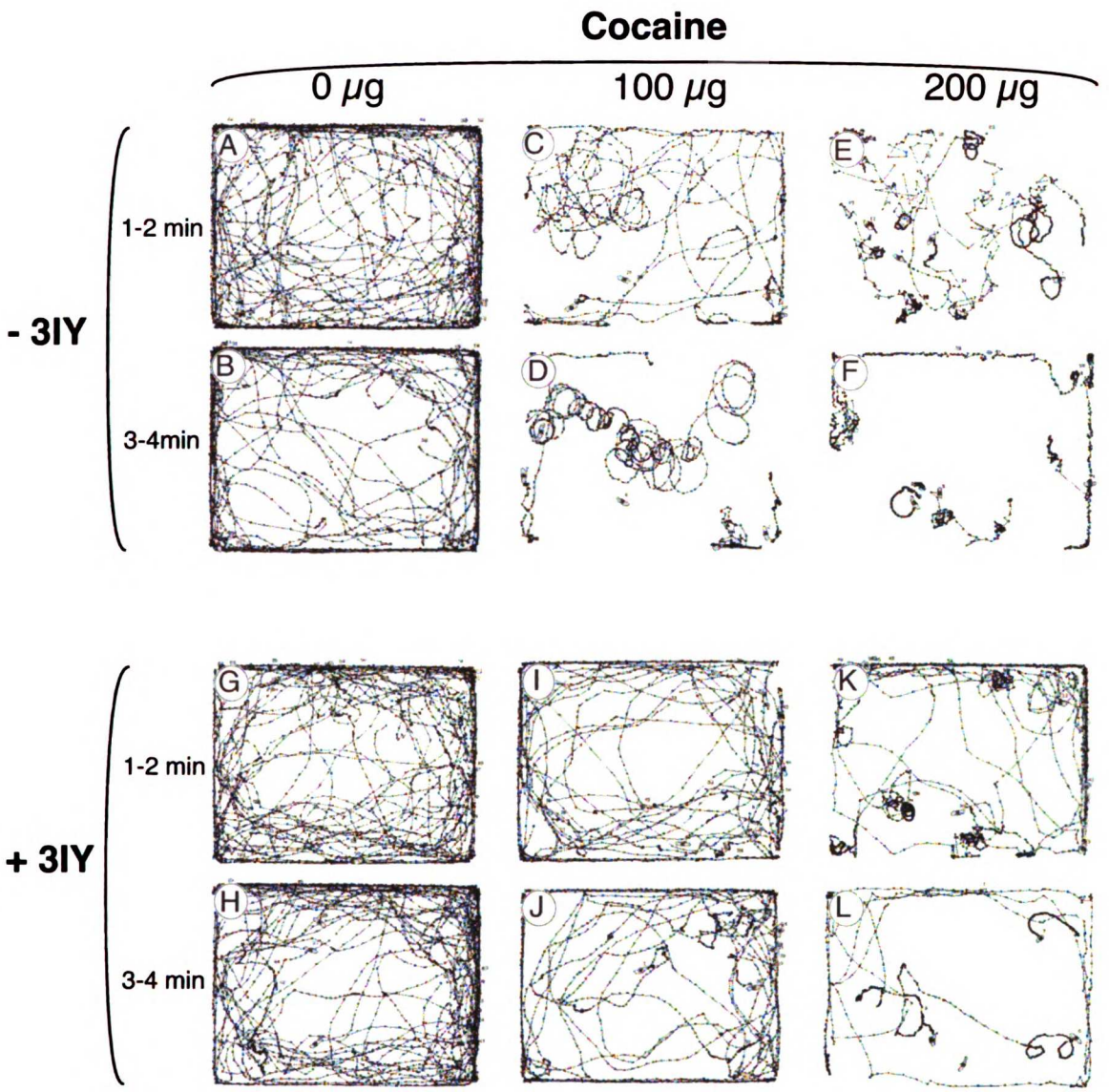
60
40
20
0
3IY – + + – – + + – – + + –
L-Dopa – – + + – – + + – – + +
B
Cocaine
200 μg
Drug Effect Score, %
60
50
40
30
20
10
0
0
10
30
[Reserpine], μM

Figure 3


Cocaine
0 μg
100 μg
200 μg
- 3IY
+ 3IY
1-2 min
3-4min
3-4 min
A
B
C
D
E
F
G
H
I
J
K
L

UMI

Figure 4

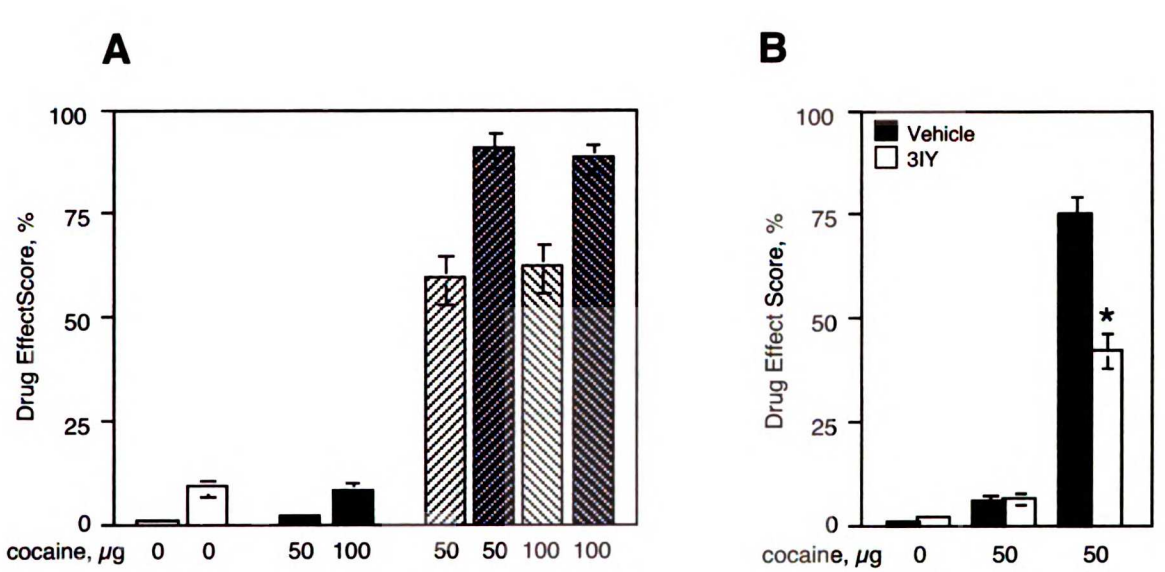

A
100
75
50
25
0
Drug EffectScore, %
cocaine, μg 0 0 50 100 50 50 100 100
nicotine, μg 1.5 3.0 0 0 1.5 3.0 1.5 3.0
B
100
75
50
25
0
Drug Effect Score, %
Vehicle
3IY
*
cocaine, μg 0 50 50
nicotine, μg 1.5 0 1.5

Figure 5

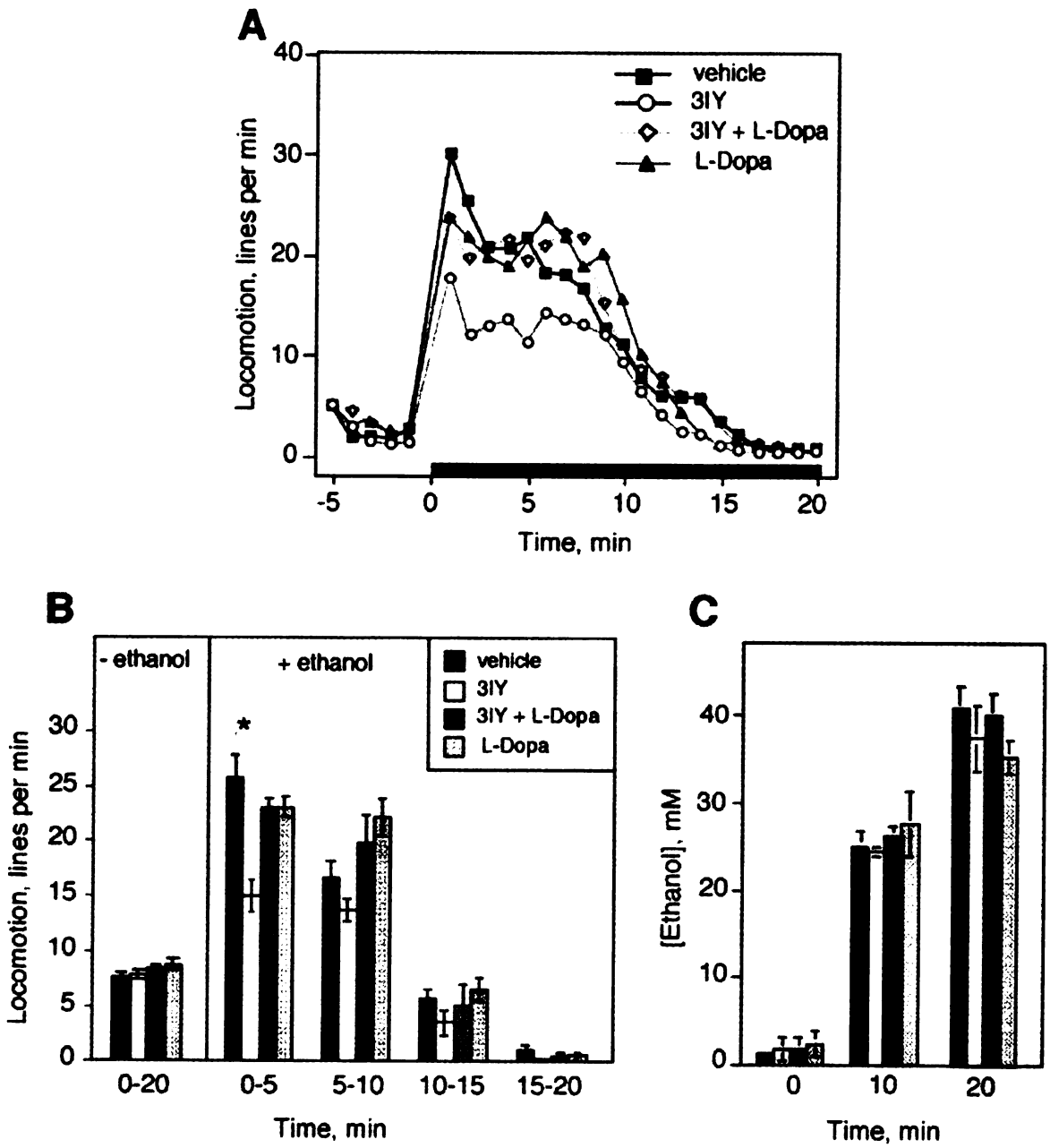

A
Locomotion, lines per min
40
30
20
10
0
-5
0
5
10
15
20
Time, min
vehicle
3IY
3IY + L-Dopa
L-Dopa
B
- ethanol
+ ethanol
vehicle
3IY
3IY + L-Dopa
L-Dopa
*
Locomotion, lines per min
30
25
20
15
10
5
0
0-20
0-5
5-10
10-15
15-20
Time, min
C
[Ethanol], mM
40
30
20
10
0
0
10
20
Time, min

Table I

Treatment	[Dopamine], μg/ml ±SEM (n)	[Serotonin], μg/ml ±SEM (n)
Vehicle	0.76 ± 0.05 (10)	0.40 ± 0.08 (8)
3IY (10 mg/ml)	0.08 ± 0.01 (10)*	0.40 ± 0.08 (8)
3IY (10 mg/ml) + L-Dopa (1 mg/ml)	0.30 ± 0.04 (9)*	0.39 ± 0.08 (8)
L-Dopa (1 mg/ml)	1.46 ± 0.29 (10)*	0.37 ± 0.06 (8)
Vehicle	0.63 ± 0.02 (3)	0.28 ± 0.02 (3)
Reserpine 10 μM	0.51 ± 0.03 (3)	0.30 ± 0.01 (3)
Reserpine 30 μM	0.44 ± 0.02 (3)*	0.31 ± 0.01 (3)

Table II

Cocaine		0 μg			100 μg		200 μg	
Treatment		-3IY (n)	+3IY (n)		-3IY (n)	+3IY (n)	-3IY (n)	+3IY (n)
Median velocity (mm/sec)	1-2 min	10.9 (5)	10.0 (5)		4.7 (8)	3.8 (8)	2.3 (8)	6.5 (8)
	3-4 min	8.2 (5)	9.6 (5)	0.602	1.2 (8)	2.1 (8)	0.9 (8)	0.9 (8)
Median Turning (°/mm)	1-2 min	10.6 (5)	9.8 (5)		19.9 (9)	12.5 (8)	38.5 (7)	20.6 (8)
	3-4 min	12.0 (5)	10.5 (5)		41.1 (6)	16.5 (8)	59.4 (9)	17.1 (5)

Chapter 2

Mutant Alleles of *Lmo* Reveal a Novel Role for *Drosophila* Circadian Pacemaker Neurons in Modulation of Cocaine–Sensitive Locomotor Behaviors

Linus T.–Y. Tsai, Roland J. Bainton, Justin Blau, Ulrike Heberlein

Summary

In a genetic screen for mutants with altered cocaine sensitivity, we identified multiple alleles of the *Drosophila* LIM-only gene (*Lmo*). Reduced *Lmo* function increases behavioral responses to cocaine, while *Lmo* overexpression causes the opposite effect, reduced cocaine responsiveness. Expression of *Lmo* in the principal *Drosophila* circadian pacemaker cells, the PDF–expressing ventral lateral neurons (LN_vs), is sufficient to confer normal cocaine sensitivity. Consistent with a role for *Lmo* in LN_v function, *Lmo* mutants also show some defects in circadian rhythms of behavior. However, the role for LN_vs in modulating cocaine responses is separable from their role as pacemaker neurons: ablation or functional silencing of the LN_vs reduces cocaine sensitivity, while loss of the principal circadian neurotransmitter PDF has no effect. Together, these results reveal a novel role for *Lmo* and the LN_v pacemaker neurons in the modulation of an acute behavioral response, sensitivity to behavioral consequences of acute cocaine administration. In addition, we show a that wild type *Lmo* functionis required for robust circadian behavioral rhythms.

Introduction

Cocaine, a naturally occurring plant alkaloid, is the prototype addictive psychomotor stimulant. It elicits a variety of acute behavioral changes ranging from mood elevation, disinhibition, and motor activation at low doses to compulsive stereotypies and psychosis at higher doses (Gawin, 1991). Long–term cocaine use generally results in tolerance to many of its subjective

effects, an increased craving towards the drug, and eventually to drug abuse and addiction.

Cocaine's primary mechanism of action is to bind and inhibit plasma membrane monoamine transporters, thereby increasing synaptic monoamine neurotransmitter levels and potentiating their action. Cocaine's direct role in increasing dopamine in the nucleus accumbens (NA) via inhibition of the dopamine transporter (DAT) has contributed to the prevalent dopamine hypothesis of drug addiction, which posits that the shared ability of drugs of abuse to increase DA in the NA underlies their reinforcing properties (Wise and Bozarth, 1985; Kuhar *et al.*, 1991). More recent animal studies, however, have shown that cocaine still elicits a robust conditioned place preference in mice with genetic deletions of DAT, suggesting that the rewarding properties of cocaine are not solely mediated by its action on DAT (Sora *et al.*, 1998). Additional studies on mice in which the serotonin transporter (SERT) or norepinephrine transporter (NET) has been deleted, together with studies using selective SERT or NET inhibitors, have suggested roles for both serotonin and norepinephrine systems in mediating cocaine's rewarding properties (reviewed in Uhl *et al.*, 2002). These data show that the molecular bases for cocaine's psychostimulant and reinforcing properties are more complicated than once thought, and that a combination of actions at multiple sites may mediate its effects. Indeed, multiple genes and signaling pathways have been implicated in the stimulant and rewarding properties of cocaine in mice (reviewed in Laakso *et al.*, 2002).

The fruit fly *Drosophila melanogaster* has been advanced as a useful model system for identifying novel genes that regulate behavioral responses to drugs of abuse including cocaine (Wolf and Heberlein, 2003). Several of cocaine's most characteristic properties have been recapitulated in flies. First, cocaine induces motor behaviors in flies, including that are remarkably similar to those observed in mammals (McClung and Hirsh, 1998; Bainton *et al.*, 2000). Second, repeated cocaine administration induces behavioral sensitization (McClung and Hirsh, 1998), a form of behavioral plasticity believed to underlie certain aspects of addiction (Robinson and Berridge, 1993; Schenk and Partridge, 1997). Finally, a key role for dopaminergic systems in mediating cocaine's effects has been demonstrated through both pharmacologic and genetic methods (Bainton *et al.*, 2000; Li *et al.*, 2000). More importantly, *Drosophila* studies have identified genes and pathways whose role in cocaine responsiveness had not been anticipated (reviewed in Hirsh, 2001; Rothenfluh and Heberlein, 2002). For instance, the *Drosophila* circadian gene *period* (*per*) was identified as a necessary mediator of cocaine sensitization (Andretic *et al.*, 1999). Subsequently, mice carrying various *per* mutations were found to have altered cocaine sensitization and conditioned place preference (Abarca *et al.*, 2002).

In order to identify novel molecules and pathways involved in behavioral responses to cocaine, we carried out a genetic screen for *Drosophila* mutants with altered acute responses to cocaine. Here, we report the phenotypic and molecular characterization of mutations in the *Drosophila* LIM-only gene, *Lmo*

(also called *Beadex*), isolated due to their increased sensitivity to cocaine–induced loss of negative geotaxis. The products of *Drosophila* and mammalian *Lmo* genes modulate the function of LIM-homeodomain (LIM-HD) proteins (Milan *et al.*, 1998; reviewed in Retaux and Bachy, 2002), which in turn regulate various aspects of nervous system development, including the specification of neural identity (Thor and Thomas, 1997; Hobert and Westphal, 2000; Shirasaki and Pfaff, 2002; Tsalik *et al.*, 2003). We find that cocaine sensitivity is inversely related to the levels of *Lmo* function: reduced function causes increased sensitivity, while increased function causes resistance. This bi-directional regulation of cocaine responsiveness is mediated by *Lmo* function in a small set of neurons, the ventral lateral neurons (LN_vs), which are the primary circadian pacemaker cells in *Drosophila* (Helfrich-Forster, 1997; Renn *et al.*, 1999). Like other mutants that affect LN_v function, *Lmo* mutants show altered circadian locomotor behaviors. However, the role of the LN_vs in regulating cocaine sensitivity and circadian behavioral rhythms is genetically separable, as mutants lacking the neuropeptide PDF—the only known functional output of these neurons—show normal cocaine sensitivity. Thus, *Lmo* defines a novel role for the circadian pacemaker cells in regulating behavioral responses to cocaine.

Results

Loss– and gain–of–function mutations in the *Lmo* locus show altered cocaine sensitivity

To identify novel molecules involved in cocaine–related behaviors, we carried out a genetic screen for mutants with altered acute responses to volatilized freebase cocaine using the crackometer, a simple assay that measures cocaine–induced loss of negative geotaxis (Bainton *et al.*, 2000). Screening of 400 first chromosome P–element insertions from the EP collection (Rorth, 1996; Berkeley *Drosophila* Genome Project) led to the identification of five mutants with reduced cocaine sensitivity and seven with increased sensitivity. Two of the mutants conferring increased cocaine sensitivity, *EP1306* and *EP1383* (Fig. 1A), carry a P-element insertion in the promoter region of the *Drosophila LIM–only* (*Lmo*) locus (Milan *et al.*, 1998; Zeng *et al.*, 1998). *Drosophila* LMO protein has been shown to regulate activity of the LIM homeodomain (LIM–HD) transcription factor *apterous* through its interactions with the LIM–HD activator Chip (Milan and Cohen, 1999; Weihe *et al.*, 2001).

Independently, we isolated a P[GAL4] insertion in the *Lmo* locus, which we dubbed *pipedream* (*pdrm*), that also shows increased cocaine sensitivity (Fig. 1A). Finally, *heldup* (*hdp*) mutant flies, which carry well-characterized loss-of-function mutations in *Lmo* (Milan *et al.*, 1998), display increased sensitivity to cocaine that is similar in magnitude to that of the *EP1306* line (Fig. 1A). This increased sensitivity is observed at all cocaine doses tested (Fig. 1B, data not shown). The *EP1306* and *hdp* alleles consistently demonstrated stronger

phenotypes than *EP1383* and *pdrm*. All mutants tested performed within the normal range in task-specific baseline behaviors (see Experimental Procedures). Finally, we were able to revert the cocaine phenotype of *EP1306* by excision of the P element (data not shown). Taken together, these data demonstrate that loss-of-function mutations in *Lmo* result in increased sensitivity to cocaine.

The dominant *Beadex* (*Bx*) mutations were first described by Morgan, Bridges, and Sturtevant in 1925, and named for the characteristic beaded wing margin (Morgan *et al.*, 1925). More recently, the *Bx* mutants were shown to be overexpression alleles of *Lmo* (Milan *et al.*, 1998; Shoresh *et al.*, 1998; Zeng *et al.*, 1998). Overexpression results from the insertion of naturally occurring transposable elements into the 3' UTR of *Lmo*, which leads to transcript stabilization (Shoresh *et al.*, 1998). The resultant overexpression of *Lmo* in the wing imaginal disc causes decreased *apterous* activity in the dorsal compartment of the wing and, consequently, to disorganization of the wing margin (Milan and Cohen, 1999; Milan and Cohen, 2000).

We tested multiple available *Bx* alleles, and found that they all caused resistance to the acute effects of cocaine at every dose tested (Fig. 2A, B). The strength of the cocaine resistance phenotype correlated well with the severity of the beaded wing-margin phenotype, $Bx^{J}>Bx^{1}=Bx^{2}=Bx^{3}$ (Fig. 2A, B and data not shown). Because in *Bx* mutants *Lmo* transcripts are stabilized in their normal spatio–temporal pattern (Shoresh et al., 1998), the observed cocaine phenotypes likely result from overexpression, rather than from misexpression of *Lmo*. Taken together with the loss-of-function effects described above (Fig. 1A, B), these data

reveal a graded behavioral response to cocaine that is inversely related to *Lmo* levels.

In order to study the *Lmo* mutant behavior in a more detailed fashion, we recorded the cocaine–induced patterns of locomotor activity of control and *Lmo* flies using a movement tracking system (Bainton *et al.*, 2000; Wolf *et al.*, 2002; see Experimental Procedures). Upon mock exposure, control flies show robust locomotor activity, generally walking in straight lines (Fig. 1C, top left panel). At relatively low doses of cocaine (75 and 100 μg), flies engage in stereotyped circling patterns of activity (Fig. 1C, top right panels). At higher doses (150-200 μg), flies begin to show spasmodic movements (seen as zig-zag patterns in movement) and severe hypokinesis (Fig. 1C and data not shown, (Bainton *et al.*, 2000). *EP1306* flies, while clearly walking more slowly, show normal patterns of activity upon mock exposure (Fig. 1C, lower left panel). Upon exposure to cocaine, these mutant flies show a shift in the dose–response relationship. At low doses (75μg), *EP1306* flies show a marked increase in stereotyped circling behavior compared to controls (Fig. 1C, lower middle panel), while at higher doses, the mutant flies show an increased likelihood of becoming spasmodic or akinetic (Fig. 1C, lower right panel). Thus, despite the reduced speed observed in the mock exposures, increases in specific cocaine–induced locomotor behaviors demonstrate that *EP1306* flies have a shifted dose–response to cocaine: mutant flies exposed to 75μg of cocaine behave similarly to control flies exposed to 100μg of the drug.

Bx^{J} flies also show changes in walking patterns that are consistent with a shift in the cocaine dose–response relationship. After exposure to 100µg of drug, most control flies show slow circling behavior and some are akinetic (Fig. 2C, top middle panel). *Bx*^{J} flies are much less affected, showing increased locomotion (mostly in straight lines), decreased slow circling, and almost no akinesia (Fig. 2C, bottom middle panel). At 125µg, control flies show very little movement (Fig. 2C, top right panel), while most *Bx*^{J} flies continue to show circling behaviors seen in control flies at lower doses (Fig. 2C, bottom right panel). *Bx*^{J}, like *EP1306* flies, show reduced activity upon mock exposure (Fig. 2C, lower left panel). Despite this reduced activity, these two alleles show very different sensitivities to cocaine: *EP1306* flies are more affected and *Bx*^{J} flies are less affected, suggesting that the reduced activity upon mock exposure is unrelated to the cocaine response. Long–term activity recordings revealed no differences between *Bx*^{J}, *EP1306*, and control lines (data not shown, see below).

In summary, using two different behavioral assays we show that flies carrying loss-of-function mutations in *Lmo* display increased sensitivity to the effects of cocaine on locomotor behaviors, while gain-of-function mutations show the converse effect, a reduced response to the drug. This inverse relationship between *Lmo* gene activity and drug responsiveness suggests that *Lmo* regulates the expression of genes that might play a direct role in controlling cocaine responses (see Discussion).

Molecular Characterization of *Lmo* Mutants

The *Lmo* locus produces at least three transcripts by differential promoter use (Fig. 3A). The RA and RC transcripts differ only in their 5'UTRs and are predicted to encode identical proteins of 313 amino acids; the RB transcript is predicted to utilize an alternative translational start site in its first exon, which would result in an addition of 71 N–terminal amino acids. The insertions that produce increased cocaine sensitivity all lie within the putative promoter region for the RA transcript, between 25–90 bp upstream of its transcriptional start site (Fig. 3A). In order to identify molecular changes caused by these insertions, we assessed *Lmo* transcript levels by real time quantitative RT-PCR in wild-type flies and *Lmo* mutants. In wild-type flies the RA transcript is about 4-fold more abundant in heads as compared to bodies, suggesting that this transcript is enriched in the nervous system (Fig. 3B; see also Fig. 4). Importantly, the RA transcript is reduced by greater than 50% in the heads, but not the bodies, of *EP1306* flies (Fig. 3B). Thus, as predicted by the location of the P element insertion, the *EP1306* mutation causes a reduction in *Lmo* transcript levels, a finding that is consistent with the observation that the *EP1306* cocaine sensitivity is similar to that seen with known loss-of-function alleles of *Lmo* (the *hlp* alleles) and opposite to that seen with gain-of-function *Bx* alleles. In addition, the finding that transcript levels are specifically reduced in the heads of *EP1306* flies, and not their bodies, suggests that this mutation affects a nervous system enriched (or specific) *Lmo* transcript (see below). Finally, none of the P-element insertions in the promoter of the RA transcript lead to a held-up wing phenotype, which is

characteristic of loss-of-function *hdp* alleles (Shoresh *et al.*, 1998; Milan and Cohen, 1999). This suggests that the P element insertions isolated in our genetic screen cause either less severe or more spatially restricted changes in *Lmo* gene expression.

Restricted expression of *Lmo* in the nervous system is sufficient for wild–type cocaine sensitivity

The *pdrm* GAL4 insertion, which acts as a promoter/enhancer detector, is expected to reproduce, at least in part, the expression pattern of the *Lmo* gene. In adult flies, the *pdrm* line shows extensive GAL4 expression in the brain as visualized with the *UAS-GFP* transgene (Fig. 4A). Prominent expression is observed in the antennal lobes (ALs) and the Kenyon cells of the mushroom bodies (MBs), which are major brain centers involved in olfaction and olfactory associative learning respectively (Stocker, 1994; Zars, 2000). In addition, GAL4 is expressed in the ventral lateral neurons (LN_vs), which are the major pacemaker cells regulating circadian locomotor rhythmicity in flies (Renn *et al.*, 1999). Finally, GAL4 expression is seen in neurosecretory cells located in the pars intercerebralis (PI), in glial cells surrounding the optic lobes, and in scattered cells throughout the ventral nerve cord (Fig. 4A and data not shown). In *pdrm* larvae and adult flies, GAL4 expression is restricted to the nervous system. GAL4 expression is not detected in larval imaginal discs, such as the wing disc (data not shown), where *Lmo* expression has been shown to play an important patterning role (Milan and Cohen, 1999). Thus, *pdrm* traps a specific subset of

Lmo regulatory elements, possibly those controlling nervous system-restricted expression of the RA transcript.

We hypothesized that the expression pattern of the *pdrm* enhancer-trap may identify the cells in which *Lmo* expression is disrupted in the P element insertion lines that cause increased cocaine sensitivity. If this is the case, *pdrm*–driven expression of *UAS–Lmo* transgenes is expected to restore normal cocaine sensitivity to *pdrm* flies. Indeed, we find that two *UAS-Lmo* transgenes rescue the cocaine–sensitivity of the *pdrm* mutation (Fig. 4B). This effect is specific to *Lmo*, as *pdrm*–driven expression of *UAS–tauGFP* and *UAS–lacZ* fails to restore normal behavior. Finally, *Lmo* mutants that do not contain a GAL4 enhancer trap, such as *EP1306*, fail to be rescued by the presence of *UAS–Lmo* transgenes (data not shown). These data demonstrate that the observed cocaine–sensitivity defect of *pdrm* flies is due to the loss of *Lmo* function, and that nervous system–specific expression of *Lmo* in cells dictated by the *pdrm* GAL4 line is sufficient to confer normal cocaine responses.

Lmo expression in PDF neurons is sufficient to confer normal cocaine responses

In order to refine further the spatial requirements for *Lmo* function, we attempted to rescue the *EP1306* phenotype by expression of *Lmo* in specific brain regions. EP lines carry a P element insertion containing multiple UAS sites to which GAL4 can bind to drive expression of adjacent genomic sequences (Rorth, 1996). Therefore, mutagenic EP insertions, if oriented appropriately, can

be used to drive expression of the disrupted gene. It was demonstrated previously that GAL4–driven *Lmo* expression could be mediated by the *EP1306* and *EP1383* insertions (Milan *et al.*, 1998; Zeng *et al.*, 1998). Consistent with this, we were able to rescue the *EP1306* phenotype in the presence of the heat-inducible hs-GAL transgene, which shows low levels of ubiquitous GAL4 expression even in the absence of heat shock (data not shown).

We then used GAL4 enhancer trap lines with expression in specific brain regions to drive *Lmo* expression from the *EP1306* insertion. We focused specifically on GAL4 lines that drive expression in the MBs, ALs, PI neurons, LN_vs, and glia, sites of expression revealed by the *pdrm* line (Fig. 4A). GAL4 lines that drive expression specifically in the MBs and the ALs failed to rescue the cocaine–sensitivity phenotype of *EP1306* (data not shown, see Materials and Methods for lines used). Similar negative results were obtained with the glial–specific *repo–GAL4* driver (Xiong *et al.*, 1994;data not shown). However, expression of *Lmo* in the LN_vs using the *pdf–GAL4* driver, which drives expression in cells that contain the neuropeptide PDF (ref), restored nearly wild-type cocaine responsiveness to *EP1306* flies (Fig. 5A, C). *pdf–GAL4* does not rescue *hdp* and *pdrm* flies, demonstrating dependence on induced *Lmo* expression provided by the *EP1306* insertion. These results show that *Lmo* expression in the PDF-expressing LN_vs neurons alone is sufficient to rescue wild–type cocaine responses, thus demonstrating that these cells underlie the increased cocaine responses observed in *Lmo* loss of function mutants.

In order to test whether LN_vs are also the locus for the resistance to cocaine observed in *Bx* mutants, we overexpressed *Lmo* in wild-type flies using the *pdf–GAL4* driver and *UAS-Lmo* transgenes. We found that these flies show a significant decrease in cocaine sensitivity (Fig. 5B). The magnitude of the induced resistance is lower than that observed in the Bx^J strain, but is similar to that of weaker *Bx* alleles. It is possible that overexpression of *Lmo* with the *pdf-GAL4* driver may not be as high as that caused by the Bx^J mutation, or alternatively, overexpression of *Lmo* in cells other than the LN_vs mediate the remaining resistance to cocaine observed in Bx^J. Nonetheless, this experiment supports a role LN_vs as a site at where *Lmo* regulates sensitivity to cocaine.

In the adult fly, *pdf*–GAL4 drives expression in small and large LNs (s–LN_vs and l–LN_vs) and in a group of neuroendocrine cells located at the very tip of the ventral nerve cord (VNC) (Park *et al.*, 2000; Fig. 5C). The LN_vs express many central clock genes and have been demonstrated, through genetic ablations and electrical silencing studies, to play a central role in maintaining circadian locomotor rhythmicity (Renn *et al.*, 1999; Nitabach *et al.*, 2002; Peng *et al.*, 2003); the function of the *pdf-GAL4* expressing cells in the VNC is unknown. The *pdrm* line drives expression in both the s–LN_vs and l–LN_vs as demonstrated by immunohistochemical analysis of *pdrm/UAS-GFP* flies with antibodies directed against GFP and the PDF precursor PAP (Fig. 5C). *pdrm* does not, however, drive expression in the PDF-expressing cells located in the VNC (data not shown). This overlap in expression of *pdrm* and *pdf–GAL4* in the LN_vs

implicates these few neurons as mediators of the altered cocaine–sensitivity observed in *Lmo* mutants.

Because of the known role of LMO as a regulator of developmentally–important transcription factors (Hobert and Westphal, 2000), we asked if the development of PDF-expressing LN_vs is disrupted in *Lmo* mutants. Projections of the s–LN_vs and l–LN_vs can be visualized with an antibody that recognizes the PDF-precursor PAP (Renn *et al.*, 1999). l–LN_vs make an elaborate network of varicosities on the surface of the optic medulla, and project across the midline to the contralateral LN_vs, while s–LN_vs, make a very specific projection to the dorsal central brain (Fig. 5C). Both sets of LN_v neurons also make extensive arborizations in the accessory medulla (reviewed in Hellfrich–Förster, 2003). In the *EP1306* and *pdrm* alleles, anti–PAP staining revealed that the number and detailed morphology of LN_v neurons are completely normal (data not shown). Thus, *Lmo* does not appear to play a role in the development of the LN_vs, although subtle developmental defects could have been missed.

Lmo is required for robust circadian locomotor rhythms

The increased sensitivity to cocaine of *Lmo* mutants suggested that mutations in *Lmo* alter LN_v function without grossly affecting LN_v development. In order to determine whether *Lmo* mutants have a general dysfunction in the LN_vs, we *Lmo* mutants in locomotor rhythm assays. The LN_vs are required for the maintenance of circadian locomotor rhythms in constant darkness (DD), and

are thus the pacemaker neurons of the fly (reviewed in Stanewsky, 2003). *Lmo* mutants *EP1306* and *EP1383* had less robust locomotor rhythms in DD than a control EP line (Fig. 6A, data not shown) and, particularly for EP1306, a higher tendency for arrhythmicity. We used the power of the rhythm as an estimate of the degree of rhythmicity and found that EP1306 flies had weaker behavioral rhythms than EP1383 flies which, in turn, were less robustly rhythmic than a control EP line (Fig. 6A,B). This allelic series is the same as previously noted for cocaine sensitivity. However there were rhythmic flies in all genotypes assayed and the period lengths of the rhythms were very similar across the three genotypes (see figure legend for details).

EP1306 and EP1383 mutant flies also had defects in their patterns of locomotor activity when assayed in cycles of 12 hours of light and 12 hours of darkness (LD) with a less pronounced bimodal distribution and a consequently decreased power of the rhythm (Fig 6B,C). In LD, EP1383 anticipate the lights-off transition ~ 1 to 1.5 hours earlier than control flies (Fig 6C). This behavior in LD is reminiscent of flies carrying null mutations in *Pdf*, the only documented neuropeptide or neurotransmitter expressed in LN_vs, and flies in which the LN_vs have been electrically inactivated (Renn *et al.*, 1999; Nitabach *et al.*, 2002). Together, these data demonstrate that disruption of *Lmo* results in less robust circadian rhythms of behavior and suggest a generalized dysfunction of the LN_vs in *Lmo* mutants.

LN_vs regulate circadian rhythmicity and cocaine sensitivity independently

The observation that *Lmo* functions in the circadian pacemaker neurons to regulate cocaine sensitivity, together with the finding that *Lmo* mutants show weak circadian locomotor rhythms, suggested that the pathways regulating cocaine sensitivity interact with the circadian clock. Alternatively, *Lmo* could regulate these two behaviors independently. We addressed these possibilities in two sets of experiments. First, we determined if cocaine–sensitivity is regulated by the circadian clock. We entrained flies to LD cycles and then tested them for cocaine sensitivity during the light and dark phases at 3–hourly intervals over 24 hours (see Experimental Procedures). We found that cocaine sensitivity is essentially the same at all times tested (Fig. 7A), demonstrating that cocaine–responsiveness is not a behavioral output of the circadian clock.

Second, we asked if PDF, the only known functional output of the LN_vs in the context of circadian rhythms, is involved in regulating cocaine sensitivity. *Pdf* mutant flies show a circadian phenotype that has been localized to the LN_vs. We found that the Pdf^{01} mutant flies, which completely lacks PDF (Renn et al., 1999), show normal cocaine responses compared to a control line (Fig. 7B). To eliminate the possibility that the absence of a phenotype was caused by genetic modifiers in the background of the Pdf^{01} strain, we also tested flies carrying the Pdf^{01} chromosome over a deficiency for the locus (see Methods). These Pdf^{01} hemizygote flies also showed normal cocaine sensitivity (Fig. 7B). Taken together, these results show that the altered cocaine sensitivity of *Lmo* flies is not secondary to their abnormal circadian rhythms. Moreover, our data show that

cocaine sensitivity and circadian behaviors, although both localized to the LN_vs, are genetically separable.

Transgenic Ablation/Silencing of LN_vs Results in Resistance to Cocaine

To better understand the mechanisms underlying the increased cocaine sensitivity of *Lmo* mutants, we next addressed the role of LN_vs in wild–type responses to cocaine. The hypersensitivity of *Lmo* mutants could result from either the disruption of an LN_v output that normally acts to negatively modulate cocaine sensitivity or from an increase in an output of LN_vs that normally enhances cocaine sensitivity. In order to differentiate between these two possibilities, we generated flies with genetic LN_v ablations via targeted expression of the cell death gene *head involution defective* (*hid*) using *pdf*–GAL4. This approach was previously used to demonstrate that LN_vs are the *Drosophila* pacemaker neurons responsible for rhythmic locomotor activity (Renn *et al.*, 1999). When tested in the crackometer, flies with LN_v ablations showed reduced sensitivity to cocaine when compared to controls (Fig. 8). These results show that LN_vs normally act to increase sensitivity to cocaine.

To confirm that the behavioral resistance observed in LN_v–ablated animals resulted from loss of neuronal signaling from these cells, rather than from compensations induced by the ablations, we functionally silenced the LN_vs either electrically, via targeted expression of the mammalian inward rectifying K+ channel (*Kir2.1*), or synaptically, via expression of tetanus toxin (*TeTx*) (Sweeney *et al.*, 1995; Baines *et al.*, 2001). These manipulations do not affect LN_v survival

or their normal projection patterns (Kaneko *et al.*, 2000; Nitabach *et al.*, 2002) Expression of either *Kir2.1* or *TeTx* caused a reduced cocaine sensitivity to a similar extent as genetic LN_v ablations (Figure 8B). Importantly, expression of a mutant inactive version of tetanus toxin (*TeTx*^{in}) did not significantly alter cocaine responses, confirming that the actions of these transgenes were specific to their ability to silence or ablate the neuron (Figure 8B). These data further demonstrate that circadian phenotype does not predict cocaine phenotype, as targeted expression of either *hid* or *Kir2.1* in LN_vs caused arrhythmia, while expression of *TetTx* did not (Renn *et al.*, 1999; Kaneko *et al.*, 2000; Nitabach *et al.*, 2002). In summary, these results confirm a novel role for LN_v activity and synaptic output in increasing behavioral responses to cocaine in a manner independent from its role in regulating circadian locomotor rhythmicity.

Discussion

In a P element screen for *Drosophila* mutants with altered acute responses to cocaine, we isolated multiple insertions in the *Lmo* locus with increased sensitivity. Behavioral characterization of gain and loss of function alleles of *Lmo* demonstrate an inverse correlation between *Lmo* expression and cocaine sensitivity. Through targeted expression of *Lmo*, we show that differential *Lmo* expression in the circadian pacemaker ventral lateral neurons (LN_vs), underlies these altered cocaine responses. Additionally, we identify *Lmo* as a novel regulator of circadian locomotor rhthmicity. Using a variety of genetic methods to ablate or functionally silence these neurons, we reveal a novel role

for the LN_vs in modulating acute locomotor responses to cocaine that is independent of their pacemaker function in modulating circadian locomotor activity. These findings add to mounting data in *Drosophila* and mice highlighting a shared role for circadian genes in cocaine–related behaviors (Andretic *et al.*, 1999; Park *et al.*, 2000b; Abarca *et al.*, 2002; Rothenfluh and Heberlein, 2002). Our discovery that cocaine actions are modulated by neurons critical for the modulation of circadian responses suggests a basis for this overlap.

Lmo Regulates Cocaine Sensitivity in *Drosophila*

We provide several lines of evidence that *Lmo* regulates acute sensitivity to cocaine. First, in a P element screen for mutants with altered responses to volatilized cocaine, we identified multiple insertions in the *Lmo* locus with increased sensitivity to cocaine. Second, *Bx* mutants, which are caused by overexpression of *Lmo*, showed reduced sensitivity to cocaine. Third, induced expression of *Lmo* restored the cocaine sensitivity of different *Lmo* mutants in multiple rescue paradigms. Lastly, induced overexpression of *Lmo* in LN_vs phenocopied *Bx* mutant resistance to cocaine.

Molecular analysis of *Lmo* transcript levels showed a specific loss of the head–enriched RA transcript in adult heads in the *EP1306* allele. This result suggested that reduced expression of *Lmo* in the brain mediated the increased cocaine sensitivity in EP1306. Using both *pipedream*'s GAL4 enhancer trap expression and *pdf*–GAL4 to drive transgenic *Lmo* expression, we further localized a requirement for *Lmo* to a few neurons within the brain, the LN_vs.

Moreover, we showed that altering expression of *Lmo* specifically in LN_vs induced changes in cocaine responsiveness. The LN_vs are a group of 8–10 neurons in each brain hemisphere that express the neuropeptide PDF. These neurons have previously been identified as the circadian pacemaker neurons of adult *Drosophila* and are involved in modulation of rhythmic locomotor behavior (Renn *et al.*, 1999; Blanchardon *et al.*, 2001). Interestingly, expression of a mouse homolog of *Lmo*, *Lmo4*, is enriched in the mouse suprachiasmatic nucleus (SCN), which serve as mammalian pacemaker cells (Amy Lasek and David Kapfhamer, personal communication). Furthermore, microarray data shows that in many tissues, *Lmo4* expression varies with circadian time (Panda *et al.*, 2002). These data suggest that there may be some evolutionary conservation of an *Lmo* role in clock neuron function.

Lmo is known to act as a regulator of LIM–HD activity and stability (reviewed in Retaux and Bachy, 2002). LIM–HD proteins are transcription factors involved in many stages of nervous system development, from neuronal generation and axon guidance to determination of neuronal subtype identity (Hobert and Westphal, 2000). Expression of these genes in postmitotic neurons suggests a role for LIM–HDs in maintenance of their differentiated state (Hobert and Westphal, 2000). The development and structure of the LN_vs, the neurons to which we localize *Lmo* action, have been studied in detail (Helfrich-Forster, 1997). The LN_vs can be further divided into two groups of 4–5 neurons based on cell body size, projection pattern, and when they arise during development. The small LN_vs (s–LN_vs), which arise in early larval development, project to the dorsal

central brain, terminating near two sets of dorsal neurons (DN_1s and DN_2s) that express clock genes at high levels. The large LN_vs (l–LN_vs) arise during pupal stages and project onto the surface of the optic medulla, as well as onto the contralateral LN_vs through fibers running in the posterior optic tract. Both s– and l–LN_vs express PDF and have dense arborizations in the accessory medulla, a neuropil proposed to be a circadian pacemaker center in cockroaches and crickets (Helfrich-Forster *et al.*, 1998). An assessment of neuron number and detailed projection patterns revealed no differences between wild type and *Lmo* mutants in either s–LN_vs or l–LN_vs. Furthermore, by both quantitative PCR and immunohistochemistry, we determined that PDF content was unchanged in *Lmo* mutants. Expression of PDF, the only known LN_v output, is restricted to the LN_vs and a few tritocerebral neurons in the brain. As PDF expression might be expected to be a very specific marker for the differentiated state of these neurons, it is unlikely that these neurons have gross differences in their terminal differentiation.

The absence of any observed structural abnormalities of LN_vs suggests that *Lmo* may play an active role in regulating cocaine responses. In fact, evidence is mounting that expression of *Lmo* is dynamically regulated within the nervous system. For instance, expression of *Lmo1*, *Lmo2*, and *Lmo3* are differentially regulated by seizure activity in specific regions of the hippocampus and forebrain in adult mice (Hinks *et al.*, 1997). Mammalian *Lmo* homologs have also been identified in gene array experiments to be under circadian regulation in the SCN and to have increased expression in the cerebral cortex during sleep

deprivation or spontaneous waking relative to sleep (Cirelli and Tononi, 2000; Panda *et al.*, 2002). Furthermore, *Lmo*1 was isolated as a transcript upregulated by DA administration in cultured astrocytes (Shi *et al.*, 2001). Lastly, Ghosh and colleagues recently described *Lmo2* and *Lmo4* as two of four genes identified in a screen for calcium–regulated activators of transcription, suggesting a role for *Lmo* genes in regulating expression changes induced by neural activity. These data are intriguing in light of the many observed changes that occur in reward pathways in the addicted state. We attempted to determine whether *Lmo* played an active role in regulating cocaine response, using a *heatshock* promoter–GAL4 (*hs*–GAL4) fusion to rescue the increased sensitivity of *EP1306* Unfortunately, we found that "leaky" expression from the *hs* promoter restored wildtype cocaine responses, even when grown at lower temperatures (18 °C), and thus have not yet addressed this question.

The Role of LN_vs in regulation of Cocaine Behaviors

We provide the first evidence in *Drosophila* that the ventral lateral neurons (LN_vs) are involved in cocaine responses in addition to their role in regulating circadian behaviors. Flies with LN_v ablations show reduced sensitivity to cocaine, establishing that these cells normally increase behavioral responses to cocaine. Further, we find that both electrical and synaptic activity within the PDF neurons are necessary for normal cocaine response. The reduced sensitivity observed in these manipulations is consistent with a role for these neurons in directly responding to cocaine, thereby mediating part of the response to

cocaine. A direct effect for cocaine on the LN_vs is also supported by the presence of functional DA receptors on a subset of LN_vs (Wegener *et al.*, 2004). Hirsh and colleagues showed that mutants in the *Drosophila* clock genes *period* (*per*), *clock*, and *cycle*, but not *timeless* (*tim*) fail to sensitize to repeated cocaine exposures (Andretic *et al.*, 1999). These genes, which are expressed at high levels in the LN_vs, have been to shown to act in the LN_vs to modulate circadian behavior (Kaneko *et al.*, 1997). It would be interesting to determine if the LN_vs also mediate the reduced sensitization observed in *Drosophila* clock mutants.

It had been previously shown that electrical activity within LN_vs is necessary to both maintain clock cycling and to drive behavioral rhythms in free–running conditions (Nitabach *et al.*, 2002). However, electrical silencing of LN_vs only subtly alters locomotor behaviors in light-dark conditions, making it unclear if LN_v activity can directly drive locomotor activity on a rapid timescale. In our experiments, flies respond to cocaine within a minute, suggesting that LN_vs can mediate fast changes in behavior. In line with this novel role for LN_vs, recently submitted work by Blau and colleagues shows that these neurons mediate a rapid behavioral response of larvae away from light (Mazzoni *et al.*, 2004).

Our experiments also indicate that LN_vs drive circadian and cocaine behaviors in distinct ways. First, we found that sensitivity to cocaine is not modulated in a circadian manner, which shows that the cocaine response is not simply output, and that locomotor rhythms and cocaine sensitivity are regulated independently. Second, we showed three manipulations that produce similar

locomotor rhythm deficits—*Lmo* mutants, *pdf* mutants, and flies in which LN_vs have been electrical silenced or ablated—show completely uncorrelated cocaine sensitivities (sensitive, wild type, and resistant, respectively). Lastly, we provide evidence that the LN_v outputs which mediate cocaine and circadian behaviors are divergent. PDF, the only known LN_v output neurotransmitter, is required for normal locomotor rhythms (Renn *et al.*, 1999). *pdf* null mutants, however have normal cocaine responses. Furthermore, while synaptic silencing of PDF–expressing cells does not disrupt circadian locomotor activity (Kaneko *et al.*, 2000), we show here that blocking synaptic activity in PDF neurons reduces cocaine responses to the same extent as ablating them. Together, these results imply the existence of an alternate, tetanus toxin–sensitive molecular output that mediates LN_v modulation of cocaine responses. Interestingly, Blau and colleagues have also hypothesized a PDF–independent output to mediate another rapid behavioral response, larval photophobicity (Mazzoni *et al.*, 2004).

These findings can be most easily explained by a model in which individual LN_vs modulate separate behavioral processes. The identification of the unique projection patterns of s– and l–LN_vs provides a potential neuroanatomical basis for this separation. Indeed, there is growing evidence that these two cell types have divergent functions, and that, specifically, l–LN_vs are not involved in circadian rhythmicity (Helfrich-Forster, 2003). First, l–LN_vs make no projections to the dorsal brain, an area also involved in locomotor rhythmicity (Helfrich-Forster, 1997). Second, unlike in s–LN_vs, molecular clock cycling is not sustained in these cells during free–running conditions (Kaneko *et al.*, 2000;

Yang and Sehgal, 2001; Shafer *et al.*, 2002). Lastly, PDF expression has been shown to be controlled by clock genes and PDF release has been shown to be modulated by the molecular clock in s–LN_vs, but not in l–LN_vs (Blau and Young, 1999; Park *et al.*, 2000a). These results suggest an independent functional role for the l–LN_vs, making them strong candidates for mediating cocaine's actions.

These results contribute to growing evidence of a link between circadian neurons and modulation of cocaine–related behaviors. In mammals, the neuropeptidergic neurons of the suprachiasmatic nuclei (SCN) have been shown in a variety of studies to be the master pacemaker for controlling circadian rhythms (reviewed in van Esseveldt *et al.*, 2000). Studies have demonstrated that the fetal mammalian SCN contains D1 receptors through which cocaine can influence entrainment of fetal biological rhythms (Simonik *et al.*, 1994; Viswanathan *et al.*, 1994; Bender *et al.*, 1997). Other studies have shown that disruption of another major component of the mammalian circadian system, the pineal gland, or its secretory product melatonin, results in altered cocaine responses (Uz *et al.*, 2002; Zhdanova and Giorgetti, 2002; Uz *et al.*, 2003).

How might LN_vs modulate cocaine responses?

We have demonstrated that LN_v electrical activity and synaptic output contribute to cocaine–induced behavioral responses. These findings raise a number of questions regarding the interaction of cocaine with these neurons and their output. We propose a simple model in which some or all LN_vs directly increase activity in response to cocaine administration (Figure 9). In this model,

increased activity of these neurons results in increased locomotor responses to cocaine. Interestingly, a recent report has shown that subsets of cultured LN_v neurons can respond to either acetylcholine or dopamine, but never to glutamate, 5–HT, octopamine, or histamine (Wegener *et al.*, 2004). The presence of dopamine receptors on some LN_vs provides a mechanism by which LN_v activity can be directly increased by cocaine administration, as cocaine's primary mechanism of action is to inhibit the reuptake of dopamine by the dopamine transporter. In this model, when the LN_vs are ablated or silenced, one site of cocaine action would be eliminated, thus reducing cocaine's effects (Figure 9B).

An outstanding question is how *Lmo* fits into this model. Our data suggests that in *Lmo* loss of function mutants, the LN_v output induced by cocaine is boosted either by increased activity of the cocaine–sensitive LN_vs (Fig 9), or by recruitment of an increased number of LN_vs that can respond to cocaine. In the Wegener study, only 31% of LN_vs responded to 100μM dopamine (DA) as demonstrated by increases in intracellular calcium levels, suggesting only a subset of LN_vs respond to dopamine. These results provide a plausible mechanism for the increased cocaine responses observed in *Lmo* mutants. Perhaps increases in DA receptor content in cells that normally express the receptor or, alternatively, expression of the DA receptor in LN_v cells that don't normally express them mediate the increased response of these cells. Changes in receptor content would not be unprecedented given LMO's interaction with LIM–HDs. LMO has been shown to be a negative regulator of LIM–HD, and a reduction in LMO leads to increased LIM–HD activity (Milan and Cohen, 1999).

Both increases and decreases LIM–HD function have been shown to affect elements of neural subtype identity including neurotransmitter and receptor expression profiles. For instance, mutations in the *Drosophila* gene *islet* result in loss of dopamine and serotonin synthesis, while ectopic expression can cause ectopic expression of TH (Thor and Thomas, 1997). The graded TH expression phenotype observed in this study are reminiscent of the graded cocaine responses of *Lmo* gain and loss of function mutants. In our model, gain–of–function *Lmo* mutants have decreased DA receptor content and, therefore, decreased cocaine sensitivity. Future work is required to determine whether DA receptor expression in *Lmo* mutants is altered, as is dopaminergic receptor expression in various *C. elegans* LIM-HD mutants (Tsalik *et al.*, 2003). In summary, via an unbiased genetic screen for mutants with altered cocaine sensitivities , we have isolated *Lmo* mutants that reveal a novel function for the *Drosophila* pacemaker cells in modulating cocaine action; furthermore, we show that this novel function is separable from the LN_v role in regulating circadian rhythmic behaviors.

Experimental Procedures

Drosophila culture, strains and genetics

All flies were maintained on standard cornmeal molasses agar at 25°C and 70% humidity under constant light. All lines used for behavioral experiments, unless noted below, were outcrossed (20 females X 20 males) for five generations to a *w1118* stock isogenic for chromosomes II and III. *Bx* alleles were outcrossed either via their dominant wing phenotype or by outcrossing to the outcrossed *EP1306* line, selecting against *EP1306*. Because Pdf^{01} and its background control strain (*w33*) are unmarked, only chromosomes I and II were replaced into the *w1118* background via crosses to balancers in this *w1118* genetic background; additionally, the GAL4 lines *repo*–GAL4 and *MHC82*–GAL4 were also not outcrossed to the *w1118* isogenic strain.

The Rorth EP collection was obtained from G. Rubin (University of California, Berkeley) (Rorth, 1996). The *pipedream* allele was originally isolated in a genetic screen of P[GAL4] insertions for altered response to the locomotor–activating effects of ETOH (Fred Wolf, unpublished data). These P[GAL4] insertions were generated as follows: virgin females carrying a P[GAL4] donor chromosome marked with Cy were mated en masse to males carrying the Δ2–3 transposase at 20°C. 3 to 5 dysgenic males were mated to 5 w, attached–X/Y (w, XX/Y) virgin females at 20°C. Individual males with w^+ eye color were selected and mated to 5 w, XX/Y virgin females to determine whether the insertion was X–linked or autosomal. X–linked P[GAL4] inserts were crossed

to 10 FM6K, y, w, B virgin females to generate a balanced stock. After outcrossing, insertion position was confirmed by inverse PCR (BDGP, unpublished, http://www.fruitfly.org/about/methods/inverse.pcr.html).

The *hdp*^{R590} allele was provided by S. Cohen (EMBL Heidelberg, Germany) (Milan *et al.*, 1998). UAS–*Lmo* lines were provided by C. Zeng (University of Wisconsin, Milwaukee) (Zeng *et al.*, 1998). The *hdp*^{rev83} strain was generated by N. Justice by imprecise excision of the *EP1306* line, screening for the *hdp* phenotype (unpublished data). *Bx* alleles and *pdf* deficiencies (*Df(3R)T1–X* and *Df(3R)T1–P*) were obtained from the *Drosophila* Stock Center (Bloomington, IN). *w33*, *pdf*^{01} and *pdf*–GAL4 were obtained from Paul Taghert (Washington University, St. Louis) (Renn *et al.*, 1999). UAS–*Kir* lines were obtained from S. Sweeney (University of California, San Francisco) (Baines *et al.*, 2001). UAS–*TetTxLC* lines were generated as previously described (Sweeney *et al.*, 1995; Scholz *et al.*, 2000). UAS–*hid* was provided by R. S. Stowers (NASA Ames Research Center) (Zhou *et al.*, 1997). UAS–*tau*GFP and UAS–*lacZ* lines were obtained from Y.–N. Jan (University of California, San Francisco).

Behavioral Assays and statistics

For all behavioral experiments, fifteen male flies were collected under CO_2 anesthesia between 0–2d post eclosion (day 12), and tested 2–3 days later. Flies were equilibrated to room temperature for >1 hr. before being exposed to cocaine. Cocaine exposures were performed as described previously (McClung

and Hirsh,1998 and Bainton *et al.*, 2000), and startle–induced negative geotaxis was assayed in a glass cylinder as described previously (Bainton *et al.*, 2000). A drug effect score was determined as the average (over 5 min) of the number of flies that remained on the bottom expressed as a percentage of the total number of flies. Significance was established for each experiment as described in figure legends. Generally, either student's paired *t*–tests assuming equal variance or one–way ANOVA with *post hoc* Bonferroni planned comparisons or Tukey–Kramer multiple comparisons were performed in GraphPad Prism 4 (GraphPad, San Diego, CA). Error bars in all experiments represent ± SEM. To maintain an experiment–wide error rate of $\alpha = 0.05$, the adjusted error rates were $p = 0.05/n$ for the n subsequent planned pair–wise comparisons in each experiment.

To observe cocaine locomotor activity patterns, 10 flies were exposed to volatilized cocaine for 1 minute, transferred to a 7.5cm X 10cm X 0.5cm acrylic box, and images were captured on an Apple G4 computer using Adobe Premiere at 10 frames/sec for five minutes. Fly locomotion was tracked using the dynamic image analysis system software (DIAS) (Solltech, Oakdale, IA). Movies were subdivided into 30s increments to produce the path traces shown, and the indicated times refer to the time after the end of cocaine exposure. Each genotype was tested at each dose multiple times ($n \geq 3$), and representative data was selected for the figures.

Baseline negative geotaxis was measured by mock exposures of flies to cocaine and subsequent assay in the crackometer as above. All genotypes

tested demonstrated behavioral scores of less than 10% in these mock exposures, with no significant differences between genotypes (n>8).

Screen and selection of controls

The ~400 EP lines were initially screened as hemizygotes crossed to a w, XX/Y tester strain in the crackometer as described above (n = 6–9). These lines distributed normally and 30 lines with phenotypes greater than 1.5 S.D. from the mean were chosen to be outcrossed into our *w1118* background and retested. An additional 70 lines were selected to be outcrossed in order to provide a population distribution from which to select control lines. These 100 outcrossed lines were rescreened for acute responses to cocaine, nicotine, and ETOH as described previously (Bainton *et al.*, 2000; Moore *et al.*, 1998, Wolf *et al.*, 2002). Behavioral responses for this selected set of lines showed a normal distribution for each assay. A group of 5 control EP lines were chosen based on having a normal acute response (< 0.5 S.D. from the mean) to all drugs tested. In all behavioral experiments involving EP lines, at least 3 of these control lines were tested to ensure that the control shown was representative of the EP population. In no case did any of the individual EP control lines show significant differences from each other. The control EP lines for each experiment is noted in the figure legend. As a P[GAL4] insertional control, 10 P[GAL4] lines normal for response to ETOH (tracking and inebriometer) and nicotine were outcrossed and tested for acute responses to cocaine (Adrian Rothenfluh, Douglas Guarnieri, Aylin Rodan, and Fred Wolf, unpublished data). None showed significant differences from

each other and one (line *8.142*) was selected for all experiments involving the *pipedream* allele.

In figures 1 and 3, all lines were crossed to a *w, XX/Y* tester strain to reduce possible effects of recessive autosomal modifiers. In figure 1, *hdp* refers to the hdp^{R590} allele, which previously has been been characterized as a loss of function allele of *Lmo* (Milan *et al.*, 1998). In addition to the genotypes shown, we tested three P element lines in the *Lmo* locus that did not produce cocaine phenotypes: *MS1096*, which lies 20 bp downstream of the first exon encoding the RA transcript, and two additional EP insertions 5' to *EP1383* (*EP0443* and *EP1394*). *MS1096* has been shown to have a very weak wing venation phenotype , but has otherwise has not been phenotypically or molecularly characterized (Milan *et al.*, 1998). *EP1394* has previously been shown to be able to overexpress *Lmo* in the presence of GAL4 (Milan *et al.*, 1998; Zeng *et al.*, 1998). We also tested two other *Bx* alleles (Bx^2 *and* Bx^3), which had similar phenotypes to Bx^1.

Lines used for P[GAL4] rescue

A small–scale screen for GAL4 lines which rescued the *EP1306* allele phenotype was performed focusing on p[GAL4] lines selected for expression in areas revealed by the *pdrm* expression pattern. P[GAL4] driver lines used were: MB line $P[GAL4]^{4.89}$ (unpublished data), AL lines $P[GAL4]^{11.50}$, $P[GAL4]^{7.78}$, $P[GAL4]^{9.22}$ (unpublished data), glial *repo*–GAL4 (Xiong *et al.*, 1994), and LNvs *pdf*–GAL4 (Renn *et al.*, 1999). Ubiquitous neural expression using elavGAL4

resulted in lethality. Negative controls c290 (no CNS expression) and *MHC82*–GAL4 (muscle driver line) also did not rescue cocaine–sensitivity (Davis *et al.*, 1998; Rodan *et al.*, 2002).

Histology

Expression pattern of enhancer trap GAL4 lines was examined by crossing to reporter lines UAS–GFPT2 and UAS–mCD8GFP. All adult and larval dissections were performed in 1X PBS, and fixed in 4% Formaldehyde in PEM for 40–40 minutes, washed in 1XPBS, then dehydrated in 50% Glycerol/PBS for 1 hour. Tissue was mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and flourescence was imaged using a Zeiss Axioskop 2 microscope or using an Biorad confocal microscope with BioRad Lasersharp 2000 software. Multiple specimens were observed for each genotype. Antibody staining was performed on CNS preparations that were dissected, fixed, and washed as above. Specimens were incubated in 1:2000 dilution of anti–PDF in 1XPBT, and with a Texas Red–coupled goat anti–guinea pig secondary antibody, diluted 1:200 (Jackson Labs, West Grove, PA).

Real–Time qPCR

2-4 d old flies were collected and frozen immediately at –80 °C. Headswere removed from bodies by vortexing and separated in a sieve. RNA was extracted from the flies by homogenizing the flies in hot phenol and NTES. Complementary DNA (cDNA) was prepared from using TaqMan Reverse

Transcription Reagents (Applied Biosystems) according to the manufacturer's specifications, with the addition of random sequence hexamers to 2.5μM. cDNA was analyzed by quantitative, real-time PCR using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The following probes and primers were designed using ABI PrimerExpress software: *Lmo*RA–For, GAAGAGAAACAACAGCAGCAACA; *LmoRA*–Rev, ATTTGCATATTTCGCACTTGTTTAGCT; *Lmo*RA–Probe, CTGCTGCCGTTGCTG; *pdf*–For, CGGATGAGGAGCGCTATGTG; *pdf*–Rev, CACGCCCACGTTGTTGAAC; *pdf*–Probe, TCGAGGAGATCCCGATTGT; *rp49* probe and primers (CT 6405) were obtained as standard primers from Applied Biosystems. TaqMan PCR reactions consisted of 50 ng of cDNA, 0.9 μM each diagnostic primer, 0.25 μM diagnostic probe, and 1x final of TaqMan Universal PCR Mastermix (Applied Biosystems) in a reaction volume of 25μl. DNA amplifications were carried out in 96-well plates (Applied Biosystem, Branchburg, New Jersey, USA). The TaqMan PCR conditions were used as described in TaqMan guidelines, with a 10-min preincubation at 95°C required for activation of AmpliTaq Gold DNA polymerase, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was analyzed in triplicate. As negative controls, we used both a no-template control for genomic DNA contamination and DNAse-treated non-transcribed total RNA as a no-amplification control. No significant amplification was observed in these samples. *rp49* transcript levels were used as an endogenous normalization control for RNA samples, and relative mRNA abundance was calculated using the comparative delta-Ct method. Reference mRNA is noted in figure legends. To determine statistical significance, two-tailed

t–test assuming equal variance were performed with a *p* value of < 0.05 taken as statistically significant. qPCR analysis was performed on at least two independent RNA preparations, and showed similar results.

Circadian Behavioral Analysis

Locomotor activity of individual flies was measured using the TriKinetics infrared beam-crossing system recording total crosses in 30 min bins. Raw activity histograms were analyzed for circadian rhythms using Actimetrics Clocklab software. Chi-square periodograms were constructed according to Sokalove and Bushell (Sokolove and Bushell, 1978), and significant circadian rhythmicity was defined as presence of a peak in periodogram power that extends above the .01 chi–prosquare significance line. Since this line is equal to a power of 75 at a period of 24 hr, flies with no periodogram peak crossing the significance line were assigned a circadian power of 75. This would tend to overestimate the circadian power of these flies, and thus is conservative with regard to assessing statistical differences in power between genotypes exhibiting frequent arrhythmicity and those that are predominately rhythmic.

For the circadian cocaine sensitivity experiment, flies wereset up and raised in LD, collected under CO_2 anesthesia during the light phase at 10–12d, and placed back in LD for 2–3d. At the indicated ZT times, flies were tested in the crackometer, under lights, within 5 minutes of being removed from LD conditions.

References

Abarca, C., U. Albrecht and R. Spanagel (2002). Cocaine sensitization and reward are under the influence of circadian genes and rhythm. Proc Natl Acad Sci U S A *99*(13): 9026-30.

Andretic, R., S. Chaney and J. Hirsh (1999). Requirement of circadian genes for cocaine sensitization in Drosophila. Science *285*(5430): 1066-8.

Baines, R. A., J. P. Uhler, A. Thompson, S. T. Sweeney and M. Bate (2001). Altered electrical properties in Drosophila neurons developing without synaptic transmission. J Neurosci *21*(5): 1523-31.

Bainton, R. J., L. T. Tsai, C. M. Singh, M. S. Moore, W. S. Neckameyer and U. Heberlein (2000). Dopamine modulates acute responses to cocaine, nicotine and ethanol in Drosophila. Curr Biol *10*(4): 187-94.

Bender, M., J. Drago and S. A. Rivkees (1997). D1 receptors mediate dopamine action in the fetal suprachiasmatic nuclei: studies of mice with targeted deletion of the D1 dopamine receptor gene. Brain Res Mol Brain Res *49*(1-2): 271-7.

Blanchardon, E., B. Grima, A. Klarsfeld, E. Chelot, P. E. Hardin, T. Preat and F. Rouyer (2001). Defining the role of Drosophila lateral neurons in the control of circadian rhythms in motor activity and eclosion by targeted genetic ablation and PERIOD protein overexpression. Eur J Neurosci *13*(5): 871-88.

Blau, J. and M. W. Young (1999). Cycling vrille expression is required for a functional Drosophila clock. Cell *99*(6): 661-71.

Cirelli, C. and G. Tononi (2000). Gene expression in the brain across the sleep-waking cycle. Brain Res *885*(2): 303-21.

Davis, G. W., A. DiAntonio, S. A. Petersen and C. S. Goodman (1998). Postsynaptic PKA controls quantal size and reveals a retrograde signal that regulates presynaptic transmitter release in Drosophila. Neuron *20*(2): 305-15.

Gawin, F. H. (1991). Cocaine addiction: psychology and neurophysiology. Science *251*(5001): 1580-6.

Helfrich-Forster, C. (1997). Development of pigment-dispersing hormone-immunoreactive neurons in the nervous system of Drosophila melanogaster. J Comp Neurol *380*(3): 335-54.

Helfrich-Forster, C. (2003). The neuroarchitecture of the circadian clock in the brain of Drosophila melanogaster. Microsc Res Tech *62*(2): 94-102.

Helfrich-Forster, C., M. Stengl and U. Homberg (1998). Organization of the circadian system in insects. Chronobiol Int *15*(6): 567-94.

Hinks, G. L., B. Shah, S. J. French, L. S. Campos, K. Staley, J. Hughes and M. V. Sofroniew (1997). Expression of LIM protein genes Lmo1, Lmo2, and Lmo3 in adult mouse hippocampus and other forebrain regions: differential regulation by seizure activity. J Neurosci *17*(14): 5549-59.

Hirsh, J. (2001). Time flies like an arrow. Fruit flies like crack? Pharmacogenomics J *1*(2): 97-100.

Hobert, O. and H. Westphal (2000). Functions of LIM-homeobox genes. Trends Genet *16*(2): 75-83.

Kaneko, M., C. Helfrich-Forster and J. C. Hall (1997). Spatial and temporal expression of the period and timeless genes in the developing nervous system of

Drosophila: newly identified pacemaker candidates and novel features of clock gene product cycling. J Neurosci *17*(17): 6745-60.

Kaneko, M., J. H. Park, Y. Cheng, P. E. Hardin and J. C. Hall (2000). Disruption of synaptic transmission or clock-gene-product oscillations in circadian pacemaker cells of Drosophila cause abnormal behavioral rhythms. J Neurobiol *43*(3): 207-33.

Kuhar, M. J., M. C. Ritz and J. W. Boja (1991). The dopamine hypothesis of the reinforcing properties of cocaine. Trends Neurosci *14*(7): 299-302.

Laakso, A., A. R. Mohn, R. R. Gainetdinov and M. G. Caron (2002). Experimental genetic approaches to addiction. Neuron *36*(2): 213-28.

Li, H., S. Chaney, I. J. Roberts, M. Forte and J. Hirsh (2000). Ectopic G-protein expression in dopamine and serotonin neurons blocks cocaine sensitization in Drosophila melanogaster. Curr Biol *10*(4): 211-4.

Mazzoni, E., C. Desplan and J. Blau (2004). Circadian pacemaker cells transmit and modulate visual
information to control a rapid behavioral response. submitted.

McClung, C. and J. Hirsh (1998). Stereotypic behavioral responses to free-base cocaine and the development of behavioral sensitization in Drosophila. Curr Biol *8*(2): 109-12.

Milan, M. and S. M. Cohen (1999). Regulation of LIM homeodomain activity in vivo: a tetramer of dLDB and apterous confers activity and capacity for regulation by dLMO. Mol Cell *4*(2): 267-73.

Milan, M. and S. M. Cohen (2000). Temporal regulation of apterous activity during development of the Drosophila wing. Development *127*(14): 3069-78.

Milan, M., F. J. Diaz-Benjumea and S. M. Cohen (1998). Beadex encodes an LMO protein that regulates Apterous LIM-homeodomain activity in Drosophila wing development: a model for LMO oncogene function. Genes Dev *12*(18): 2912-20.

Morgan, T. H., C. C. Bridges and A. H. Sturtevant (1925). *The Genetics of Drosophila*. Gravenhage, The Netherlands.

Nitabach, M. N., J. Blau and T. C. Holmes (2002). Electrical silencing of Drosophila pacemaker neurons stops the free-running circadian clock. Cell *109*(4): 485-95.

Panda, S., M. P. Antoch, B. H. Miller, A. I. Su, A. B. Schook, M. Straume, P. G. Schultz, S. A. Kay, J. S. Takahashi and J. B. Hogenesch (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. Cell *109*(3): 307-20.

Park, J. H., C. Helfrich-Forster, G. Lee, L. Liu, M. Rosbash and J. C. Hall (2000a). Differential regulation of circadian pacemaker output by separate clock genes in Drosophila. Proc Natl Acad Sci U S A *97*(7): 3608-13.

Park, S. K., S. A. Sedore, C. Cronmiller and J. Hirsh (2000b). Type II cAMP-dependent protein kinase-deficient Drosophila are viable but show developmental, circadian, and drug response phenotypes. J Biol Chem *275*(27): 20588-96.

Peng, Y., D. Stoleru, J. D. Levine, J. C. Hall and M. Rosbash (2003). Drosophila free-running rhythms require intercellular communication. PLoS Biol *1*(1): E13.

Renn, S. C., J. H. Park, M. Rosbash, J. C. Hall and P. H. Taghert (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. Cell *99*(7): 791-802.

Retaux, S. and I. Bachy (2002). A short history of LIM domains (1993-2002): from protein interaction to degradation. Mol Neurobiol *26*(2-3): 269-81.

Robinson, T. E. and K. C. Berridge (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain Res Brain Res Rev *18*(3): 247-91.

Rodan, A. R., J. A. Kiger, Jr. and U. Heberlein (2002). Functional dissection of neuroanatomical loci regulating ethanol sensitivity in Drosophila. J Neurosci *22*(21): 9490-501.

Rorth, P. (1996). A modular misexpression screen in Drosophila detecting tissue-specific phenotypes. Proc Natl Acad Sci U S A *93*(22): 12418-22.

Rothenfluh, A. and U. Heberlein (2002). Drugs, flies, and videotape: the effects of ethanol and cocaine on Drosophila locomotion. Curr Opin Neurobiol *12*(6): 639-45.

Schenk, S. and B. Partridge (1997). Sensitization and tolerance in psychostimulant self-administration. Pharmacol Biochem Behav *57*(3): 543-50.

Scholz, H., J. Ramond, C. M. Singh and U. Heberlein (2000). Functional ethanol tolerance in Drosophila. Neuron *28*(1): 261-71.

Shafer, O. T., M. Rosbash and J. W. Truman (2002). Sequential nuclear accumulation of the clock proteins period and timeless in the pacemaker neurons of Drosophila melanogaster. J Neurosci *22*(14): 5946-54.

Shi, J., W. Cai, X. Chen, K. Ying, K. Zhang and Y. Xie (2001). Identification of dopamine responsive mRNAs in glial cells by suppression subtractive hybridization. Brain Res *910*(1-2): 29-37.

Shirasaki, R. and S. L. Pfaff (2002). Transcriptional codes and the control of neuronal identity. Annu Rev Neurosci *25*: 251-81.

Shoresh, M., S. Orgad, O. Shmueli, R. Werczberger, D. Gelbaum, S. Abiri and D. Segal (1998). Overexpression Beadex mutations and loss-of-function heldup-a mutations in Drosophila affect the 3' regulatory and coding components, respectively, of the Dlmo gene. Genetics *150*(1): 283-99.

Simonik, D. K., S. R. Robinson and W. P. Smotherman (1994). Cocaine alters cyclic motor activity in the fetal rat. Dev Psychobiol *27*(8): 489-501.

Sokolove, P. G. and W. N. Bushell (1978). The chi square periodogram: its utility for analysis of circadian rhythms. J Theor Biol *72*(1): 131-60.

Sora, I., C. Wichems, N. Takahashi, X. F. Li, Z. Zeng, R. Revay, K. P. Lesch, D. L. Murphy and G. R. Uhl (1998). Cocaine reward models: conditioned place preference can be established in dopamine- and in serotonin-transporter knockout mice. Proc Natl Acad Sci U S A *95*(13): 7699-704.

Stanewsky, R. (2003). Genetic analysis of the circadian system in Drosophila melanogaster and mammals. J Neurobiol *54*(1): 111-47.

Stocker, R. F. (1994). The organization of the chemosensory system in Drosophila melanogaster: a review. Cell Tissue Res *275*(1): 3-26.

Sweeney, S. T., K. Broadie, J. Keane, H. Niemann and C. J. O'Kane (1995). Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects. Neuron *14*(2): 341-51.

Thor, S. and J. B. Thomas (1997). The Drosophila islet gene governs axon pathfinding and neurotransmitter identity. Neuron *18*(3): 397-409.

Tsalik, E. L., T. Niacaris, A. S. Wenick, K. Pau, L. Avery and O. Hobert (2003). LIM homeobox gene-dependent expression of biogenic amine receptors in restricted regions of the C. elegans nervous system. Dev Biol *263*(1): 81-102.

Uhl, G. R., F. S. Hall and I. Sora (2002). Cocaine, reward, movement and monoamine transporters. Mol Psychiatry *7*(1): 21-6.

Uz, T., M. Akhisaroglu, R. Ahmed and H. Manev (2003). The pineal gland is critical for circadian Period1 expression in the striatum and for circadian cocaine sensitization in mice. Neuropsychopharmacology *28*(12): 2117-23.

Uz, T., J. I. Javaid and H. Manev (2002). Circadian differences in behavioral sensitization to cocaine: putative role of arylalkylamine N-acetyltransferase. Life Sci *70*(25): 3069-75.

van Esseveldt, K. E., M. N. Lehman and G. J. Boer (2000). The suprachiasmatic nucleus and the circadian time-keeping system revisited. Brain Res Brain Res Rev *33*(1): 34-77.

Viswanathan, N., D. R. Weaver, S. M. Reppert and F. C. Davis (1994). Entrainment of the fetal hamster circadian pacemaker by prenatal injections of the dopamine agonist SKF 38393. J Neurosci *14*(9): 5393-8.

Wegener, C., Y. Hamasaka and D. R. Nassel (2004). Acetylcholine increases intracellular Ca2+ via nicotinic receptors in cultured PDF-containing clock neurons of Drosophila. J Neurophysiol *91*(2): 912-23.

Weihe, U., M. Milan and S. M. Cohen (2001). Regulation of Apterous activity in Drosophila wing development. Development *128*(22): 4615-22.

Wise, R. A. and M. A. Bozarth (1985). Brain mechanisms of drug reward and euphoria. Psychiatr Med *3*(4): 445-60.

Wolf, F. W. and U. Heberlein (2003). Invertebrate models of drug abuse. J Neurobiol *54*(1): 161-78.

Wolf, F. W., A. R. Rodan, L. T. Tsai and U. Heberlein (2002). High-resolution analysis of ethanol-induced locomotor stimulation in Drosophila. J Neurosci *22*(24): 11035-44.

Xiong, W. C., H. Okano, N. H. Patel, J. A. Blendy and C. Montell (1994). repo encodes a glial-specific homeo domain protein required in the Drosophila nervous system. Genes Dev *8*(8): 981-94.

Yang, Z. and A. Sehgal (2001). Role of molecular oscillations in generating behavioral rhythms in Drosophila. Neuron *29*(2): 453-67.

Zars, T. (2000). Behavioral functions of the insect mushroom bodies. Curr Opin Neurobiol *10*(6): 790-5.

Zeng, C., N. J. Justice, S. Abdelilah, Y. M. Chan, L. Y. Jan and Y. N. Jan (1998). The Drosophila LIM-only gene, dLMO, is mutated in Beadex alleles and might represent an evolutionarily conserved function in appendage development. Proc Natl Acad Sci U S A *95*(18): 10637-42.

Zhdanova, I. V. and M. Giorgetti (2002). Melatonin alters behavior and cAMP levels in nucleus accumbens induced by cocaine treatment. Brain Res *956*(2): 323-31.

Zhou, L., A. Schnitzler, J. Agapite, L. M. Schwartz, H. Steller and J. R. Nambu (1997). Cooperative functions of the reaper and head involution defective genes in the programmed cell death of Drosophila central nervous system midline cells. Proc Natl Acad Sci U S A *94*(10): 5131-6.

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Figure Legends

Figure 1: *Lmo* loss–of–function mutants show increased sensitivity to cocaine

(A) Cocaine phenotypes of various *Lmo* mutants. Indicated genotypes were assayed at a 150 µg dose for cocaine sensitivity in the crackometer as described in the Experimental Procedures. Compared to their control, Ctl-1 (*EP1631*), *EP1383* ($p = 0.016$, $n = 20$) and *EP1306* ($p = 3 \times 10^{-5}$; $n = 20$) showed significantly increased sensitivity to cocaine. Similarly, compared to their respective controls, Ctl-2 (*8.142*) and Ctl-3 (*w1118*), *pdrm* ($p = 2 \times 10^{-6}$, $n = 20$) and *hdp* ($p = 0.0003$, $n = 20$) were significantly more sensitivite to cocaine. Asterisks denote significant differences from control (student's paired *t*–test assuming equal variance). **(B)** Cocaine–sensitive *Lmo* mutants demonstrated increased cocaine responses across the dose–response curve. *EP1306* (filled squares) and *pdrm* (filled circles) and their controls Ctl–1 (open squares) and Ctl–2 (open circles) were exposed to the indicated doses of cocaine. At each dose, the responses for *EP1306* and *pdrm* were significantly higher than their controls ($p < 5 \times 10^{-5}$, $n = 16$–20). **(C)** *Lmo* mutant *EP1306* showed alterations in cocaine–induced locomotor patterns of activity. Flies were exposed to 0, 75, or 100µg of cocaine, as indicated, for one minute. Representative traces ($n \geq 4$) shown are 30s of recorded activity of ~10 flies starting 1 minute after end of the cocaine exposure. Top panels show response of control flies to indicated amounts of cocaine; bottom panels show activity of *EP1306* flies after cocaine

administration. Compared to control flies, *EP1306* shows increased circling at 75µg (middle panels) and increased akinesia at 100µg (right panels).

Figure 2: *Lmo* gain–of–function *Bx* alleles show reduced sensitivity to cocaine

(A) Cocaine phenotypes of *Bx* mutants. The *Bx* alleles, which are gain–of–function alleles of *Lmo*, showed significant reductions in sensitivity to cocaine compared to Ctl (w1118) ($p = 0.0003$ for Bx^1 and $p = 5 \times 10^{-9}$ for Bx^J; $n = 12$). Asterisks denote significant differences from control (student's paired *t*–test assuming equal variance). **(B)** The cocaine–resistant Bx^J allele (filled circles) showed reduced sensitivity compared to Ctl (open circles) across the dose–response curve ($p < 2 \times 10^{-5}$, $n = 16$–20 for all doses except for 250µg, where $p = 0.0015$, $n = 8$. **(C)** Bx^J showed alterations in cocaine–induced locomotor patterns of activity. Flies were exposed to 0, 100, or 125µg of cocaine, as indicated, for one minute. Representative traces ($n \geq 3$) shown are 30s of recorded activity of ~10 flies starting 30s (100µg) or 1 minute (mock and 125 µg) after end of the cocaine exposure. Top panels show response of control flies to indicated amounts of cocaine; bottom panels show activity of Bx^J flies after cocaine administration. Bx^J flies exposed to cocaine show a decrease in stereotyped circling behaviors at 100µg (middle panels), and a reduced likelihood of akinesia at 125µg (right panels).

Figure 3: *Lmo* gene locus and specific loss of *Lmo*–RA transcript in heads of *EP1306* mutants

(A) A genomic map of the *Lmo* locus, displaying the P element insertional positions of three cocaine–sensitive mutants and the approximate positions of *Bx* insertions. Three different first exons can be utilized, forming the basis for three alternative transcripts. Exon 1A is separated from the alternative exon 1 start sites by a large ~30kb intron. *EP1306*, *EP1383*, and *pdrm* insert 25, 73 and 91 bp respectively, upstream of the exon 1A transcriptional start site. Arrows within the EP elements refer to the orientation of the insertion and the expected direction of inducible expression off UAS sites contained within the EP element. RB transcript has a translational start that adds 70AA to the N–terminus of the protein encoded by the RA and RC transcripts. *Bx* alleles are insertions of natural transposons into the 3'UTR of the *Lmo* gene that have been shown to stabilize *Lmo* transcript (Shoresh *et al.*, 1998). **(B)** Expression of the *Lmo* RA transcript is enriched in *Drosophila* heads, and is reduced specifically in the *EP1306* mutant. RNA was isolated from heads and bodies as described in the After cDNA synthesis, real–time quantitative PCR was performed using primers specific to the RA transcript of *Lmo* in addition to primers to a reference transcript, the ribosomal protein *rp49*, as detailed in the Experimental Procedures. Measurements were normalized by the *Lmo*(RA) – *rp49* average value for all samples, and relative mRNA abundance was calculated using the comparative delta-Ct method. Relative abundance is expressed as –fold increase over CTL-1 body mRNA. In Ctl (*EP1631*) flies, there is a ~4 fold

enrichment of the RA transcript in the head. *EP1306* flies have a 2.5 fold reduction in RA transcript levels in the head compared to Ctl. No detectable amplification was seen in RNAse–treated controls (data not shown). Asterisk denotes significant differences from control (student's paired *t*–test assuming equal variance; $p = 9 \times 10^{-5}$, $n = 3$). Error bars represent standard error of the mean.

Figure 4: *pipedream*'s GAL4 expression is sufficient to drive *Lmo* transgene–mediated rescue of cocaine sensitivity

(A) *pipedream*'s GAL4 expression pattern. UAS–*GFP* reveals GAL4 expression pattern of the *pipedream* enhancer trap insertion in a representative brain ($n > 5$). Prominent GFP fluorescence can be seen in all mushroom body (MB) lobes and mushroom body calyces. Antennal Lobe staining (AL) probably represents local interneurons rather than projection neurons, as no axonal projections from the AL to the MB or lateral horn can be visualized. The large cell bodies of the peptidergic ventral lateral neurons and neurons of the Pars Intercerebralis (PI) are also easily visualized. **(B)** *Lmo* expression restores wild–type cocaine responses. Expression of either of two *Lmo* transgenes was driven by *pipedream* or a control GAL4 line (*8.142*). As a control for non–specific effects of transgene overexpression, UAS–*GFP* and UAS–*lacZ* transgenes were also driven by *pipedream*. Specific expression of *Lmo* restored wild–type cocaine responses. One–way ANOVA revealed a significant effect of genotype in the UAS–*lacZ* ($F = 17.4; p < 5 \times 10^{-6}$), UAS–*GFP* ($F = 19.47$; $p < 1 \times 10^{-6}$), or no

transgene groups ($F = 4.1$; $p < 1 \times 10^{-6}$), but not in either UAS–*Lmo* transgene groups ($F = 1.58$;$p = 0.22$ and $F = 1.21$;$p = 0.31$). For the UAS–*GFP* and UAS–*lacZ* transgenes, *post hoc* pair–wise planned comparisons, with the critical p value adjusted to $\alpha = 0.0167$, showed significant differences between *pipedream* and both UAS–transgene alone or Ctl GAL4 line + UAS–transgene groups ($p < 0.002$). Pair–wise comparisons between *pipedream* and either no GAL4 or Ctl GAL4 in the presence of UAS–Lmo^1 ($p = .99$, $p = .14$) or UAS–Lmo^2 ($p = .09$, $p = 0.99$) showed no significant differences, indicating full rescue of *pipedream* cocaine sensitivity. For all genotypes, $n = 16 – 20$.

Figure 5: *Lmo* expression in PDF neurons alters cocaine responses

(A) *pdf*–GAL4–driven expression of *Lmo* using the *EP1306* element rescues the *EP1306* insertional phenotype. *Lmo* mutants *EP1306* and *pipedream*, as well as a control (*EP1413*), were tested with and without *pdf*–GAL4. One–way ANOVAs with *post hoc* planned comparisons in the – *pdf*–GAL4 group confirmed both *EP1306* ($p = 0.001$) and *pipedream* ($p = 0.003$) had significantly increased sensitivity to cocaine, as shown previously (see Fig. 1A)($n = 15–26$). In the + *pdf*–GAL4 group, one–way ANOVAs with *post hoc* planned comparisons showed a significant difference between the *pipedream* genotype and control genotypes (Ctl + *pdf*–GAL4 and *pdf*–GAL4 alone)($p < 0.003$, $n = 24–27$), while *EP1306* now showed no significant difference ($p = 0.026$, $n = 27–36$). For the – *pdf*–GAL4 comparisons, the critical p value was adjusted to $\alpha = 0.025$, and for the +

pdf–GAL4 comparisons, $\alpha = 0.0125$ for the number of planned comparisons. Furthermore, within genotype comparisons (+/– *pdf*–GAL4) using *t*–tests indicate a significant difference only in the *EP1306* group ($p = 0.002$). Asterisks denote significant differences between –*pdf*–GAL4 and +*pdf*–GAL4 phenotypes. **(B)** Flies overexpressing *Lmo* in PDF cells show decreased sensitivity to cocaine. Flies carrying both *pdf*–GAL4 and either of two UAS–*Lmo* transgenes were compared to flies carrying either UAS–*Lmo* or *pdf*–GAL4 alone. One way ANOVA revealed a significant effect of genotype for both UAS–*Lmo* transgene groups. Post-test planned comparisons, with the critical *p* value adjusted to $\alpha = 0.025$, showed significant differences between the *pdf*–GAL4 + UAS–*Lmo* groups and either pdf–GAL4 ($p < 0.02$, $n = 16$) or UAS–*Lmo* alone ($p < 0.005$, $n = 16$). Asterisks denote significant differences. **(C)** Confocal images demonstrate overlap of *pipedream* and PDF expression in the LN_vs. In the left panel, UAS–mCD8GFP reveals *pipedream* expression pattern (green) in the adult brain. α–PAP staining reveals the PDF–expressing LN_vs. Right panels are close–ups of the cell bodies of the LN_vs. The top panel shows α–PAP staining (magenta); the middle panel shows *pipedream* expression (green); and the bottom panel shows the merged images, with overlap in white.

Figure 6: Wild–type *Lmo* is required for robust circadian rhythms of locomotor activity

Locomotor activity of control (*EP0443*) and *Lmo* hypomorphic mutant (*EP1383* and *EP1306*) flies was carried out in either DD or LD as previously described (Nitabach *et al.*, 2002). **(A)** Representative actograms of control and the stronger

Lmo mutant (*EP1306*) in DD. Control flies show robust circadian rhythms with clear distinctions between activity during the subjective day and inactivity during the subjective night. The pattern of activity in *EP1306* flies was more stochastic. **(B)** Quantitation of the power of the rhythm (see Experimental Procedures), which gives an indication of the strength of the activity rhythm in LD and DD for Ctl, *EP1383*, and *EP1306*. The average power decreases with the strength of the *Lmo* allele in both LD and DD. The x-axis has higher values than in LD because LD rhythms are reinforced by the light transitions and are consequently more robust than rhythms in DD (n = 29–32). Error bars show standard error of the mean. One-way ANOVA revealed a significant difference between EP lines in DD ($p<0.0001$). Post-hoc pairwise comparisons using Tukey's HSD test and Mann-Whitney *U* test revealed significant differences between each pairwise comparison of EP lines ($p<0.01$). Number of flies analyzed: Ctl: 31; *EP1383*: 32; *EP1306*: 29. For LD experiment, one-way ANOVA revealed a significant difference between EP lines ($p<0.0001$). Post-hoc pairwise comparisons using Tukey's HSD test and Mann-Whitney *U* test revealed that *EP1306* flies had significantly less robust rhythms than *EP1383* and Ctl ($p<0.01$). Number of flies analyzed: Ctl: 13; *EP1383*: 15; *EP1306*: 13.Overall activity levels in DD (counts / minute) are not significantly different between any of the three genotypes (Ctl: 0.37 $\pm$ 0.02; *EP1383*: 0.28 $\pm$ 0.05; *EP1306*: 0.42 $\pm$ 0.02). Although 14/29 *EP1306* flies were considered arrhythmic by ClockLab software and either arrhythmic or weakly rhythmic by visual inspection, the average period of the 15 flies with a detectable rhythm was 23.5 hours $\pm$ 0.4 which lies in the wild–type

range for circadian behavior and was similar to *EP1383* (23.6 ± 0.4) and Ctl (23.9 ± 0.2). There was no significant difference in the overall levels of activity per day between Ctl and EP1383 or EP1306 (Mann Whitney *U* Test, data not shown). **(C)** Average daily activity in LD cycles for Ctl, *EP1383*, and *EP1306*. Ctl flies show characteristically low levels of activity during the afternoon siesta while *EP1383* flies show higher activity during the siesta and an earlier anticipation of the lights–off transition. *EP1306* flies show even higher activity during the siesta and reduced activity during LD transitions.

Figure 7: Cocaine responses are not a circadian output and *pdf* mutants show wild–type cocaine sensitivity

(A) Cocaine responses do not vary with the circadian clock. Flies were raised under LD conditions and assayed for cocaine phenotypes in the crackometer at the indicated zeitberger times. One–way ANOVA revealed no significant effect of time of day ($F = 0.53$; $p = 0.82$, $n = 32$). **(B)** Flies lacking the LN neuropeptide PDF (pdf^{01}) display wild–type cocaine sensitivity. pdf^{01} mutants, as well as pdf^{01} hemizygotes (pdf^{01}/Df) flies, were tested for cocaine phenotypes in the crackometer and showed wild–type responses. Individual pair–wise comparisons using student's *t*–tests revealed no significant differences between control (+/+ and +/*Df*) and *pdf* mutant genotypes ($p = 0.69$, $n = 20$; $p = 0.97$, $n = 8$)

Figure 8: Silencing or ablating PDF cells induces resistance to cocaine

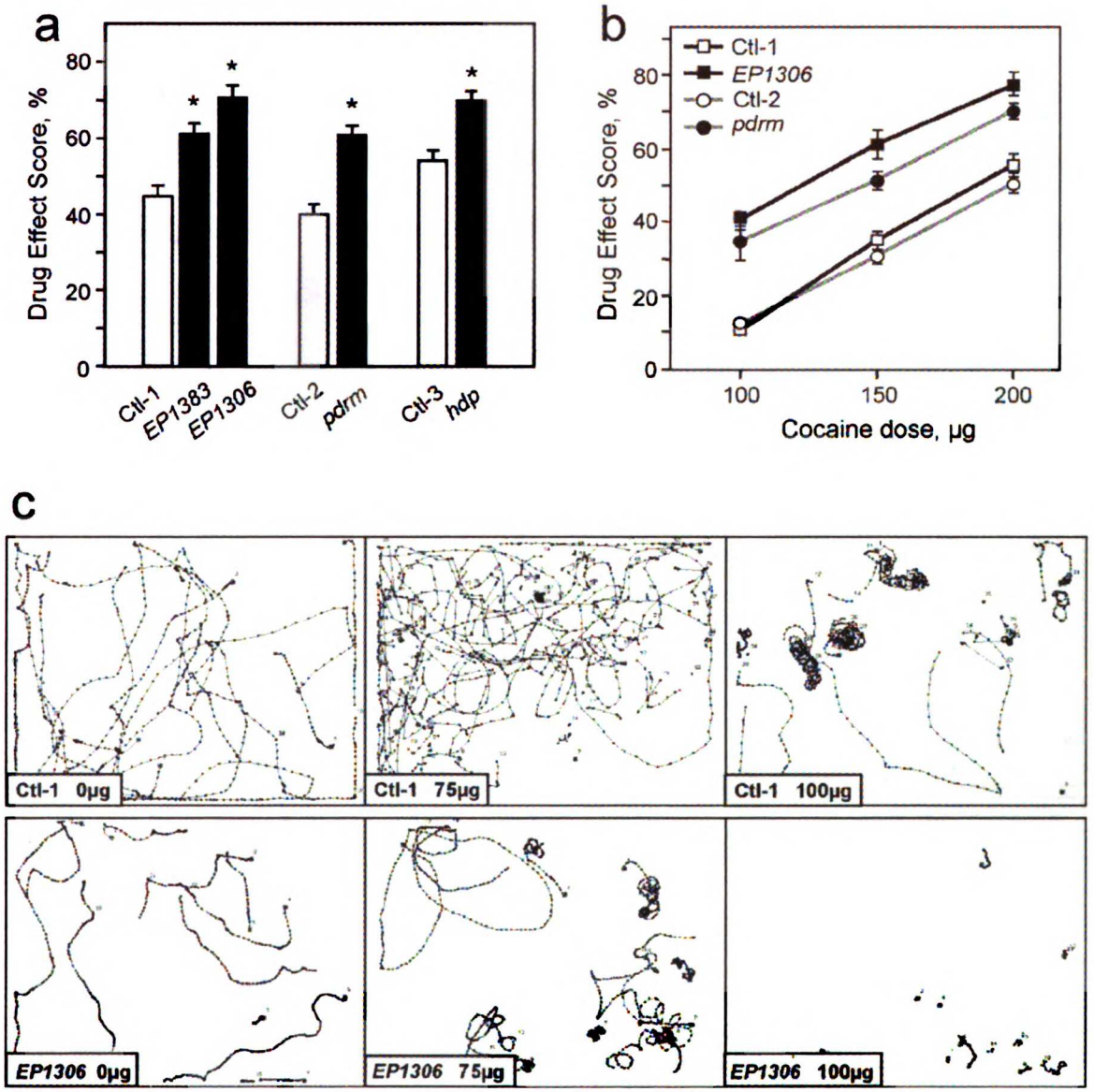
Ablation of PDF cells with *pdf*–GAL4 and UAS-*hid* reduced sensitivity to cocaine compared to parental lines (*pdf*–GAL4 alone and UAS–*hid* alone). Electrical (UAS–*Kir2.1*^{8} or UAS–*Kir2.1*^{7}) or synaptic (UAS–*TeTx*) silencing of PDF cells with *pdf*–GAL4 phenocopied PDF cell ablations. One way ANOVAs with *post hoc* planned comparisons reveal a significant effect of genotype for UAS–*hid* (p < ,0.003 n = 20), UAS–*Kir2.1*^{7} (p < 0.008, n = 32), UAS–*Kir2.1*^{8} (p < 0.002, n = 28), and UAS–*TeTx* (p < 0.00003, n = 28) groups, but not for an inactive tetanus toxin control (*TeTx*^{in}) (p > 0.045 , n = 28). Critical p value adjusted to α = 0.025. Asterisks denote significant differences in both planned comparisons (*pdf*–GAL4 alone and UAS–transgene alone versus *pdf*–GAL4 + UAS–transgene). Variations in *pdf*–GAL4 alone phenotype for each transgene set reflect testing the various transgenes on different days.

Figure 9: Electrical activity and synaptic output of LN_vs increase cocaine sensitivity

Simple schematic models of *Lmo* and LN_v function in various experimental states. In wild type, LN_vs modulate locomotor responses via electrical activity and synaptic transmission. We propose a model in which cocaine acts to directly increase LN_v activity. Upon cocaine administration, synaptic dopamine concentrations are increased (via cocaine's inhibiton of the plasma membrane dopamine transporter). Activation of dopamine receptors on the LN_v (dark arrowheads) stimulates electrical activity and subsequent synaptic output. This

activity increases the behavioral response of the fly to cocaine. LN_v ablations eliminate LN_v contribution to the cocaine response, reducing cocaine sensitivity. In our nodel, *Lmo* loss–of–function mutants (Lmo^{LOF}), which have increased cocaine sensitivity, have increased activity/output during the cocaine response. This increased activity may be mediated by increases in dopamine receptor content on the LN_v or by recruitment of other LN_vs that normally do not express dopamine receptors into the cocaine response (see text for details). Lmo^{GOF} mutants have reduced LN_v output and reduced cocaine sensitivity. This could also result from a reduction in dopamine receptor density.

Figure 1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Ctl-1
EP1383
EP1306
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pdrm
Ctl-3
hdp
b
Ctl-1
EP1306
Ctl-2
pdrm
Drug Effect Score, %
100
150
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Cocaine dose, µg
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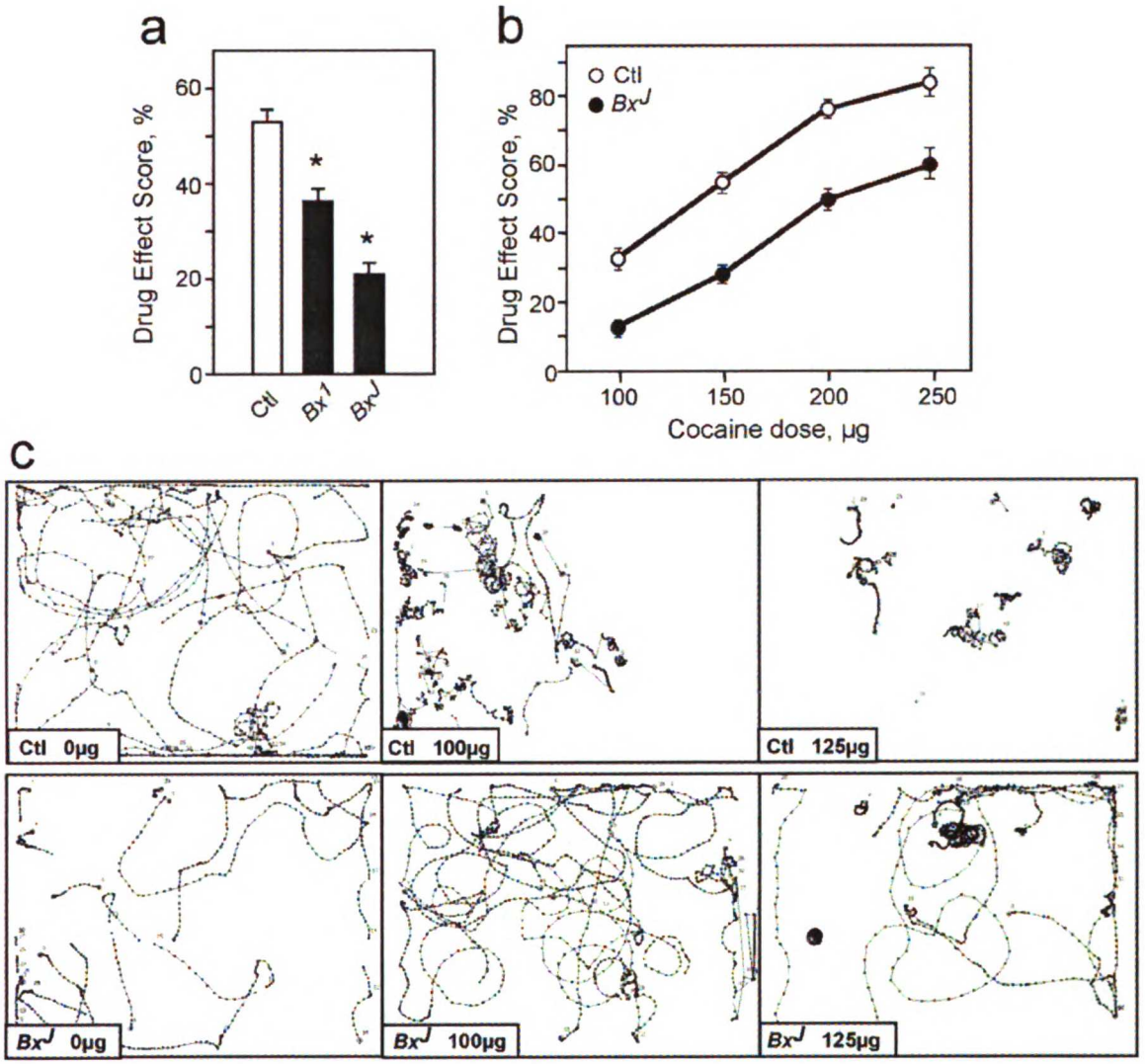
Figure 2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*
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Ctl
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b
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60
40
20
0
100
150
200
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Cocaine dose, µg
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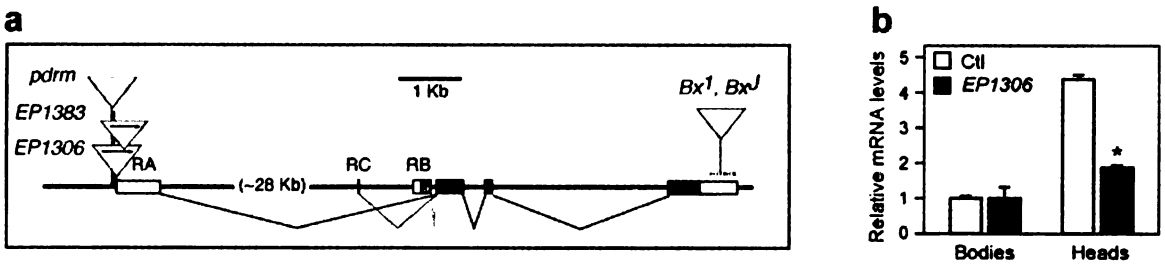
BxJ 100µg
BxJ 125µg

Figure 3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pdrm
EP1383
EP1306
RA
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(~28 Kb)
RC
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Bx1, BxJ
b
Ctl
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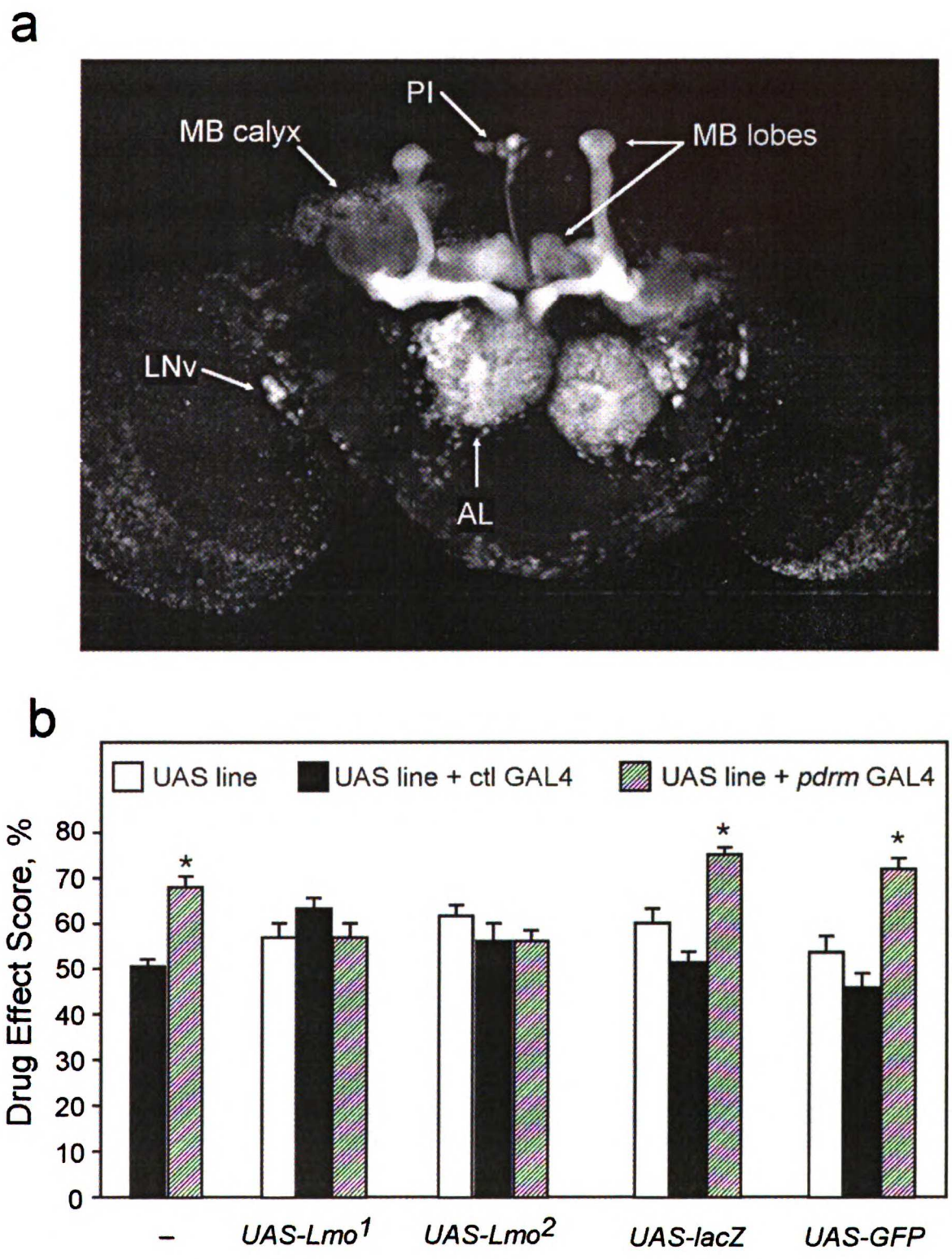
Relative mRNA levels
0
1
2
3
4
5
Bodies
Heads

Figure 4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10
20
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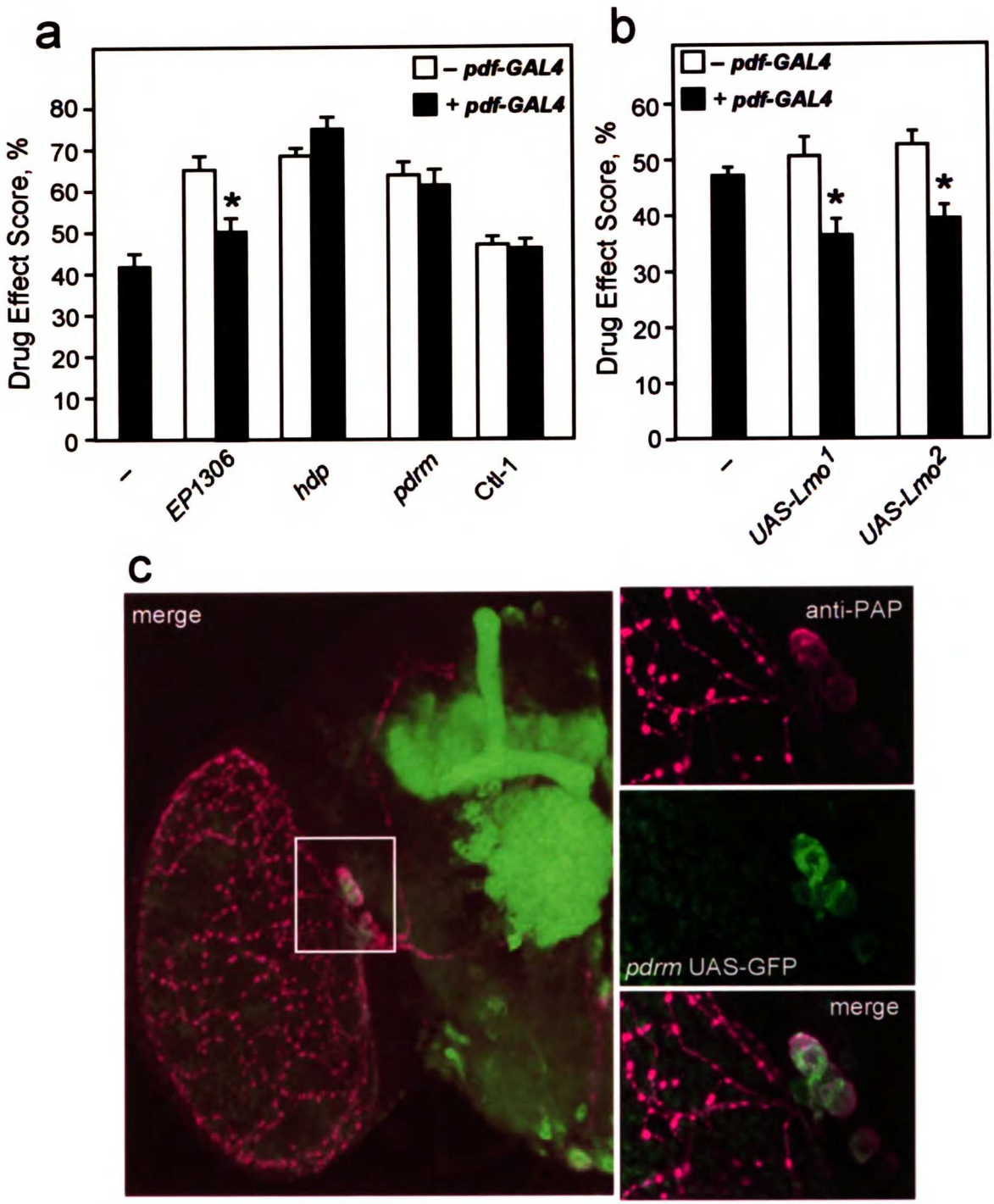
UAS-Lmo1
UAS-Lmo2
UAS-lacZ
UAS-GFP

Figure 5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pdrm
Ctl-1
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pdrm UAS-GFP
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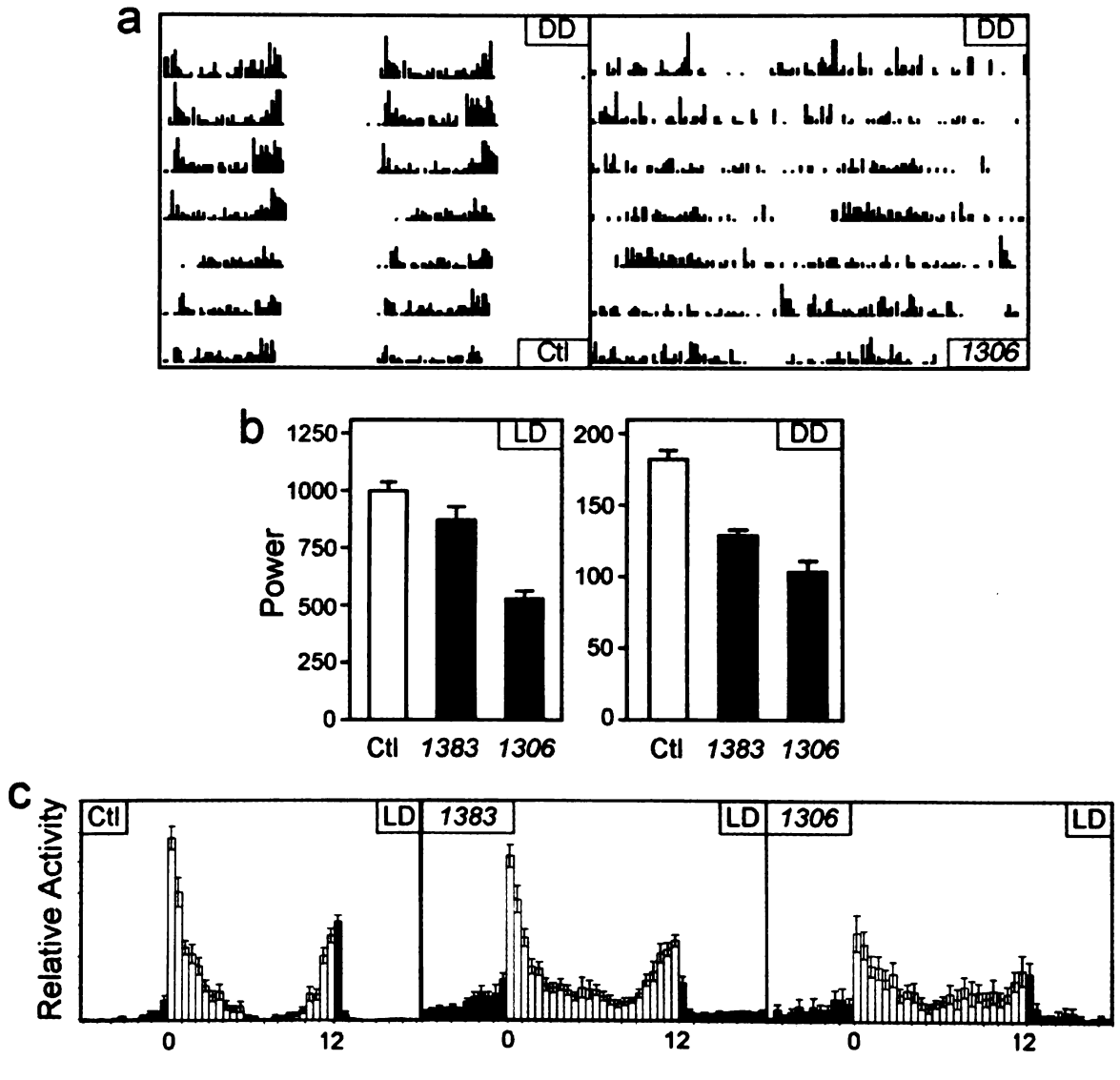
Figure 6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Ctl
1383
1306
Ctl
1383
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Relative Activity
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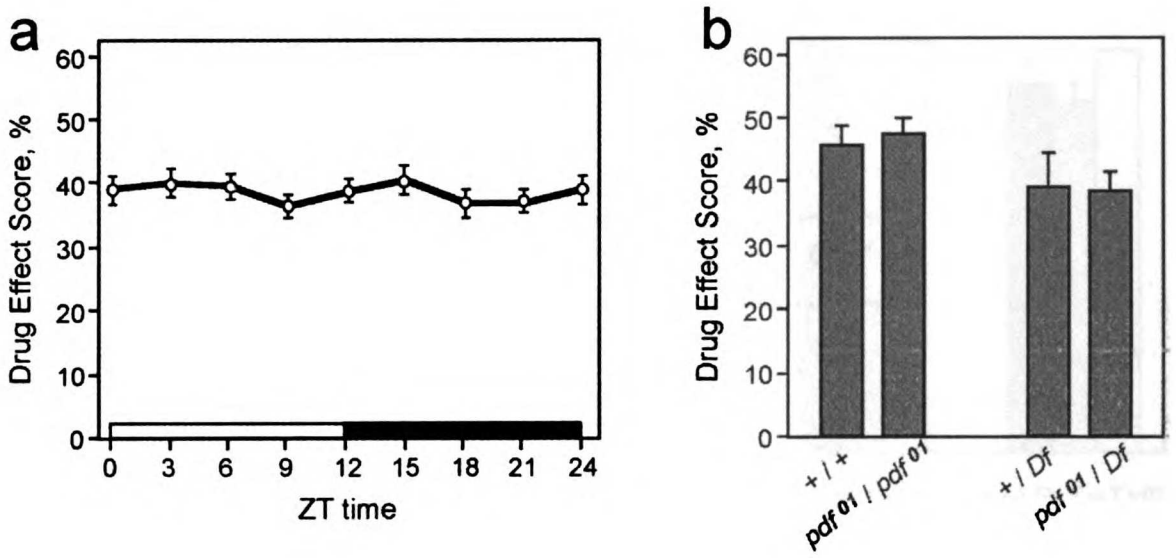
Figure 7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ZT time
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Drug Effect Score, %
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30
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0
+ / +
pdf 01 / pdf 01
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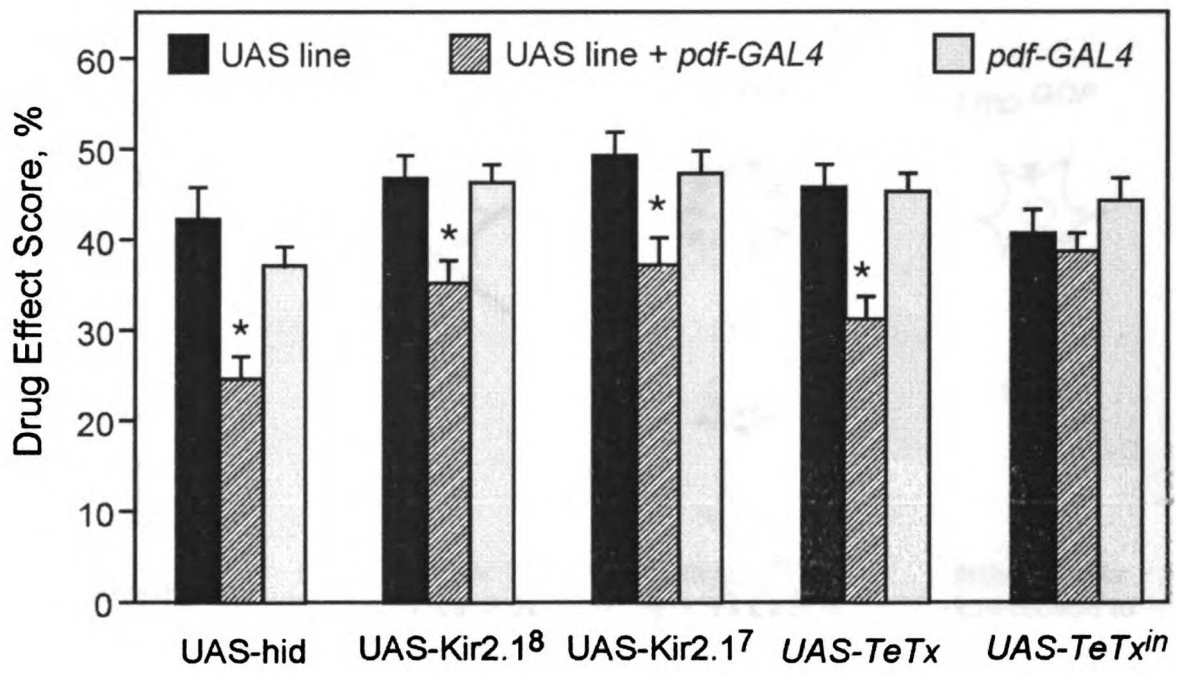
pdf 01 / Df

Figure 8


UAS line
UAS line + pdf-GAL4
pdf-GAL4
Drug Effect Score, %
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50
40
30
20
10
0
*
*
*
*
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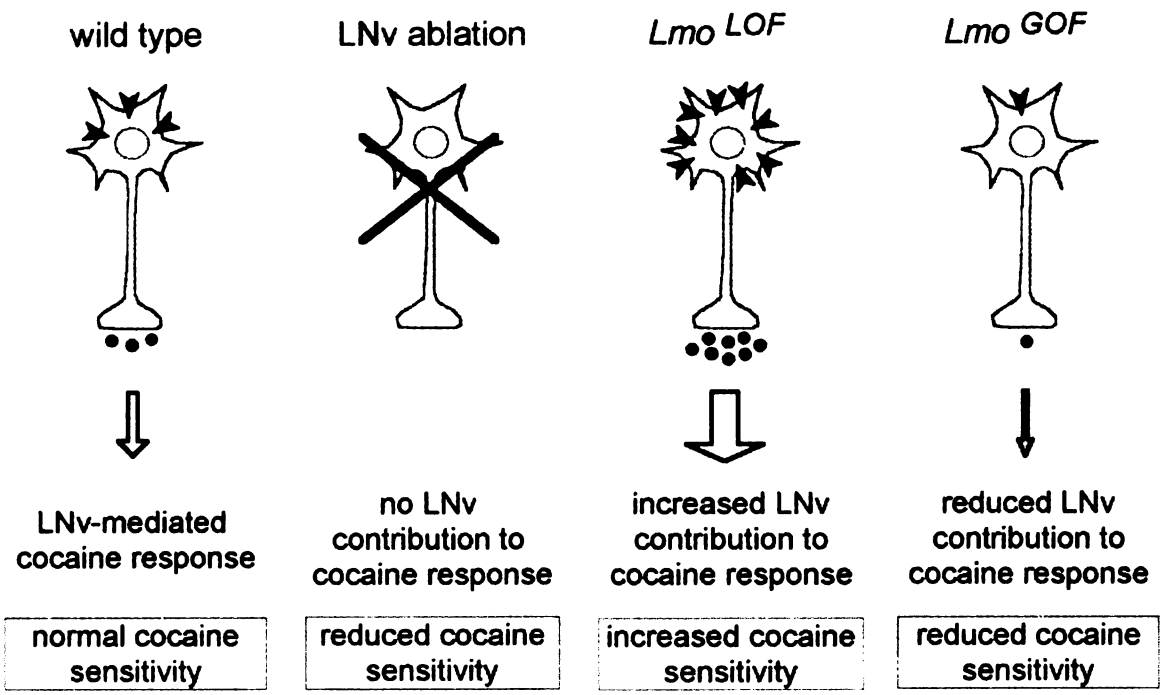
UAS-Kir2.1^8
UAS-Kir2.1^7
UAS-TeTx
UAS-TeTx^in

Figure 9


wild type
LNv ablation
Lmo LOF
Lmo GOF
LNv-mediated cocaine response
no LNv contribution to cocaine response
increased LNv contribution to cocaine response
reduced LNv contribution to cocaine response
normal cocaine sensitivity
reduced cocaine sensitivity
increased cocaine sensitivity
reduced cocaine sensitivity

Conclusions and Future Directions

Conclusions

Drug addiction is a behavioral disease, and as such, is a consequence of altered brain function. An attempt to understand the biological underpinnings of this aberrant brain function requires two broad areas of inquiry. One of these is the identification of genetic factors that contribute to the risk, development, or maintenance of the diseased state. The other is the definition and characterization of the units of behavioral function, which are the neuronal networks that produce and maintain the aberrant behavior. Further, an understanding of how genes act within these defined circuits will be necessary to complete the picture. Each of these lines of inquiry undoubtedly benefits from the others, and thus a comprehensive approach in which genes, circuits, and behaviors are all examined should be a goal.

Drosophila melanogaster is an excellent model system in which to pursue such an approach. *Drosophila* has long been valued for its accessibility to both forward and reverse genetic analysis; and its complex and meaningful behavioral repertoire, together with emergent technologies that allow for spatiotemporal control of expression and active monitoring of neuronal activity, should push it to the forefront of the analysis of complex behaviors such as responses to drugs of abuse. Here, I will discuss how *Drosophila* has, and can be, used to analyze complex behaviors in the specific context of the findings presented in chapters one and two and in the context of possible future experiments based on these findings.

Behaviors

There is no point in arguing that fly behaviors are as complex or nuanced as human behaviors. If they were, flies might be running us through the inebriometer. However, as a reductionist behavioral system, flies show exquisitely performed motor outputs that are modulated by sensory inputs, experience, and motivational state. Important for the study of drugs of abuse, flies show acute locomotor responses to drug administration that are similar to those seen in mammals. Ethanol induces a marked increase in locomotor activity in flies at ethanol concentrations that result in disinhibition and euphoria in humans (Wolf *et al.*, 2002). At higher concentrations, ethanol causes incoordination, loss of postural control, and finally sedation at concentrations that cause analogous behaviors in humans (Singh and Heberlein, 2000). Cocaine stimulates locomotor stereotypies at low doses and at higher doses causes spasmodic activity, tremors, and finally akinesia (McClung and Hirsh, 1998). Nicotine stimulates a marked hyperactivity, moving to spasmodic activity and akinesia at increasing doses (Bainton *et al.*, 2000). Additionally, repeated administration of drugs of abuse elicit changes in behavioral responses that are also observed in mammalian models: repeated cocaine administration induces behavioral sensitization, while repeated ethanol administration results in tolerance (McClung and Hirsh, 1998; Scholz *et al.*, 2000). High throughput assays that measure acute locomotor activation and the plastic changes that result from repeated drug administration have been already developed (McClung and Hirsh, 1998; Bainton *et al.*, 2000; Scholz *et al.*, 2000; Wolf *et al.*, 2002).

An important question is whether the behaviors that can be measured in flies have any relevance to the process of addiction. In mammals, it has been proposed that locomotor activation models the reinforcing properties of drugs (Wise and Bozarth, 1987). Building on this theory, the sensitization of locomotor responses has been proposed as an important step in the development and maintenance of addiction (Robinson and Berridge, 1993). All drugs of abuse tested induce some type of locomotor activation in flies as well as in mammals, and sensitization to psychomotor stimulant drugs has been demonstrated in flies as well. In mammals, a shared dopaminergic system mediates both the reinforcing and locomotor–stimulating properties of drugs (reviewed in Spanagel and Weiss, 1999; Berke and Hyman, 2000); a similar role for a shared dopaminergic system in locomotor responses to drugs has been described in flies (Bainton *et al.*, 2000; Li *et al.*, 2000). These strong parallels suggest that existing behavioral assays should provide meaningful insight into the human addictive process.

However, recently, the locomotor activating and reinforcing properties of drugs have been separated through the analysis of targeted gene deletions (Xu *et al.*, 1994; Miner *et al.*, 1995; Giros *et al.*, 1996; Rocha *et al.*, 1998). Although the importance of this divergence to the development of addiction in the wildtype state remains unclear, the development of assays to directly assess the reinforcing properties of drugs in flies remains a high priority. Promisingly, an ethanol preference assay for adult flies similar to drug discrimination paradigms in rodents has been described recently (Cadieu *et al.*, 1999). Furthermore,

associative learning paradigms akin to conditioned place preference have long been used by many groups to study olfactory learning and memory (Roman and Davis, 2001). It seems only a matter of time before those assays are adapted to the study of drugs.

Until the successful development of models of reinforcement in flies, a feasible approach may be to use rodent reinforcement paradigms to test the role of genes discovered in flies. As has been demonstrated time and time again, homologies between fly and mammalian genes extend past sequence and into functional homologies, even for genes involved in behavior (reviewed in Rothenfluh and Heberlein, 2002; Stanewsky, 2003). The increasing ease of generating gene deletions in mice thus makes it realistic to screen for simple endophenotypes in flies, while testing the relevance of identified genes in mouse models. Caveats of this approach will be discussed below.

Genes

Seymour Benzer's pioneering studies in *Drosophila* were the first to demonstrate that mutations in single genes could affect behaviors, transforming the field of behavioral genetics from one that had focused on natural genetic variation to the search and characterization of single genes (Benzer, 1967; Tully, 1996). Since that time, *Drosophila*, along with *C. elegans*, have been the dominant genetic model systems in which to conduct phenotype–driven behavioral screens. Subsequent work in *Drosophila* has significantly advanced the understanding of many mammalian behaviors, including circadian rhythms,

learning and memory, and more recently, the actions of drugs of abuse (Sokolowski, 2001).

The ability to perform phenotype–driven screens is by far the most important advantage of using *Drosophila* as a model system, so it bears some further discussion. Starting an analysis of a gene's role in behavior with an alteration in the behavior itself provides many benefits. First, this approach makes no assumptions about the genetic basis of behavior; as this method is free of extant information about the behavior, the identification of novel genes and pathways can be achieved. The obvious drawback of this approach is trying to determine the relevance of identified mutations to the behavioral process being modeled. For instance, screens for alcohol mutants have identified mutants that were either severely uncoordinated or had altered drug absorptions; and olfactory learning screens have identified mutants that were unable to smell. Both primary and secondary screens must be designed well to make sure the observed phenotype represents involvement in the desired behavior of study.

A second benefit of the phenotype–first approach has been the discovery that many of the behavioral mutants that have provided the most insight have been those that do not completely inactivate the gene (Greenspan, 1997; Sokolowski, 2001). In fact, for many behavioral genes, complete deletions are lethal owing to involvement of the gene in non–behavioral processes. Unsurprisingly, these hypomorphic behavioral mutations are more indicative of the more subtle genetic influences that occur in nature (Greenspan, 1997). Along these lines, *Drosophila* provides many advantages. First, P

element–mediated mutagenesis tends to create hypomorphs, rather than complete deletions. Second, we have observed in our mutant analyses, that P elements often affect only specific splice variants within the same locus. There is precedent for specific transcripts having a unique role in behavior, as a promoter–specific P1 transcript of the essential gene *fruitless* is necessary for the specification of neural circuits involved in courtship behaviors (Goodwin *et al.*, 2000). Lastly, recent technological advances provide effective methods of conditionally knocking out fly genes under both temporal and spatial control (Lee and Luo, 1999; Lee and Carthew, 2003; McGuire *et al.*, 2004). These novel methods may thus eliminate the pleiotropic effects of whole animal knockouts.

Lastly, any discussion of gene identification techniques must now include a mention of how genomic approaches such as gene expressing profiling can be used. Expression–profiling studies are somewhat of a mix of reverse and forward genetics. Like forward genetic screens, they allow for identification of novel genes or pathways in an unbiased fashion. However, in order to test the validity of whether these uncovered genes are involved in behaviors, reverse genetic techniques must be employed. With regards to *Drosophila* behavior, such whole genome approaches have already been used to good effect in the study of circadian rhythms, implicating previously unknown pathways and genes in these behaviors that now must be tested (Claridge-Chang *et al.*, 2001; McDonald and Rosbash, 2001; Ceriani *et al.*, 2002; Ueda *et al.*, 2002). For drug behaviors, many expression–profiling studies are already underway, though none have been completed. In general, flies have been exposed to drug either acutely

or chronically and changes in gene expression compared between groups or with unexposed flies. At this time, studies that analyze changes in gene expression underlying plastic behaviors such as tolerance and sensitization hold the most promise. However, as more mutants are isolated with altered acute sensitivities, comparative genomic studies can then be utilized to understand acute responses as well. An example from mouse models of how these techniques will be used is illustrative. Yao *et al.* compared the expression profiles of multiple mutants with increased sensitivity to cocaine, as well as mice who were sensitized to repeated doses of cocaine; they showed that mutations of one of the overlapping genes, PSD-95, showed alterations in cocaine sensitivity as well (Yao *et al.*, 2004). The complementary use of these reverse genetic techniques, for the characterization of genes identified in forward genetic screens, provides the *Drosophila* model system with the best of both genetic worlds.

Circuits

Behavior is produced by the activity of neural circuits, and to understand a given behavior, the circuits that mediate the behavior must be identified and characterized. *Drosophila* provides some advantages and disadvantages in this regard. *Drosophila* neuroanatomy is very poorly understood when compared to that of either the mammalian brain or the *C. elegans* neural network, whose 302 neurons' connections have been largely characterized. Characterization of the major *Drosophila* brain neuropils by Golgi impreganations has provided some basic insight (Hanesch *et al.*, 1989; Ito *et al.*, 1998). However, a comprehensive

analysis of neural connectivity, transmitter content, and, most importantly, function will require dedicated efforts from the fly community.

Many novel techniques are being used to map out the brain connectivity in the fly that I'll describe below. One way has been to mark individual neurons within a GAL4 expression pattern of interest using the "mosaic analysis with a repressible cell marker" (MARCM) system (Lee and Luo, 1999). This technique allows the tracing of individual neurons of interest and their projection patterns, similar to that of Golgi impregnation (Marin *et al.*, 2002). The GAL4/UAS expression system is another powerful tool that has been used to map neuroanatomical circuits involved in courtship conditioning, learning, and ethanol responses (Joiner and Griffith, 1999; Zars *et al.*, 2000a; Zars *et al.*, 2000b; reviewed in Rodan, 2002). Transgenes that can block synaptic transmission or electrically silence neurons allow for the functional silencing of neurons within a circuit to determine if their involvement in the behavior of interest.

One common problem that has been encountered in these types of studies is that many GAL4 lines have very broad expression. Lethality induced by broad transgene expression limits the number of GAL4 lines that can be tested behaviorally. Additionally, it is often a challenge to determine which of a broad number of expressing neurons is really involved in the studied behavior. A few approaches have been taken to overcome this issue. Whereas GAL4 expression patterns of interest were initially chosen for their broad expression in neuropils of interest, they are now being chosen for their specificity. Similarly, many groups are constructing elements in which GAL4 is placed under the

control of promoters of interest allowing for more specific control over expression. So far, it is these promoter fusions that have been used effectively to identify specific neuroanatomical loci affecting drug behaviors (Li *et al.*, 2000; Rodan, 2002). Lastly, techniques to control both spatial and temporal expression at the same time have been developed, allowing the silencing of specific neurons only during adulthood. This is done in one of two ways: either by co–expression of a temperature–sensitive GAL4 repressor (GAL80) or by expression of temperature–sensitive *shibire* (Kitamoto, 2001; McGuire *et al.*, 2004). The latter acts dominantly to inactivate neurons when shifted to higher temperatures. These approaches allow flies to develop normally before the neurons of the adult expression pattern are inactivated.

Until recently, one serious drawback in the understanding of how neural circuits work within the fly brain has been the inability to monitor the electrical properties of neurons within these circuits. However, promising advances in both imaging of neural activity and electrophysiology within the brain of the behaving animal have been developed (Wang *et al.*, 2001; Perez-Orive *et al.*, 2002; Wang *et al.*, 2003; Wilson *et al.*, 2004). A striking example of the rapid pace at which neural circuits can be dissected is that of the functional organization of the *Drosophila* antennal lobe. Within just a few years, the antennal lobe has moved from being a poorly understood brain neuropil to an electrophysiology–amenable system in which to study the transformation of sensory information between successive neurons in a relay hierarchy into a meaningful representation for the animal. Using the MARCM system, the antennal lobe projections to higher

olfactory centers were mapped and shown to be highly stereotyped; the observed spatial convergence and divergence of antennal lobe projections suggested an integration of olfactory information mediated by the projection pattern architecture (Marin *et al.*, 2002). Calcium imaging of antennal lobe glomerular activity has shown a functional map of odor activity in which specificity of a glomerular response is determined by the odorant receptors in the primary sensory neurons that innervate that glomerulus (Wang *et al.*, 2003). Although this imaging study also suggested that no modulation occurred within the glomerulus itself, electrophysiological studies have shown quite conclusively that the second–order projection neurons display much more complex activity than those of afferents innervating those projection neurons, implying that the antennal lobe synapse is not simply a relay synapse (Wilson *et al.*, 2004). These types of studies herald the entry of *Drosophila* into the arena of neural circuit dissection. Applying these techniques to study drugs should allow for the identification and characterization of circuits that underlie drug responses and for a future examination of the functional changes in those circuits that are altered in mutants.

Integration of Genes, Circuits and Behavior

The identification and characterization of *Lmo* has benefited from the comprehensive approach provided by the *Drosophila* model system. Starting first with a behavior, forward genetic screening isolated multiple alleles of *Lmo*, a gene that we never would have chosen to study using reverse genetic methods.

Analysis of gene function first directed our attention to the *Drosophila* pacemaker lateral neurons as a neuroanatomical locus underlying cocaine sensitivity. In return, the analysis of how lateral neurons act to modulate cocaine behaviors has suggested that mutations in *Lmo* increase lateral neuron output, thereby providing insight into *Lmo* gene function. And to complete the loop, the examination of *Lmo* function in lateral neurons brought the project back to the study of a behavior, locomotor rhythmicity. Together, these lines of inquiry have synergized to shed light on a novel role for *Lmo* and lateral neurons in the modulation of cocaine– and circadian–regulated behaviors .

Future Directions: More Genes, More Circuits, More Behavior

In this last section, I will present some possible future directions for the study of *Lmo* and lateral neurons in light of the above discussion. Some of the discussion will be speculative; however, most of the proposed experiments and lines of research can be achieved with tools available now.

Behavior

An obvious behavioral question to address is whether *Lmo* mutants retain the ability to sensitize to cocaine. Circadian genes have previously been implicated in cocaine sensitization in both flies and mice (Andretic *et al.*, 1999; Abarca *et al.*, 2002). Furthermore, the finding that *timeless* mutants do not show a cocaine phenotype has been interpreted to mean that these sensitization deficits represent clock–independent functions of these circadian genes (Andretic

et al., 1999; Rothenfluh and Heberlein, 2002). We have shown a similar clock–independent role for *Lmo* in cocaine responses and predict a similar sensitization defect for *Lmo* mutants.

Another question is whether *Lmo* has a general role in drug responses or if *Lmo* phenotypes are specific to cocaine. We have shown previously that the actions of multiple drugs of abuse are at least partially mediated by dopaminergic systems (Chapter 1). Is this true for *Lmo* and lateral neurons (LN_vs) as well? *Lmo* mutants have already been tested for nicotine and alcohol phenotypes. Loss–of–function alleles show normal nicotine and inebriometer phenotypes. However they show a marked reduction in ethanol–induced hyperactivity when tested in the locomotor tracking assay. Interestingly, gain–of–function *Bx* alleles also show reduced ethanol–induced hyperactivity. In addition, *Bx* alleles are also markedly resistant in the inebriometer. Because the cocaine and ethanol phenotypes cannot be correlated, it seems unlikely that they are mediated by the same mechanisms. However, it will be interesting to determine if restoring *Lmo* expression in LN_vs can rescue ethanol phenotypes of loss–of–function alleles or if induction of *Lmo* in LN_vs can phenocopy gain–of–function phenotypes. Similarly, a role for LN_vs in ethanol responses may be tested by ablations or functional silencing manipulations.

Lastly, in order to assess the relevance of these findings to addiction, we plan to test mouse knockouts of *Lmo* homologs for cocaine phenotypes. Mice have four homologs for *Lmo*, while no other homologs have been identified in *Drosophila*. One difficulty will be to determine which homolog(s) to test. Each of

these homologs has been shown to have both unique and divergent expression and to be regulated independently by seizure activity, the sleep–wake cycle, or by the circadian clock (Hinks *et al.*, 1997; Cirelli and Tononi, 2000; Panda *et al.*, 2002). *Lmo–4* is a particularly intriguing candidate because it is expressed at high levels in the suprachiasmatic nuclei (SCN), the mammalian pacemaker neurons analogous to the LN_vs (Amy Lasek and David Kapfhamer, unpublished observations), and may cycle within the SCN (Ceriani *et al.*, 2002). Unfortunately, homozygote *Lmo–4* knockouts are perinatally lethal, and thus not testable (Hahm *et al.*, 2004; Tse *et al.*, 2004). However, *Lmo–4* knockout heterozygotes show only some slight developmental abnormalities, but are viable, and should be tested for cocaine phenotypes (Hahm *et al.*, 2004). *Lmo-1* and *Lmo-3* knockouts are also available; and although they are individually viable, double knockouts are perinatally lethal, suggesting a redundancy of function that must be taken into account in the interpretation of any behavioral data from these mice (Tse *et al.*, 2004).

These knockout mice illustrate many of the difficulties of moving from phenotype–driven fly models into reverse genetic mouse models to determine the relevance of the fly data (Gerlai, 2001). First, mice often have multiple homologs for each single fly gene. Often these homologs share some overlapping expression patterns that provide for functional redundancies; this possibility may have to be addressed by engineering multiple knockouts. Additionally, the pleiotropic nature of gene deletions often makes it impossible to test the role of a gene of interest due to its essential role in development. As

conditional knockout technology improves, however, this obstacle may be overcome.

Genes

Further study of the *Lmo* gene will focus on four major questions. First, does *Lmo* have an active, acute function in regulating cocaine responses? One way to address this would be to use the novel TARGET temperature–sensitive GAL80 system to control the timing of rescue and/or overexpression of *Lmo* in the LN_vs (McGuire *et al.*, 2004). This system will allow the suppression of GAL4–mediated expression throughout development. Within six hours of temperature shift, however, GAL4–mediated expression returns to expression levels comparable to GAL4 expression alone. If wildtype responses could be restored in mutants expressing *Lmo* transiently, an active role for *Lmo* in regulation of cocaine responses would be confirmed.

A second basic question is which LIM–HD mediates *Lmo*'s effects on the regulation of cocaine sensitivity? *Lmo* has been shown to be a negative regulator of LIM–HD–mediated transcription (Milan *et al.*, 1998). Reductions in *Lmo* expression would be predicted to result in increased LIM–HD activity while increased expression would do the opposite. Antibodies to all the LIM–HDs exist and a first step will be to look at co–expression of these LIM–HDs with *Lmo* within the LN_vs. Once a candidate is defined, transgenic flies expressing dsRNAi for that candidate within the LN_vs can be behaviorally characterized.

Since the behavioral phenotypes of *Lmo* mutants presumably result from the altered expression of genes regulated by LIM–HDs or other transcription

factors, the third major question is what gene expression changes are responsible for the differences in cocaine phenotype? In this case, a genomics strategy is ideal to identify these changes. Important for this strategy, there are multiple gain– and loss–of function alleles. This way, non–specific "noise" can be minimized by selecting for genes whose expression changes consistently within each group. We can further minimize noise by selecting genes whose expression changes in *opposite* ways in the loss–of–function versus gain–of–function alleles. These genes would be leading candidates for mediating *Lmo* cocaine phenotypes.

Lastly, a big question regarding *Lmo* gene function is how it interacts with the clock and other circadian genes. We have shown behaviorally that the role of LN_vs in cocaine sensitivity is not a clock output. However, it remains a possibility that *Lmo* itself still functions downstream of the clock. This can be easily tested by determining if *Lmo* transcript levels cycle with the circadian clock. Using microarrays, McDonald and Rosbash showed that more genes are regulated by clock genes than are regulated by the clock (McDonald and Rosbash, 2001). It will be interesting to determine if those genes that are specifically regulated by clock genes, but not the clock, are also regulated by *Lmo*. This would indicate a possible transcriptional complex involving *Lmo* and the central clock genes (which are all transcription factors) that would be important for regulating cocaine responses.

Circuits

Future characterization of LN_v involvement in cocaine sensitivity should seek to answer five somewhat overlapping questions. First, what are the roles of these cells during the cocaine response? Second, what are the outputs of these neurons? Third, how heterogeneous is this cell population? Next, how are circadian and cocaine functions separated? Lastly, what properties of these neurons change in *Lmo* mutants? The key to addressing these questions will be the application of methods to monitor neuronal activity to these cells. These cells are ideal candidates for electrophysiological or in vivo calcium–imaging analysis because they are relatively large (~6-12 microns), near the surface of the brain, and have specific markers, allowing for their easy identification (Rachel Wilson, personal communication).

These neurons have already been very well characterized by the circadian community. The projection patterns of these neurons have been studied in detail (Helfrich-Forster, 1997; Helfrich-Forster, 2003). The small LN_vs (s–LN_vs) project to the dorsal central brain, terminating near two sets of dorsal neurons (DN_1s and DN_2s) that express clock genes at high levels. These neurons arise early in larval development, and their projection patterns are maintained into adulthood. The DN_1s and DN_2s mainly send projections to contralateral DN_1 and DN_2 groups, with some scattered projections to the esophageal area and to the area of the pars intercerebralis. The large LN_vs (l–LN_vs) arise during pupal stages and project onto the surface of the optic medulla, as well as onto the contralateral LN_vs through fibers running in the posterior optic tract. Furthermore, both small

and large LN_vs have dense arborizations in the accessory medulla, a neuropil proposed to be a circadian pacemaker center in cockroaches and crickets (Helfrich-Forster *et al.*, 1998). There is increasing evidence that the l–LN_vs are not involved in circadian rhythmicity (reviewed in Helfrich-Forster, 2003). First, they make no projections to the dorsal brain, an area also involved in locomotor rhythmicity (Helfrich-Forster, 1997). Second, unlike in s–LN_vs, molecular clock cycling is not sustained in these cells during free–running conditions (Kaneko *et al.*, 2000; Yang and Sehgal, 2001; Shafer *et al.*, 2002). Lastly, PDF expression is controlled by clock genes and PDF release by the molecular clock in s–LN_vs, but not in l–LN_vs (Blau and Young, 1999; Park *et al.*, 2000). These results suggest an independent functional role for the l–LN_vs, making them strong candidates for mediating cocaine's actions.

Our findings suggest that LN_vs have non–PDF outputs that are involved in modulating cocaine sensitivity. Future experiments will examine what some of these outputs may be. This may be accomplished by in situs, or by immunohistological examination of what neurotransmitters or neuropeptides are expressed; however antibodies have not been made against many neuropeptides. Alternatively, because these lateral neurons are accessible it should be possible to extract RNA from individual cells and to examine the specific neuropeptide expression profile of individual lateral neurons. Over thirty neuropeptide genes have been identified either by sequence annotation or peptidomic methods; this set of transcripts is not an unreasonable number to

examine (Hewes and Taghert, 2001; Baggerman *et al.*, 2002). Examination of individual neurons may also shed light on the heterogeneity of the LN_vs.

To answer many of the above questions, the electrical properties of LN_vs need to be assessed, preferably by patch clamp techniques (Wilson *et al.*, 2004). In culture, subsets of LN_vs have been shown to respond to nicotine or dopamine by increasing intracellular calcium (Wegener *et al.*, 2004). A comprehensive assessment of the *in vivo* responses of both small and large lateral neurons to dopamine and nicotine should provide insight into the heterogeneity between and/or within these two major groups of neurons. Using similar techniques, the role of these cells in cocaine responses can also be tested. An observation and comparison of LN_v responses to direct and global application of cocaine would address the question of whether cocaine alters LN_v activity and whether those changes in activity are the result of a direct effect of cocaine on LN_vs. Findings that LN_vs do not respond to cocaine would imply a role in modulating other circuits that mediate cocaine responses.

Lastly, it will be important to apply these techniques to *Lmo* mutants to examine how lateral neuron function changes to produce altered cocaine sensitivity. We provide evidence that an increased activity of LN_vs mediates the increased sensitivity of *Lmo* mutants. In our model, we hypothesize that this increased activity results from either increased dopamine receptor content within neurons that normally express them, or recruitment of other LN_vs into the cocaine response via the expression of dopamine receptors in LN_vs that normally don't express them. These possibilities can be examined in a number of ways: 1)

examining DA receptor content by immunohistochemical techniques; 2) examining responses of cultured *Lmo* mutant lateral neurons to dopamine, as demonstrated previously (Wegener *et al.*, 2004); or 3) by application of electrophysiological techniques as described above.

Summary

In conclusion, a complete understanding of cocaine's actions in flies will require a comprehensive scientific approach with multiple lines of investigation. First, at the level of the whole organism, relevant assays that can measure cocaine behaviors must be developed. Second, at the level of the cell, the neurons that mediate these behaviors must be identified. Third, at the molecular level, the pathways and molecules that mediate drug action must be isolated and characterized. Finally, an integration of these methodologies (ie, the study of specific pathways in defined neurons in relevant assays) should accelerate our understanding of this complex behavioral process. The results presented here show the power of such an integrated approach to the study of behavior.

References

Abarca, C., U. Albrecht and R. Spanagel (2002). Cocaine sensitization and reward are under the influence of circadian genes and rhythm. Proc Natl Acad Sci U S A *99*(13): 9026-30.

Andretic, R., S. Chaney and J. Hirsh (1999). Requirement of circadian genes for cocaine sensitization in Drosophila. Science *285*(5430): 1066-8.

Baggerman, G., A. Cerstiaens, A. De Loof and L. Schoofs (2002). Peptidomics of the larval Drosophila melanogaster central nervous system. J Biol Chem *277*(43): 40368-74.

Bainton, R. J., L. T. Tsai, C. M. Singh, M. S. Moore, W. S. Neckameyer and U. Heberlein (2000). Dopamine modulates acute responses to cocaine, nicotine and ethanol in Drosophila. Curr Biol *10*(4): 187-94.

Benzer, S. (1967). Behavioral mutants of *Drosophila* isolated by counter–current distribution. Proc Natl Acad Sci U S A *58*: 1112-1119.

Berke, J. D. and S. E. Hyman (2000). Addiction, dopamine, and the molecular mechanisms of memory. Neuron *25*(3): 515-32.

Blau, J. and M. W. Young (1999). Cycling vrille expression is required for a functional Drosophila clock. Cell *99*(6): 661-71.

Cadieu, N., J. Cadieu, L. El Ghadraoui, A. Grimal and Y. Lamboeuf (1999). Conditioning to ethanol in the fruit fly-a study using an inhibitor of ADH. J Insect Physiol *45*(6): 579-586.

Ceriani, M. F., J. B. Hogenesch, M. Yanovsky, S. Panda, M. Straume and S. A. Kay (2002). Genome-wide expression analysis in Drosophila reveals genes controlling circadian behavior. J Neurosci *22*(21): 9305-19.

Cirelli, C. and G. Tononi (2000). Gene expression in the brain across the sleep-waking cycle. Brain Res *885*(2): 303-21.

Claridge-Chang, A., H. Wijnen, F. Naef, C. Boothroyd, N. Rajewsky and M. W. Young (2001). Circadian regulation of gene expression systems in the Drosophila head. Neuron *32*(4): 657-71.

Gerlai, R. (2001). Gene targeting: technical confounds and potential solutions in behavioral brain research. Behav Brain Res *125*(1-2): 13-21.

Giros, B., M. Jaber, S. R. Jones, R. M. Wightman and M. G. Caron (1996). Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature *379*(6566): 606-12.

Goodwin, S. F., B. J. Taylor, A. Villella, M. Foss, L. C. Ryner, B. S. Baker and J. C. Hall (2000). Aberrant splicing and altered spatial expression patterns in fruitless mutants of Drosophila melanogaster. Genetics *154*(2): 725-45.

Greenspan, R. J. (1997). A kinder, gentler genetic analysis of behavior: dissection gives way to modulation. Curr Opin Neurobiol *7*(6): 805-11.

Hahm, K., E. Y. Sum, Y. Fujiwara, G. J. Lindeman, J. E. Visvader and S. H. Orkin (2004). Defective neural tube closure and anteroposterior patterning in mice lacking the LIM protein LMO4 or its interacting partner Deaf-1. Mol Cell Biol *24*(5): 2074-82.

Hanesch, U., K.-F. Fischbach and M. Heisenberg (1989). Neuronal Architecture of the central complex in *Drosophila melanogaster*. Cell Tissue Res *257*: 343-366.

Helfrich-Forster, C. (1997). Development of pigment-dispersing hormone-immunoreactive neurons in the nervous system of Drosophila melanogaster. J Comp Neurol *380*(3): 335-54.

Helfrich-Forster, C. (2003). The neuroarchitecture of the circadian clock in the brain of Drosophila melanogaster. Microsc Res Tech *62*(2): 94-102.

Helfrich-Forster, C., M. Stengl and U. Homberg (1998). Organization of the circadian system in insects. Chronobiol Int *15*(6): 567-94.

Hewes, R. S. and P. H. Taghert (2001). Neuropeptides and neuropeptide receptors in the Drosophila melanogaster genome. Genome Res *11*(6): 1126-42.

Hinks, G. L., B. Shah, S. J. French, L. S. Campos, K. Staley, J. Hughes and M. V. Sofroniew (1997). Expression of LIM protein genes Lmo1, Lmo2, and Lmo3 in adult mouse hippocampus and other forebrain regions: differential regulation by seizure activity. J Neurosci *17*(14): 5549-59.

Ito, K., K. Suzuki, P. Estes, M. Ramaswami, D. Yamamoto and N. J. Strausfeld (1998). The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in Drosophila melanogaster Meigen. Learn Mem *5*(1-2): 52-77.

Joiner, M. A. and L. C. Griffith (1999). Mapping of the anatomical circuit of CaM kinase-dependent courtship conditioning in Drosophila. Learn Mem *6*(2): 177-92.

Kaneko, M., J. H. Park, Y. Cheng, P. E. Hardin and J. C. Hall (2000). Disruption of synaptic transmission or clock-gene-product oscillations in circadian pacemaker cells of Drosophila cause abnormal behavioral rhythms. J Neurobiol *43*(3): 207-33.

Kitamoto, T. (2001). Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons. J Neurobiol *47*(2): 81-92.

Lee, T. and L. Luo (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron *22*(3): 451-61.

Lee, Y. S. and R. W. Carthew (2003). Making a better RNAi vector for Drosophila: use of intron spacers. Methods *30*(4): 322-9.

Li, H., S. Chaney, I. J. Roberts, M. Forte and J. Hirsh (2000). Ectopic G-protein expression in dopamine and serotonin neurons blocks cocaine sensitization in Drosophila melanogaster. Curr Biol *10*(4): 211-4.

Marin, E. C., G. S. Jefferis, T. Komiyama, H. Zhu and L. Luo (2002). Representation of the glomerular olfactory map in the Drosophila brain. Cell *109*(2): 243-55.

McClung, C. and J. Hirsh (1998). Stereotypic behavioral responses to free-base cocaine and the development of behavioral sensitization in Drosophila. Curr Biol *8*(2): 109-12.

McDonald, M. J. and M. Rosbash (2001). Microarray analysis and organization of circadian gene expression in Drosophila. Cell *107*(5): 567-78.

McGuire, S. E., Z. Mao and R. L. Davis (2004). Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. Sci STKE *2004*(220): pl6.

Milan, M., F. J. Diaz-Benjumea and S. M. Cohen (1998). Beadex encodes an LMO protein that regulates Apterous LIM-homeodomain activity in Drosophila wing development: a model for LMO oncogene function. Genes Dev *12*(18): 2912-20.

Miner, L. L., J. Drago, P. M. Chamberlain, D. Donovan and G. R. Uhl (1995). Retained cocaine conditioned place preference in D1 receptor deficient mice. Neuroreport *6*(17): 2314-6.

Panda, S., M. P. Antoch, B. H. Miller, A. I. Su, A. B. Schook, M. Straume, P. G. Schultz, S. A. Kay, J. S. Takahashi and J. B. Hogenesch (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. Cell *109*(3): 307-20.

Park, J. H., C. Helfrich-Forster, G. Lee, L. Liu, M. Rosbash and J. C. Hall (2000). Differential regulation of circadian pacemaker output by separate clock genes in Drosophila. Proc Natl Acad Sci U S A *97*(7): 3608-13.

Perez-Orive, J., O. Mazor, G. C. Turner, S. Cassenaer, R. I. Wilson and G. Laurent (2002). Oscillations and sparsening of odor representations in the mushroom body. Science *297*(5580): 359-65.

Robinson, T. E. and K. C. Berridge (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain Res Brain Res Rev *18*(3): 247-91.

Rocha, B. A., F. Fumagalli, R. R. Gainetdinov, S. R. Jones, R. Ator, B. Giros, G. W. Miller and M. G. Caron (1998). Cocaine self-administration in dopamine-transporter knockout mice. Nat Neurosci *1*(2): 132-7.

Rodan, A. R. (2002). Functional Dissection of Neuroanatomical Loci Regulating Ethanol Sensitiviy in *Drosophila melanogaster*. Thesis in Genetics. San Francisco, University of California, San Francisco: 249.

Roman, G. and R. L. Davis (2001). Molecular biology and anatomy of Drosophila olfactory associative learning. Bioessays *23*(7): 571-81.

Rothenfluh, A. and U. Heberlein (2002). Drugs, flies, and videotape: the effects of ethanol and cocaine on Drosophila locomotion. Curr Opin Neurobiol *12*(6): 639-45.

Scholz, H., J. Ramond, C. M. Singh and U. Heberlein (2000). Functional ethanol tolerance in Drosophila. Neuron *28*(1): 261-71.

Shafer, O. T., M. Rosbash and J. W. Truman (2002). Sequential nuclear accumulation of the clock proteins period and timeless in the pacemaker neurons of Drosophila melanogaster. J Neurosci *22*(14): 5946-54.

Singh, C. M. and U. Heberlein (2000). Genetic control of acute ethanol-induced behaviors in Drosophila. Alcohol Clin Exp Res *24*(8): 1127-36.

Sokolowski, M. B. (2001). Drosophila: genetics meets behaviour. Nat Rev Genet *2*(11): 879-90.

Spanagel, R. and F. Weiss (1999). The dopamine hypothesis of reward: past and current status. Trends Neurosci *22*(11): 521-7.

Stanewsky, R. (2003). Genetic analysis of the circadian system in Drosophila melanogaster and mammals. J Neurobiol *54*(1): 111-47.

Tse, E., A. J. Smith, S. Hunt, I. Lavenir, A. Forster, A. J. Warren, G. Grutz, L. Foroni, M. B. Carlton, W. H. Colledge, T. Boehm and T. H. Rabbitts (2004). Null mutation of the Lmo4 gene or a combined null mutation of the Lmo1/Lmo3 genes causes perinatal lethality, and Lmo4 controls neural tube development in mice. Mol Cell Biol *24*(5): 2063-73.

Tully, T. (1996). Discovery of genes involved with learning and memory: an experimental synthesis of Hirschian and Benzerian perspectives. Proc Natl Acad Sci U S A *93*(24): 13460-7.

Ueda, H. R., A. Matsumoto, M. Kawamura, M. Iino, T. Tanimura and S. Hashimoto (2002). Genome-wide transcriptional orchestration of circadian rhythms in Drosophila. J Biol Chem *277*(16): 14048-52.

Wang, J. W., A. M. Wong, J. Flores, L. B. Vosshall and R. Axel (2003). Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. Cell *112*(2): 271-82.

Wang, Y., N. J. Wright, H. Guo, Z. Xie, K. Svoboda, R. Malinow, D. P. Smith and Y. Zhong (2001). Genetic manipulation of the odor-evoked distributed neural activity in the Drosophila mushroom body. Neuron *29*(1): 267-76.

Wegener, C., Y. Hamasaka and D. R. Nassel (2004). Acetylcholine increases intracellular Ca2+ via nicotinic receptors in cultured PDF-containing clock neurons of Drosophila. J Neurophysiol *91*(2): 912-23.

Wilson, R. I., G. C. Turner and G. Laurent (2004). Transformation of olfactory representations in the Drosophila antennal lobe. Science *303*(5656): 366-70.

Wise, R. A. and M. A. Bozarth (1987). A psychomotor stimulant theory of addiction. Psychol Rev *94*(4): 469-92.

Wolf, F. W., A. R. Rodan, L. T. Tsai and U. Heberlein (2002). High-resolution analysis of ethanol-induced locomotor stimulation in Drosophila. J Neurosci *22*(24): 11035-44.

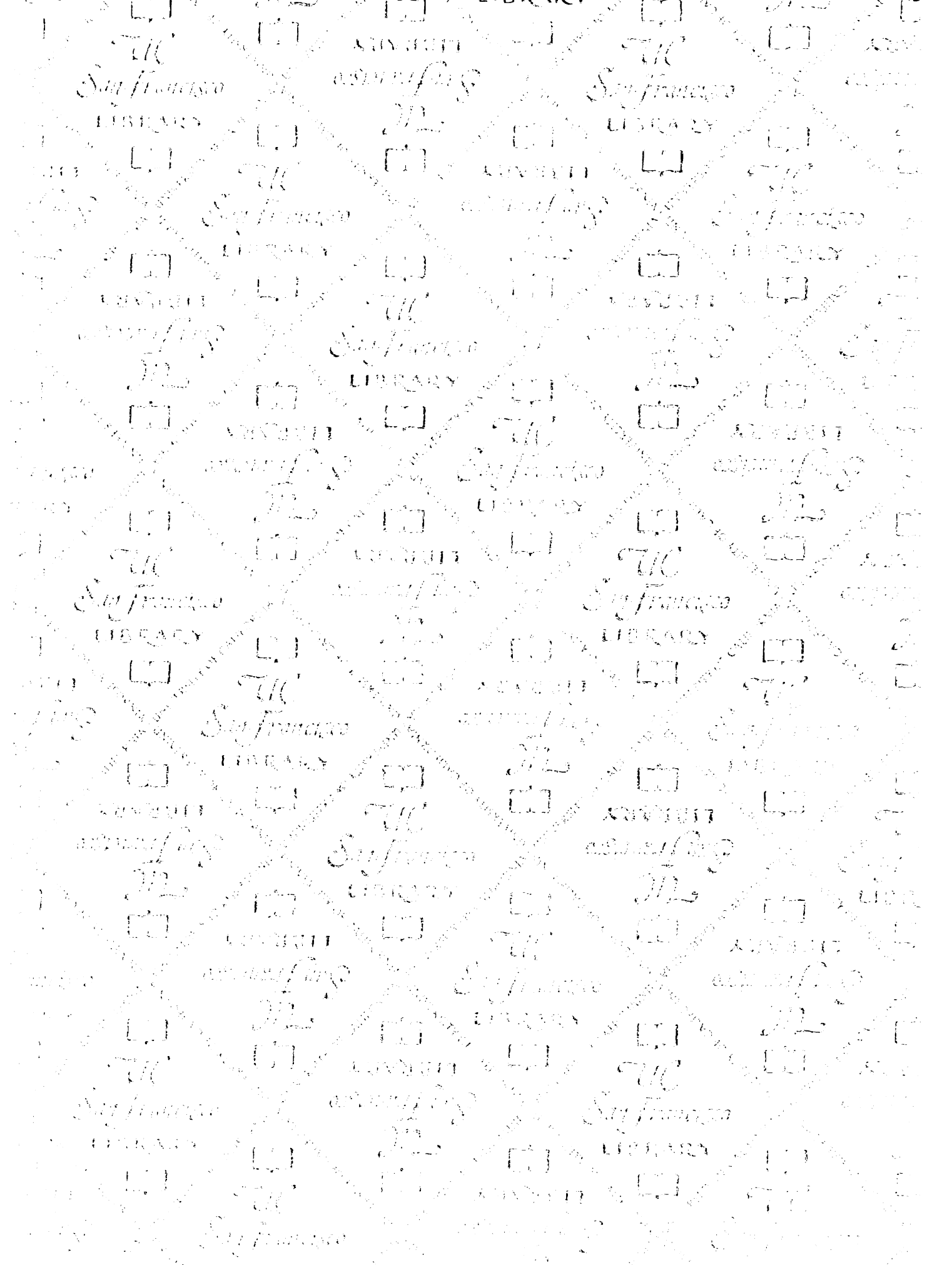
Xu, M., X. T. Hu, D. C. Cooper, R. Moratalla, A. M. Graybiel, F. J. White and S. Tonegawa (1994). Elimination of cocaine-induced hyperactivity and dopamine-mediated neurophysiological effects in dopamine D1 receptor mutant mice. Cell *79*(6): 945-55.

Yang, Z. and A. Sehgal (2001). Role of molecular oscillations in generating behavioral rhythms in Drosophila. Neuron *29*(2): 453-67.

Yao, W. D., R. R. Gainetdinov, M. I. Arbuckle, T. D. Sotnikova, M. Cyr, J. M. Beaulieu, G. E. Torres, S. G. Grant and M. G. Caron (2004). Identification of PSD-95 as a regulator of dopamine-mediated synaptic and behavioral plasticity. Neuron *41*(4): 625-38.

Zars, T., M. Fischer, R. Schulz and M. Heisenberg (2000a). Localization of a short-term memory in Drosophila. Science *288*(5466): 672-5.

Zars, T., R. Wolf, R. Davis and M. Heisenberg (2000b). Tissue-specific expression of a type I adenylyl cyclase rescues the rutabaga mutant memory defect: in search of the engram. Learn Mem *7*(1): 18-31.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