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Review

Drosophila, a genetic model system to study cocaine-related behaviors: A review with focus on LIM-only proteins

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ABSTRACT

In the last decade, the fruit fly *Drosophila melanogaster*, highly accessible to genetic, behavioral and molecular analyses, has been introduced as a novel model organism to help decipher the complex genetic, neurochemical, and neuroanatomical underpinnings of behaviors induced by drugs of abuse. Here we review these data, focusing specifically on cocaine-related behaviors. Several of cocaine's most characteristic properties have been recapitulated in *Drosophila*. First, cocaine induces motor behaviors in flies that are remarkably similar to those observed in mammals. Second, repeated cocaine administration induces behavioral sensitization a form of behavioral plasticity believed to underlie certain aspects of addiction. Third, a key role for dopaminergic systems in mediating cocaine's effects has been demonstrated through both pharmacological and genetic methods. Finally, and most importantly, unbiased genetic screens, feasible because of the simplicity and scale with which flies can be manipulated in the laboratory, have identified several novel genes and pathways whose role in cocaine behaviors had not been anticipated. Many of these genes and pathways have been validated in mammalian models of drug addiction. We focus in this review on the role of LIM-only proteins in cocaine-induced behaviors.

1. 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 actions. In mammalian animal models, the acute response to cocaine is predominantly observed as enhanced locomotor activity and stereotypic behaviors. This locomotor-stimulant effect of cocaine is mediated primarily by an inhibition of the dopamine transporter (DAT), as mice lacking DAT show enhanced levels of baseline activity that are insensitive to cocaine administration (Giros et al., 1996). Regulation of the rewarding effects of cocaine are, however, more complex, involving, in addition to DAT, the serotonin transporter (SERT) (Sora et al., 1998, 2001) as well as many additional genes. How the acute stimulant effects of cocaine relate to the long-term changes that underlie addiction is poorly understood. However, emerging evidence suggests that the mechanisms that regulate the acute stimulant effects of psychostimulants are also involved in determining their rewarding properties (Laakso et al., 2002). For example, the locomotor activity of mice lacking both DAT and SERT is unaltered by cocaine administration; these mice also fail to develop conditioned preference for cocaine, an assay that measures the rewarding effects of the drug (Sora et al., 2001). Conversely, mice lacking FosB or overexpressing ΔfosB, which are highly sensitive to the psychomotor stimulant effects of cocaine, also show enhanced place preference for cocaine (Hiroi et al., 1997; Kelz et al., 1999). It is therefore likely that a mechanistic understanding of the relatively simple process of acute drug-induced locomotor stimulation may provide valuable clues about the molecular mechanisms underlying drug reward, reinforcement, and addiction.

2. *Drosophila* as a model

Drosophila, one of the most intensively studied organisms in biology, has provided crucial insights into developmental and

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cellular processes that are conserved with mammals, including humans. Flies have a relatively sophisticated nervous system (approximately 300,000 neurons) and are capable of many complex behaviors (Hall, 1994, 1998; DeZazzo and Tully, 1995; Sokolowski, 2001). They are easy and inexpensive to rear in the laboratory and their life cycle is only approximately two weeks. The major advantage of flies is the simplicity and scale with which they can be manipulated genetically. A century of fundamental genetic analysis has led to the generation of a large number of sophisticated genetic tools (Lindsley, 1992; Rubin and Lewis, 2000), and the last few decades have witnessed the development of many powerful molecular genetic techniques that allow germ-line transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982), the use of transposable elements as mutagens (Engels, 1983), homologous recombination (Rong and Golic, 2000), and double stranded RNA-mediated gene expression interference (RNAi) (Carthew, 2001; Kalidas and Smith, 2002). Moreover, an analysis of the *Drosophila* euchromatin sequence revealed a high degree of molecular similarity between flies and mammals (Adams et al., 2000; Myers et al., 2000; Rubin et al., 2000). For example, *Drosophila* has most – if not all – major neurotransmitters, molecules involved in synaptic vesicle release and recycling, receptors and channels for neurotransmission, and signal transduction mechanisms involved in neural function in mammals (Littleton and Ganetzky, 2000; Lloyd et al., 2000). However, there are several notable differences. For example, flies use acetylcholine instead of glutamate as the major excitatory CNS neurotransmitter, and glutamate instead of acetylcholine at the neuromuscular junction. In addition, flies lack noradrenaline with octopamine fulfilling its many roles (Roeder, 1999). Importantly, genes implicated directly or indirectly in the actions of abused drugs are, for the most part, conserved. In addition to the above-mentioned genetic tools, multiple techniques exist to alter the function of specific populations of nervous system cells, thus allowing the definition of the neuroanatomical loci that regulate behaviors of interest (Brand et al., 1994; Kitamoto, 2001; Osterwalder et al., 2001; Roman et al., 2001; Stebbins et al., 2001; Stebbins and Yin, 2001; McGuire et al., 2003, 2004). In recent years, these powerful tools have been applied to the study of behaviors induced by drugs of abuse, including alcohol, cocaine, and nicotine, in *Drosophila* (Rothenfluh and Heberlein, 2002; Guarnieri and Heberlein, 2003; Wolf and Heberlein, 2003). As a consequence, the molecular genetic, neuroanatomical and neurochemical bases for responses to abused drugs in *Drosophila* are beginning to be understood, revealing a large degree of mechanistic conservation with mammalian systems.

3. Behavioral effects of cocaine administration in Drosophila

McClung and Hirsh (McClung and Hirsh, 1998) first showed that upon exposure to free-base cocaine, volatilized off a heated nichrome filament (Fig. 1A), flies show a range of unusual behaviors: low doses induce continuous grooming, intermediate doses lead to circling (Fig. 1C) and other aberrant walking behavior, while higher doses cause fast and uncontrolled movements (Fig. 1D), and eventually akinesia and even death. Continuous grooming and circling, behaviors referred to as stereotypies, are also observed in rodents upon cocaine administration. In addition to a direct visual scoring of cocaine-induced behaviors elicited by volatilized free-base cocaine (McClung and Hirsh, 1998), several additional assays have been developed to administer cocaine and quantify its effects: (1) the "crackometer" quantifies the loss of negative geotaxis and positive phototaxis (two strong innate behaviors in flies) caused by volatilized cocaine exposure (Bainton et al., 2000), (2) a video-tracking system (Bainton et al., 2000) (Fig. 1B–D) and the automated Drosophila Activity Monitoring System (Dimitrijevic et al., 2004) allow the measurement of speed and patterns of locomotion induced by exposure to volatilized cocaine and cocaine injection, (3) a sophisticated "bottom-counting" semi-automated tracking system allows the high-throughput analysis of affected individuals (George et al., 2005), and finally, (4) freebase cocaine dissolved in ethanol can be sprayed onto flies using a graphic arts airbrush modified to reproducibly control the drug dosage (Lease and Hirsh, 2005). In addition to these stereotyped responses to acute cocaine exposure, repeated cocaine exposure makes flies increasingly sensitive to the behavioral effects of the drug (McClung and Hirsh, 1998). This behavioral sensitization takes time to develop (it is strongest approximately 6 h after the first exposure) and is long lasting (dissipating approximately 2 days after a single exposure). Sensitization has been demonstrated by the direct observation method (McClung and Hirsh, 1998), the Drosophila Activity Monitoring System (Dimitrijevic et al., 2004) and the "bottom-counting" system (George et al., 2005). Thus, different routes of cocaine administration have been shown to induce a series of behaviors in Drosophila that closely mimic mammalian cocaine-induced behaviors. In addition, several behavioral assays have been developed to quantify such behaviors in flies.

4. Role of biogenic amine systems in *Drosophila* cocaine-induced behaviors

Flies synthesize the biogenic amines dopamine, octopamine and tyramine, as well as serotonin and possibly additional trace amines (Monastirioti, 1999). They also contain proteins involved in the pre-synaptic machinery needed for their synthesis, release and reuptake, as well as receptors and signaling pathways that mediate their pre- and post-synaptic effects (Littleton and Ganetzky, 2000; Lloyd et al., 2000). Interference with dopamine synthesis with the tyrosine hydroxylase (TH) inhibitor 3-iodo-tyrosine leads to reduced effectiveness of cocaine (Bainton et al., 2000). In addition, the application of cocaine solution directly to the ventral nerve cord of decapitated flies induces grooming and aberrant locomotion, an effect that is blocked by pre-administration of a dopamine D1 receptor antagonist (Torres and Horowitz, 1998). Similar behavioral effects are seen after direct application of monoamines or dopamine receptor agonists to the ventral nerve cord in decapitated flies (Yellman et al., 1998). The finding that inhibition of synaptic transmission in dopaminergic and serotonergic neurons leads to cocaine hypersensitivity is therefore somewhat surprising (Li et al., 2000). However, because these neurons are silenced throughout development, compensatory adaptations, such as hypersensitivity of the post-synaptic receptors, may be responsible for the increased cocaine sensitivity. The latter is supported by the finding that direct application of the dopamine receptor agonist quinpirole to the ventral nerve cord of these flies induces an enhanced locomotor response (Li et al., 2000).

Flies (and insects in general) synthesize tyramine from tyrosine by the action of tyramine decarboxylase (Tdc); tyramine is converted into octopamine by tyramine-β-hydroxylase (Tβh) (Monastirioti, 1999). Flies that lack both neural tyramine and octopamine because of mutation in one of the tyramine decarboxylase-encoding genes, Tdc2, have dramatically reduced basal locomotor activity levels and are hypersensitive to an initial dose of cocaine (Hardie et al., 2007). In contrast, flies that contain no measurable neural octopamine and an excess of tyramine due to a null mutation in the *tyramine-β-hydroxylase* gene exhibit normal locomotor activity and cocaine responses (Hardie et al., 2007). Finally, a *Drosophila* isoform of the vesicular monoamine transporter VMAT (DVMAT-A) is expressed in both dopaminergic and serotonergic neurons in the adult *Drosophila* brain. Overexpression of DVMAT-A in these cells potentiates spontaneous stereotypic grooming and locomotion, effects that can be reversed by blocking DVMAT activity, and administration of a dopamine receptor

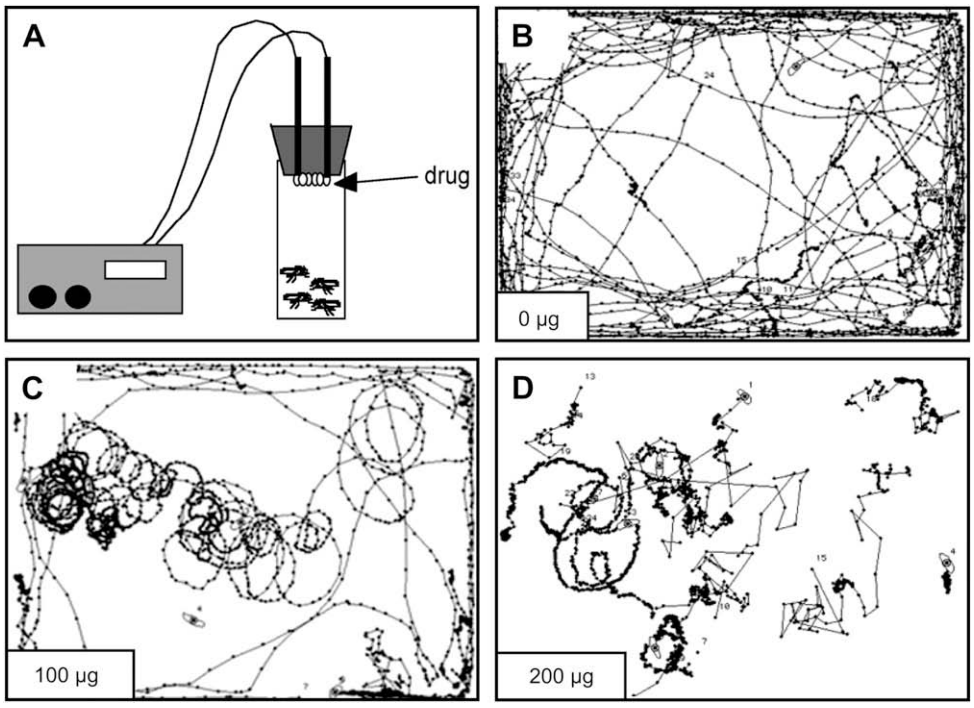

Fig. 1. Cocaine delivery and cocaine-induced locomotor behaviors in *Drosophila*. (A) Cartoon of the system used to volatilize free-base cocaine (McClung and Hirsh, 1998). Cocaine (dissolved in ethanol) is deposited on the nichrome coil and volatilized upon heating the coil. Flies "inhale" the volatilized cocaine and are then transferred to an observation chamber in which their behavior is filmed and analyzed using specialized software (Wolf et al., 2002). (B–D) Computer-generated traces of the locomotor behavior of a group of five flies exposed to volatilized free-base cocaine. Each panel corresponds to a 1-min period starting 2 min after the end of the cocaine exposure. (B) Mock exposure, (C) 100 μg cocaine, and (D) 200 μg cocaine. (Bainton et al., 2000; reprinted with permission.)

antagonist. In addition, DVMAT-A overexpression decreases the fly's sensitivity to cocaine, suggesting that the synaptic machinery responsible for this behavior may be downregulated by DVMAT-A overexpression (Chang et al., 2006). Taken together, these results indicate that dopaminergic (and possibly serotonergic) systems and the trace-amine tyramine mediate acute cocaine-induced behaviors. Tyramine has also been implicated in cocaine sensitization (McClung and Hirsh, 1999), although more recent data from the Hirsh laboratory indicate that this connection is tenuous (Hardie et al., 2007). The recent cloning of mammalian G-coupled receptors that respond specifically to trace amines such as tyramine (Borowsky et al., 2001; Bunzow et al., 2001; Miller et al., 2005), suggests that tyramine may act as a neurotransmitter/modulator in mammals. Interestingly, one of these mammalian receptors can be directly activated by amphetamine (Bunzow et al., 2001). These trace-amine receptors may also alter cocaine-induced behaviors indirectly through modification of monoamine transporter function (Miller et al., 2005) or through inhibition of dopaminergic neuron function (Geracitano et al., 2004). It will be interesting to determine if these receptors are involved in cocaine-related behaviors in mammals.

5. Novel genes involved in cocaine-induced behaviors in *Drosophila*

The data summarized above show that several of cocaine's most characteristic properties have been recapitulated in flies. First, cocaine induces motor behaviors in flies 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; Dimitrijevic et al., 2004) 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 pharmacological and genetic methods (Bainton et al., 2000; Li et al., 2000; Chang et al., 2006). More importantly, *Drosophila* studies have identified genes and pathways whose role in cocaine responsiveness had not been anticipated. For instance, flies with mutations in the circadian genes *period* (*per*), *clock*, *cycle* and *doubletime* reduce or eliminate behavioral sensitization to cocaine (Andretic et al., 1999). Subsequently, mice carrying mutations in one of the mouse *per* genes, *mper1*, were found to have defective cocaine sensitization and conditioned place preference (Abarca et al., 2002), highlighting a high degree of conservation in specific gene-behavior relationships between *Drosophila* and mammals.

In addition, several novel genes regulating cocaine-induced behavior have been identified by unbiased genetic screens for fly mutants with abnormal cocaine responses using the "crackometer". The crackometer is a foot-long narrow cylinder into which naive or drug-treated flies are introduced; naive flies climb quickly to the top of the column due to their innate propensity for negative geotaxis and positive phototaxis, while cocaine-treated flies remain near the bottom of the cylinder. A "drug effect score" (DES) is calculated based on the fraction of flies that fail to climb, with a high DES reflecting a strong effect of the drug on climbing behavior (Bainton et al., 2000). The G-protein-coupled receptors encoded by the *moody* gene function in a subtype of glia to regulate the permeability of the blood–brain barrier (BBB) (Bainton et al., 2005; Schwabe et al., 2005). The enhanced sensitivity to cocaine observed in *moody* mutants does not appear to be due to altered cocaine accessibility to the nervous system, but, rather, to some homeostatic effect that a slightly leaky BBB has on nervous system physiology (Bainton et al., 2005). Consistent with Moody functioning in G-protein signaling, the fly homolog of RGS4, *loco*, encoding a regulator/inhibitor of G-protein signaling (Granderath et al., 1999), was identified as a mutant with decreased cocaine sensitivity that also shows a defective BBB (Bainton et al., 2005;

Schwabe et al., 2005). Interestingly, the levels of RGS4 are down-regulated in the prefrontal cortex and striatum of rodents after acute and chronic administration of psychostimulants or morphine (Bishop et al., 2002; Gold et al., 2003; Schwendt et al., 2006, 2007). However, mice lacking RGS4 show normal responses to opioids (Grillet et al., 2005); further studies with these mice may uncover a role for RGS4 in the behavioral effects of psychostimulants. Finally, in mammals, claudin-5, a cell-adhesion molecule found in tight junctions of epithelial cells that form the BBB, has been implicated in normal BBB function (Nitta et al., 2003). Moreover, the human claudin-5 and RGS4 loci have been associated with vulnerability to schizophrenia (Chowdari et al., 2002; Sun et al., 2004). These observations warrant a closer examination of the role of the BBB, and the molecules that regulate its permeability, in nervous system function and the etiology of mental illness, including drug addiction.

white rabbit, encoding a Rho-family GTPase activating protein (Rho-GAP) was also identified by an unbiased genetic screen for mutants with reduced cocaine-, nicotine- and ethanol-sensitivity (Rothenfluh et al., 2006). Small GTPases of the Rho family act as molecular switches transducing extracellular signals to changes in the actin cytoskeleton (Etienne-Manneville and Hall, 2002; Meyer and Feldman, 2002). Their ability – and that of the molecules, such as Rho-GAPs, that affect their activity – to dynamically regulate the actin cytoskeleton and, consequently, the reorganization of axonal and dendritic branches (Bonhoeffer and Yuste, 2002; Luo, 2002) makes them ideally suited to regulate synaptic plasticity and behavior. Indeed, defects in these processes have been linked with certain forms of mental retardation, conditions commonly associated with abnormalities in dendrite and dendritic spine morphology. Moreover, several genes implicated in non-syndromic mental retardation are directly linked to Rho-type GTPase signaling (Ramakers, 2002; Ropers and Hamel, 2005). Importantly, cocaine has been shown to affect proper actin dynamics, which in turn has been postulated to modulate cocaine-induced reinstatement of drug seeking (Toda et al., 2006).

6. Role of the *Drosophila* LIM-only gene, *dLmo*, in cocaine sensitivity

A genetic screen for *Drosophila* mutants with altered acute responses to cocaine identified mutations in the *Drosophila* LIM-only gene, *dLmo*, isolated due to their increased sensitivity to cocaine-induced loss-of-negative geotaxis in the "crackometer" (Tsai et al., 2004). The LIM motif, a cysteine-rich zinc-coordinating domain that mediates protein–protein interactions, was originally discovered as a component of LIM–homeodomain (LIM–HD) transcription factors (Dawid et al., 1998). LMO proteins are nuclear proteins composed almost entirely by two tandem LIM domains. The dLMO protein modulates the function of the LIM–HD factor encoded by the *apterous* gene by competing with the binding of Apterous with its cofactor Chip (Fig. 2) (Milan et al., 1998). Flies carrying loss-of-function mutations in *dLmo* display increased sensitivity to the effects of cocaine, while gain-of-function mutations (in which *dLmo* is over-expressed), show the converse effect, a reduced response to the drug (Tsai et al., 2004). This inverse relationship between *dLmo* gene activity and drug responsiveness suggests that *dLmo* regulates the expression of genes that might play a direct role in controlling cocaine responses.

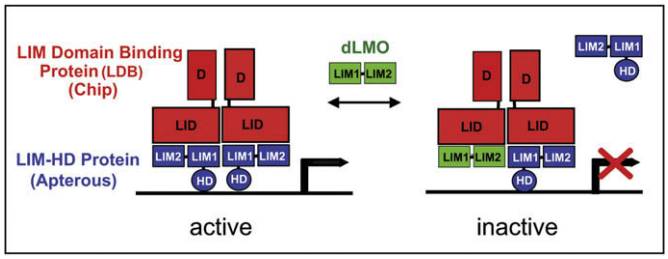

Fig. 2. Mechanism of *dLmo* function. In *Drosophila* one LMO protein is encoded by the *dLmo* locus, and has been studied primarily for its role in the development of the fly wing, which has led to a model for its function (Milan et al., 1998; Shoresh et al., 1998; Zeng et al., 1998). dLMO protein inhibits the activity of the LIM–HD transcription factor Apterous through its interactions with the co-activator Chip (the fly homolog of Clim/Ldb1/NL1) (Fernandez-Funez et al., 1998; Milan and Cohen, 1999; van Meyel et al., 1999; Weihe et al., 2001). Binding of Chip to Apterous leads to its dimerization and activation. dLMO competes for Chip binding; consequently, increased dLMO levels lead to inhibition of Apterous/LIM–HD function, while reduced dLMO levels cause increased Apterous/LIM–HD activity.

dLmo expression is prominent in several distinct brain regions, including the antennal lobes and the mushroom bodies, which are major brain centers involved in olfaction and olfactory conditioning, respectively (Stocker, 1994; Zars, 2000). In addition, *dLmo* is expressed prominently in the ventral lateral neurons (LN_vs) (Fig. 3), the major pacemaker cells regulating circadian locomotor rhythmicity in flies (Renn et al., 1999). Targeted expression of *dLmo* in these different brain regions revealed that the altered cocaine responses are due to differential *dLmo* expression in the LN_vs (Tsai et al., 2004). LN_vs express the neuropeptide pigment-dispersing factor (PDF), the primary functional output of the LN_vs in the regulation of circadian rhythms (Renn et al., 1999). Consistent with a dysfunction of these pacemaker neurons in *dLmo* mutants, the mutant flies also show somewhat altered circadian locomotor rhythms (Tsai et al., 2004), adding to mounting evidence in *Drosophila* and mice supporting a role for circadian genes in cocaine-related behaviors. The finding that cocaine actions are modulated by neurons critical for normal circadian locomotor rhythmicity suggests a basis for this overlap.

The observation that *dLmo* functions in the PDF-expressing LN_vs to regulate cocaine sensitivity, together with the finding that *dLmo* mutants show abnormal circadian locomotor rhythms, suggested that the pathways regulating cocaine sensitivity may be under the control of the circadian clock. However, experiments aimed to address this issue disproved this idea. First, cocaine sensitivity is essentially the same at all times of day, and second, mutations that eliminate PDF expression show normal cocaine sensitivity (Tsai et al., 2004). These data imply that the altered cocaine sensitivity of *dLmo* flies is not a secondary consequence of their abnormal circadian rhythms. Moreover, while the regulation of cocaine sensitivity and circadian behaviors both localize to the pacemaker neurons, the two behaviors are genetically separable. This appears to be in contrast to findings with rodents, where several cocaine-related behaviors show diurnal rhythms that are regulated by clock genes (Abarca et al., 2002; Uz et al., 2002; Akhisaroglu et al., 2004; Kurtuncu et al., 2004; Sleipness et al., 2005, 2007).

The cocaine hypersensitivity of *dLmo* mutants could result from either the disruption of an LN_v output that acts normally to dampen cocaine sensitivity or from an increase in an output of LN_vs that normally enhances cocaine sensitivity (Fig. 4). These possibilities were differentiated by testing flies that either lack LN_vs (generated by targeted expression of a cell death gene) and flies in which LN_vs are selectively silenced (generated by targeted expression of tetanus toxin or a hyperpolarizing potassium channel). These genetic manipulations cause reduced sensitivity to cocaine, the opposite effect elicited by loss-of-*dLmo* function (Tsai et al., 2004), implying that *dLmo* acts to inhibit the contribution of LN_vs to cocaine sensitivity (Fig. 4A).

Taken together, these data show that *dLmo* function in PDF-expressing LN_vs regulates acute sensitivity to volatilized cocaine in *Drosophila*. First, loss-of-function mutations in *dLmo* show increased cocaine sensitivity, a defect that can be reversed by

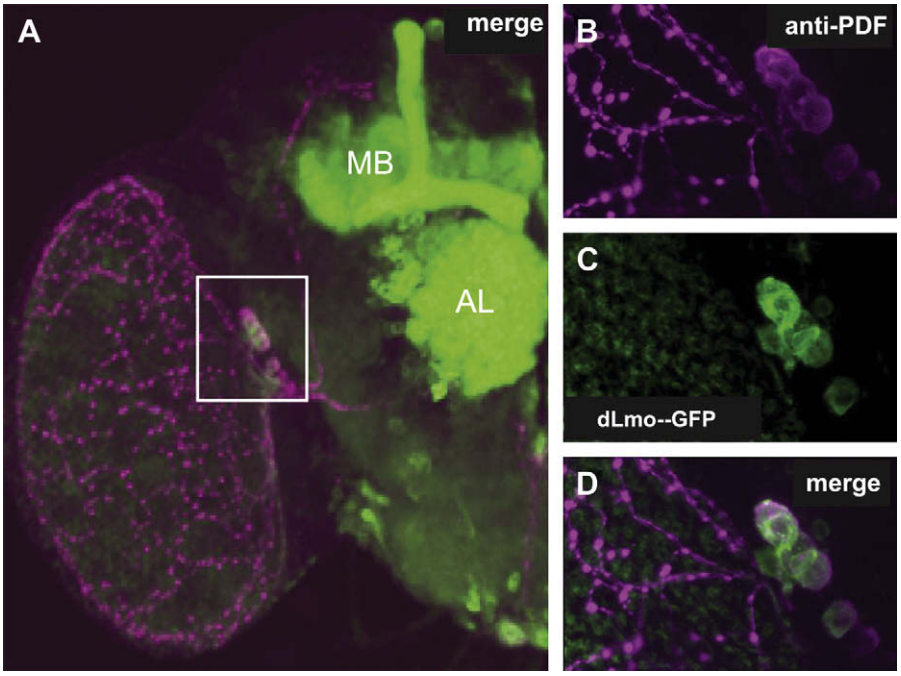

Fig. 3. Expression of *dLmo* in PDF-positive circadian pacemaker cells (LN_vs) in *Drosophila*. (A) Confocal image of an adult brain hemisphere (midline to the right), in which GFP expression is under the control of *dLmo* regulatory sequences (Tsai et al., 2004), double-stained with antibodies that recognizes PDF (magenta) and GFP (green). Overlap between GFP and PDF expression is observed in the LN_vs (white box). GFP expression is also seen in the mushroom body (MB) and antennal lobe (AL). (B–D) Higher magnification views of the LNvs (white box in panel A). (Reprinted from Tsai et al. (2004). PLoS Biol. 2, e408, made available under the terms of the Creative Commons Attribution License 2.5.)

induced expression of *dLmo* in the LN_vs. Second, gain-of-function mutations in which *dLmo* is over-expressed, show reduced cocaine sensitivity; this resistance is mimicked by overexpression of *dLmo* specifically in the LN_vs. The role of *dLmo* in cocaine sensitivity, although mapped to the circadian pacemaker neurons, is separable from its role in circadian regulation. Interestingly, expression of a mouse homolog of *dLmo*, *Lmo4*, is highly enriched in the suprachiasmatic nucleus (SCN) (AWL, DK, and UH, unpublished observations), the mammalian central pacemaker. Furthermore, microarray analysis revealed that in many mouse tissues, *Lmo4* expression varies with circadian time (Panda et al., 2002). These data suggest an evolutionarily conserved role for LMOs in circadian systems, a possibility that has not yet been addressed experimentally in rodents. How do LN_vs modulate cocaine responses? The observation that LN_v electrical activity and synaptic output contribute to cocaine-induced behavioral responses raises

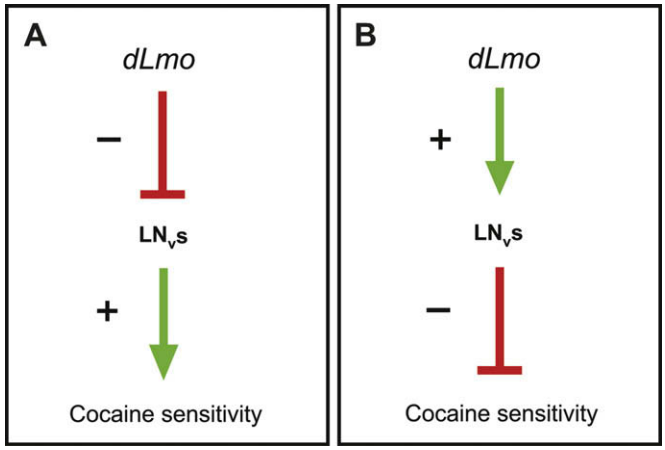

Fig. 4. Model for Lnv and *dLmo* function in *Drosophila*. *dLmo* could function by (A) disrupting an LNv output that acts normally to promote cocaine sensitivity, or (B) increasing an output of LNvs that normally dampens cocaine sensitivity. In both cases, loss-of-*dLmo* would result in enhanced cocaine sensitivity. These possibilities can be differentiated by testing flies that either lack LN_vs (generated by targeted expression of a cell death gene) or flies in which LN_vs are selectively silenced (generated by targeted expression of tetanus toxin or a hyperpolarizing potassium channel). These genetic manipulations cause reduced sensitivity to cocaine, the opposite effect elicited by loss-of-*dLmo* function (Tsai et al., 2004), implying that *dLmo* acts to inhibit the contribution of LN_vs to cocaine sensitivity (panel A).

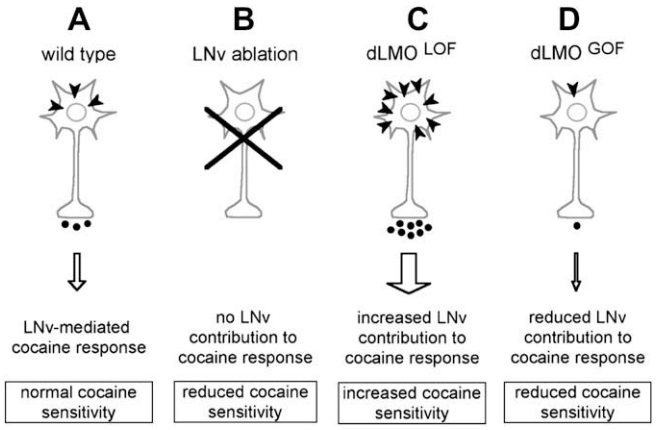

Fig. 5. A model for LN_v and dLMO regulation of cocaine sensitivity in *Drosophila*. (A) In wild type, LN_vs modulate locomotor responses via electrical activity and synaptic transmission. In this model, cocaine would act to directly increase LN_v activity. Upon cocaine administration, synaptic dopamine concentrations would be increased (via cocaine's inhibition of the plasma membrane dopamine transporter), and activation of presumed dopamine receptors on the LN_v (dark arrowheads) would stimulate electrical activity and subsequent synaptic output. This activity would then contribute to the behavioral response of the fly to cocaine. (B) LN_v ablations eliminate LN_v contribution to the cocaine response, reducing cocaine sensitivity. (C) *dLmo* loss-of-function mutants ($dLmo^{LOF}$), which have increased cocaine sensitivity, would have increased activity/output during the cocaine response. This increased activity may be mediated by increases in dopamine receptor content on the LN_v. (D) $dLmo^{GOF}$ mutants would have reduced LN_v output and reduced cocaine sensitivity. This could result from a reduction in dopamine receptor density. (Reprinted from Tsai et al. (2004). PLoS Biol. 2, e408, made available under the terms of the Creative Commons Attribution License 2.5.)

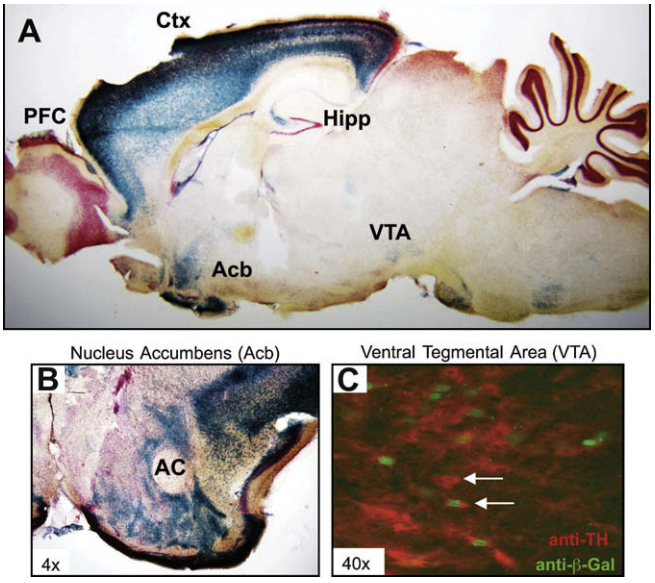

Fig. 6. Expression of *Lmo4* in the adult mouse brain. (A) Sagittal brain section of an adult *Lmo4Gt*/+ mouse stained with X-Gal (blue) to detect β-galactosidase expression (expressed as a Lmo4-β-galactosidase fusion protein). Intense β-galactosidase expression is observed in neocortex (Ctx; layers III and V), hippocampus (Hipp; CA3 regions and subiculum), and nucleus accumbens (Acb); low expression is also seen in the ventral tegmental area (VTA) and some hypothalamic nuclei. Expression in basolateral amygdala and caudate putamen is not seen in this specific sagittal section. (B) Coronal section showing β-galactosidase expression in the Acb of an *Lmo4Gt*/+ adult mouse (AC = anterior commissure). (C) Expression of LMO4-β-galactosidase fusion protein in the VTA of an *Lmo4Gt*/+ mouse. Brain sections containing VTA were processed for β-galactosidase (green) and tyrosine hydroxylase (TH, red) antibody reactivity. Colocalization of LMO4 fusion protein and TH is observed in some (arrow), but not all (arrowhead) TH-positive dopaminergic neurons.

a number of possibilities regarding the interaction of cocaine with these neurons and their output. It is possible, for example, that the activity of LN_vs directly increases upon cocaine administration, which in turns results in cocaine-induced changes in behavior (Fig. 5A). In agreement with this possibility is the finding that isolated cultured LN_vs respond to either dopamine or acetylcholine, but not to other neurotransmitters (Wegener et al., 2004), suggesting that LN_vs express dopamine receptors and may therefore be sensitive to the enhanced synaptic dopamine levels produced upon inhibition of DAT by cocaine in pre-synaptic neurons. However, it is also possible that other, yet to identified dopamine receptor-expressing neurons function pre- or post-synaptically to the LN_vs (or in a parallel pathway) to regulate cocaine sensitivity. If dopamine receptors are expressed in LN_vs, we speculate that their activity could be directly increased by cocaine administration. When the LN_vs are ablated or silenced (Fig. 5B), one site of cocaine action would be eliminated, thus reducing cocaine's effect. How would *dLmo* fit into this model? It is possible that in *dLmo* loss-of-function mutants, LN_v output is boosted, possibly due to increased expression of dopamine (or other) receptors, leading to enhanced cocaine sensitivity (Fig. 5C). Conversely, increased *dLmo* expression in LN_vs would result in reduced receptor content and, consequently, in dampened cocaine sensitivity (Fig. 5D). LMO-induced changes in receptor expression are not inconceivable given LMO's interaction with LIM–HD proteins. Changes in LIM–HD protein function have been shown to affect aspects of neuronal subtype identity, including neurotransmitter and receptor expression profiles. For example, mutations in the *Drosophila* LIM–HD gene *islet* cause a loss-of-dopamine and serotonin syntheses, while ectopic expression leads to ectopic expression of TH (Thor and Thomas, 1997). This graded effect of *islet* function on dopamine levels and TH expression is reminiscent of the graded cocaine responses observed in *dLmo* gain- and loss-of-function mutants. In addition, expression of a *Drosophila* dopamine receptor in larval neurons requires the function of the LIM–HD gene *apterous* (Park et al., 2004). The observation that dopamine receptor expression is also altered in various *Caenorhabditis elegans* LIM–HD mutants (Tsalik et al., 2003), suggests an evolutionarily conserved role of LIM–HD proteins and possibly LMOs in the regulation of neurotransmitter identity and responsiveness of specific neurons. Mammalian LMOs interact with multiple transcription factors, which in turn regulate various aspects of nervous system development, including the specification of neural identity (see below) (Hobert and Westphal, 2000; Shirasaki and Pfaff, 2002). Thus, LMOs are ideally suited to modulate the neurochemical identity and sensitivity of the nervous system to various stimuli, including drugs of abuse.

7. Role of *Lmo4* in cocaine-related behaviors in mice

Mammalian genomes encode four *Lmo* genes, *Lmo1–4*, which have been studied primarily for their roles in oncogenesis. *Lmo1* and *2* were first discovered as chromosomal translocations in T-cell leukemias (Rabbitts, 1998). Moreover, high expression of *Lmo3* is correlated with unfavorable prognosis in neuroblastomas (Aoyama et al., 2005b), while *Lmo4* plays a role in a variety of cancers (Visvader et al., 2001; Sum et al., 2005; Taniwaki et al., 2006; Murphy et al., 2008). In addition, high levels of LMO proteins contribute to increased cell proliferation, suggesting that the function of LMOs in diverse tissues is to inhibit cellular differentiation. *Lmo* genes are highly expressed during development, and their roles in

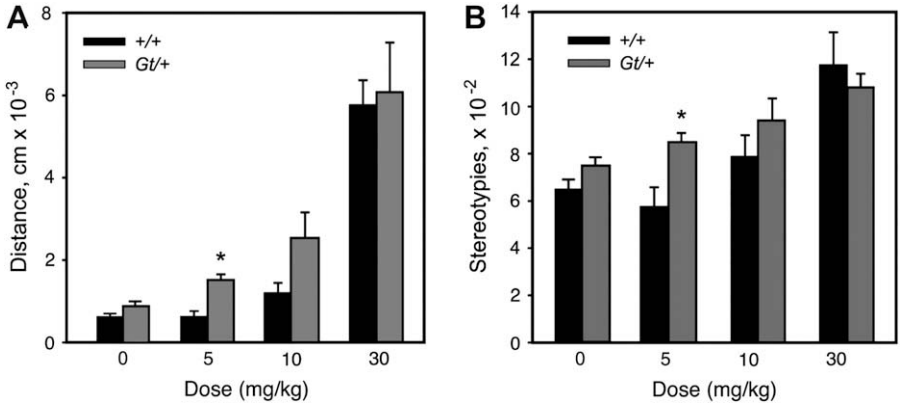

Fig. 7. Cocaine-sensitivity phenotypes of *Lmo4Gt* heterozygous mice. (A) Mice heterozygous for the *Lmo4Gt* insertion (*Gt*/+, gray bars) show increased locomotor activation compared to wild-type littermates (+/+, black bars) upon administration of 5 mg/kg, but not 10 nor 30 mg/kg, of cocaine. Data show total activity for 15 min after injection. Asterisk indicates significant differences between groups (Student's *t*-test, $P < 0.05$, $n = 6–13$). (B) Stereotypic counts in response to acute cocaine injections. Data were analyzed as in (A).

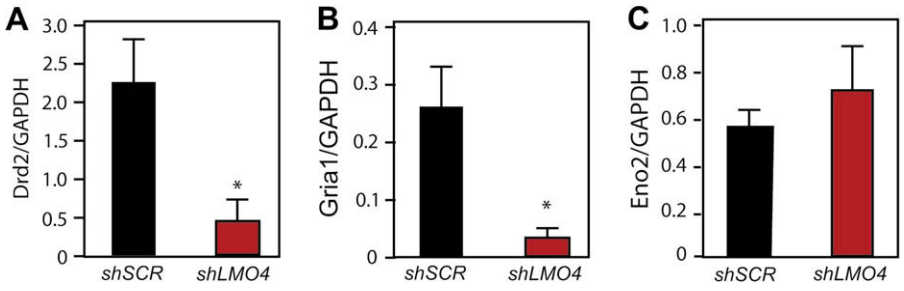

Fig. 8. Altered expression of *Drd2* and *Gria1* upon *Lmo4* downregulation in mouse Acb. (A–C) Quantitative RT-PCR analysis of mRNA isolated from Acb infected with lentiviruses expressing either an shRNA that targets *Lmo4* (shLMO4) or a "scrambled" control shRNA (shSCR) that is predicted to be inert. Infected cells were laser capture microdissected from brain sections taken 12 days after stereotaxic injection of virus into Acb. Expression of *Drd2* (A) and *Gria1* (B) are significantly different between shSCR- and shLMO4-infected samples, in contrast to the neuronal marker *Eno2* (C), which shows no difference. Error represents SEM ($n = 5$ in each group; $^*P < 0.05$ by Student's *t*-test).

embryogenesis have been investigated using knockout mice (Warren et al., 1994; Hahm et al., 2004; Tse et al., 2004). Mice containing a homozygous null mutation in *Lmo4* die just after birth, and exhibit defective neural tube closure (Hahm et al., 2004; Tse et al., 2004; Lee et al., 2005). Clues as to the function of *Lmo4* in neurons have come from cultured neuroblastoma cells, where its overexpression prevents neurite outgrowth, while its down-regulation promotes neurite formation (Vu et al., 2003), suggesting an inhibitory role in neuritogenesis. *Lmo4* is widely expressed in the brain during early embryogenesis, and becomes more specifically localized during late embryogenesis, with particularly high levels in the cortex, hippocampus, subthalamic nuclei, globus pallidus, nucleus accumbens, and caudate putamen (Hermanson et al., 1999). Expression of *Lmo4* in the developing mouse cerebral cortex is necessary for the organization of the barrel field in the somatosensory cortex and the proper patterning of thalamocortical connections, as demonstrated in mice with conditional knockout of *Lmo4* in the cortex (Kashani et al., 2006). In addition, *Lmo4* is asymmetrically expressed in the right perisylvian cerebral cortex in humans, suggesting a potential role in the development of human brain asymmetry (Sun et al., 2005). Expression of *Lmo* genes in the adult mouse central nervous system closely mimics expression seen during development, with high expression in the cortex, caudate putamen, amygdala, and hippocampus (Hinks et al., 1997; Hermanson et al., 1999) (see below).

As in *Drosophila*, mammalian LMO proteins have been shown to play a role in transcriptional regulation (Retaux and Bachy, 2002; Matthews and Visvader, 2003). LMOs interact with the mammalian homolog of *Drosophila* Chip, known as Ldb1 (Grutz et al., 1998; Deane et al., 2003). Ldb proteins, in turn, interact with DNA-binding LIM homeodomain (LIM–HD) proteins to regulate transcription. Large multimeric transcriptional complexes containing LMOs, Ldbs, and LIM–HD proteins are thought to specify neuronal cell fate, for example the choice between motor neuron or interneuron in the developing spinal cord (Thaler et al., 2002). In this context, high levels of LMO proteins are hypothesized to repress transcription mediated by LIM–HD factors by competing with LIM–HD factors for Ldb binding (Fig. 2). However, mammalian LMO proteins also interact with multiple other transcription factors, and can either activate or inhibit their function (Visvader et al., 1997; Wadman et al., 1997; Chan and Hong, 2001; Aoyama et al., 2005a; Singh et al., 2005; Kashani et al., 2006). The promiscuous binding of LMO proteins to many different types of transcription factors indicates that these proteins can exist in large transcriptional complexes and, depending on context, can promote transcriptional activation or repression. Generally, however, the transcriptional modifying property of LMO proteins tends to keep cells in an undifferentiated state, and in neurons appears to negatively regulate dendrite formation or axonal outgrowth. In addition, evidence showing that expression and activity of LMOs are dynamically regulated within the nervous system is growing. For instance, expression of the murine *Lmo1*, *Lmo2*, and *Lmo3* genes is differentially regulated by seizure activity in specific regions of the hippocampus and forebrain of adult mice (Hinks et al., 1997). In addition, gene array experiments have revealed that expression of mouse *Lmo4* is under circadian regulation in the SCN and increased in the cerebral cortex during sleep deprivation (Cirelli and Tononi, 2000; Panda et al., 2002), and *Lmo3* was isolated as a transcript upregulated by dopamine administration in cultured astrocytes (Shi et al., 2001). Lastly, *Lmo2* and *Lmo4* were isolated in a screen for calcium-regulated activators of transcription (Aizawa et al., 2004), suggesting a role for LMOs in regulating gene expression changes induced by neural activity.

As detailed above, experiments in *Drosophila* revealed a role for *dLmo* in the acute response to cocaine, suggesting the possibility that one or more mammalian *Lmo* genes may mediate behavioral responses to cocaine. This possibility was tested by examining the role of *Lmo4* in behavioral responses to cocaine. A mouse strain, *Lmo4Gt*, was generated from embryonic stem cells (Bay Genomics) that contain a gene-trap insertion in the 4th intron of *Lmo4* (Lasek et al., submitted for publication). Consistent with the reported lethality of null mutations in *Lmo4*, homozygous *Lmo4Gt* mice do not survive, indicating the generation of a null (or strong hypomorphic) allele. *Lmo4Gt/+* heterozygous mice are viable and fertile with no obvious behavioral defects, despite expressing ~50% of wild-type *Lmo4* transcript levels in the brain as determined by quantitative RT-PCR (Lasek et al., submitted for publication). The *Lmo4Gt* line produces an LMO4-β-galactosidase fusion protein, whose expression is regulated by endogenous *Lmo4* cis-regulatory elements. Intense expression of β-galactosidase in adult *Lmo4Gt/+* mice is restricted to specific brain regions, including most regions implicated in drug-related behaviors (Nestler, 2000), such as the prefrontal cortex, nucleus accumbens (Acb), and ventral tegmental area (Fig. 6A–C), data that is consistent with *in situ* hybridization studies (Hermanson et al., 1999). *Lmo4Gt/+* mice show a modest but significant increase in the acute locomotor-stimulant and stereotypic responses to a low dose of cocaine (5 mg/kg) (Fig. 7A, B); however, no significant differences are observed upon exposure to moderate or high cocaine doses (10, 15, or 30 mg/kg). A robust enhancement in behavioral sensitization to repeated cocaine exposures is also observed in *Lmo4Gt/+* mice (Lasek et al., submitted for publication). The increase in acute cocaine sensitivity observed in heterozygous *Lmo4Gt* mice parallels data on *dLmo* and cocaine responses in *Drosophila*, as decreased *dLmo* levels lead to enhanced acute cocaine sensitivity.

Identification of transcriptional targets of LMO4 will likely provide relevant insights into its mechanisms of action in regulating cocaine-induced behaviors. Since dopamine and glutamate systems regulate the behavioral responses to cocaine (see, for example, Ikegami and Duvauchelle, 2004; Kalivas et al., 2005; Haile et al.,

2007; Kalivas, 2007; Kalivas and O'Brien, 2008), the expression of dopamine and glutamate receptor mRNAs was examined in nucleus accumbens (Acb) tissue expressing a small hairpin RNA targeting *Lmo4* (shLMO4) (Lasek et al., submitted for publication). Significant reductions in mRNA levels for the dopamine D2 receptor (*Drd2*, Fig. 8A) and the GluR1 subunit of the AMPA receptor (*Gria1*, Fig. 8B) are observed in Acb tissue expressing shLMO4 (where *Lmo4* expression is decreased by approximately 50%). Expression of dopamine D1, D3 and D5 receptors and of the GluR2 subunit of the AMPA receptor are not significantly altered upon *Lmo4* downregulation (AWL, unpublished results). Interestingly, overexpression of a dominant-negative pore-dead form of GluR1 in the Acb of rats causes an enhanced acute response to cocaine (Bachtell et al., 2008). This is consistent with a predicted enhanced behavioral response to cocaine observed upon downregulation of GluR1 in the Acb of mice with decreased *Lmo4* expression. In contrast, *Drd2* knockout mice exhibit reduced locomotor activity and responsiveness to acute cocaine administration (Chausmer et al., 2002) The transcriptional activation of *Drd2* by *Lmo4* in Acb (implied by the reduced expression of *Drd2* upon *Lmo4* downregulation) is therefore not consistent with the enhanced cocaine sensitivity seen upon *Lmo4* downregulation. *Drd2* knockout mice, however, exhibit increased cocaine self-administration at high cocaine doses, suggesting a role for *Drd2* in limiting drug intake (Caine and Koob, 1994). In addition, human cocaine addicts and non-human primates chronically self-administering cocaine show decreased D2-receptor levels in striatum (Volkow et al., 1993; Nader et al., 2002), and polymorphisms of the D2 dopamine receptor resulting in reduced receptor levels have been linked to cocaine addiction (Noble et al., 1993; Persico et al., 1996). Thus, *Lmo4* either directly or indirectly regulates the profile of receptors expressed by Acb neurons, an effect that likely alters the function of neural circuits that mediate behavioral responses to cocaine and possibly other abused drugs. It will be interesting to determine if the expression of *Drosophila* dopamine and/or glutamate receptors is regulated by *dLmo* and whether such potential regulation is responsible for the cocaine-sensitivity defects observed in *dLmo* mutant flies.

8. Conclusions

It has now been well-established that drugs of abuse act in flies and mammals by similar mechanisms. Behaviors are remarkably similar, and some similarities and the molecular and neurochemical levels have already emerged. Unbiased genetic screens in *Drosophila* – aided by the ease, quickness, and low expense of fly studies – have been used to identify novel potential candidate genes regulating drug-related behaviors. These screens have now implicated a variety of signaling pathways and biological processes in drug-related behaviors, many of which appear to have evolutionarily conserved roles in mammals. The role of *dLmo* and *Lmo4* in regulating cocaine-related behaviors in flies in mice, respectively, provides an example of such conservation. Moreover, the observation that LMO4 regulates dopamine and glutamate receptor expression in the nucleus accumbens provides a possible mechanism through which LMOs may regulate cocaine-related behaviors.

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